THE EFFECT OF TYROSYL MODIFICATION AND HIGH pH ON THE ACTIVITY OF α-LACTALBUMIN

By

CHARLES RICHARD MERRIMAN

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Northwestern State College

Alva, Oklahoma

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Thesis Approved:

Kurt E. Ebner
Thesis Advisor

Franklin E. Leach

Kermit Carraway

Roger E. Koeppe

Ernest M. Hodnett

N. D. Durham
Dean of the Graduate College

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α-A	α-Lactalbumin A
α-B	α-Lactalbumin B
α-LA	α-Lactalbumin
BαLA	Bovine α-lactalbumin
CD	Circular dichroism
CHO	Carbohydrate
DFP	Diisopropylfluorophosphate
DIT	Diiodotyrosine
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
E	Enzyme
EDTA	Disodium ethylenidiamine tetraacetate
GPαLA	Guinea pig α-lactalbumin
HA	Hydroxyl apetite
HαLA	Human α-lactalbumin
MIT	Monoiodotyrosine
P	Phosphate
PEP	Phosphoenolpyruvate
TLC	Thin layer chromatography
TLCK	1-chloro-3-tosylamide-7-amino-2-heptanone
TNBS	Trinitrobenzene sulfonic acid
TPCK	1-tosylamide-2-phenylethylchloromethyl ketone

CHAPTER I

INTRODUCTION

Lactose synthesis requires the presence of two proteins, a galactosyltransferase and α-lactalbumin (1, 2). Hill et al. (3) suggested that α-lactalbumin acts as a "specifier" protein altering the galactosyl acceptor specificity of the galactosyltransferase from N-acetylglucosamine to glucose, but studies by Morrison and Ebner (4) indicate that α-lactalbumin may best be described as a modifier protein since it lowers the apparent K_m for glucose in the galactosyltransferase reaction.

This interesting function of α-lactalbumin plus the fact that α-lactalbumin can be isolated to purity in large quantities has led to many investigations of its biochemical character.

Mawal et al (5) have shown by affinity chromatography that enzyme-reactant complexes are formed between α-lactalbumin and galactosyltransferase. Thus a study of the amino acids in α-lactalbumin responsible for interaction with the galactosyltransferase should provide information as to the mechanism by which α-lactalbumin modifies the galactosyltransferase molecule. At present one of the best methods available for determining which amino acids are critical in the binding domain of α-lactalbumin is chemical modification. If specific chemical modification of a particular amino acid results in a loss of biological activity, the amino acid is considered an "essential group."

Several major problems arise when using this technique. A very serious problem is selecting a reagent which will give a minimum of nonspecific side reactions. Most of the commonly used reagents have been tested with model peptides and proteins of known structure to determine the specificity of reaction. New modification techniques must be examined to determine which amino acid residues are modified and in addition the reaction mixture must be examined for side reactions such as polymerization of the protein.

Another potential serious problem is determining if the loss of activity observed after a chemical modification is due to modifying an essential residue or due to a structural change which prevents the protein from assuming its active conformation.

Modification of α-lactalbumin by Denton and Ebner (6) indicated that the tyrosyl residues of α-lactalbumin are critical for its activity in lactose synthetase. The use of other tyrosyl reagents was undertaken in an effort to further substantiate these findings and to determine if possible which of the four tyrosyl residues are essential for activity.

N-acetylimidazole was chosen as a reagent to modify α-lactalbumin because the acetylation of the tyrosyl residues can be easily reversed. It is a very mild reagent and is very selective (7), attacking only tyrosine and lysine. N-acetylimidazole suffers the disadvantage that it attacks all four tyrosyl residues in α-lactalbumin. Thus it is not possible to determine the number or position of the essential tyrosyl residue or residues. Since most chemical tyrosyl reagents react with all the exposed tyrosines, an enzymatic method of modification was also used to achieve some degree of selectivity. Selectivity should occur

if any of the tyrosyl residues are sterically hindered and are thus inaccessible to the modifying enzyme.

Lactoperoxidase was chosen to incorporate iodide into α-lactalbumin. It is a large, stable enzyme that catalyzes the iodination of tyrosyl and histidyl residues under very mild conditions (8). Hydrogen peroxide is utilized at very low concentrations as the oxidizing agent for the reaction.

Often in the course of a scientific investigation the most significant discovery is aside from the predefined objective of the investigation. For example, the investigations of Denton and Ebner (6) led to the interesting and very puzzling observation that polymerization of α-lactalbumin occurred during nitration and iodination. Their study, plus those of other investigators (9, 10) have established polymerization as a significant side reaction under modification conditions.

The studies on the inactivation of α-lactalbumin at high pH were likewise an outgrowth of studies on the acetylation of α-lactalbumin. This observation should be of general concern to other investigations involved with the structure and function of α-lactalbumin.

CHAPTER II

LITERATURE REVIEW

The Biological Activity of α-Lactalbumin

The natural abundance of milk and the ease of acquisition has led to many investigations of its biochemical character. One of the more interesting characteristics to be discovered is the carbohydrate content of milk. The disaccharide lactose (4-O-β-D-galactosyl-α-D-glucose) is the only major carbohydrate constituent of milk and the capacity for lactose biosynthesis is restricted to the mammary gland and perhaps a few plants (11, 12) though this is doubtful. Both the galactosyl and glucosyl moieties of lactose are derived from blood glucose. Three enzymes synthesize lactose by catalyzing the following reactions:

$$\text{UTP} + \text{Glucose} \rightleftharpoons \text{UDP-glucose} + \text{PP}_i \qquad (2.1)$$

$$\text{UDP-glucose} \rightleftharpoons \text{UDP-galactose} \qquad (2.2)$$

$$\text{UDP-galactose} + \text{glucose} \longrightarrow \text{lactose} + \text{UDP} \qquad (2.3)$$

Reaction 2.1 is catalyzed by the enzyme UDP-glucose pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridyltransferase, E.C.2.7.7.9.). Reaction 2.2 is catalyzed by the enzyme UDP-galactose-4-epimerase (E.C.5.1.3.2.), and reaction 2.3 is catalyzed by lactose synthetase (E.C.2.4.1.22). The first enzyme in the sequence has been characterized from the bovine mammary gland by Aksamit and Ebner (13). The second enzyme has also been isolated from mammary tissue and characterized by Tsai, et al. (14) and Fitzgerald, et al. (15). The third enzyme was first demon-

strated by Watkins and Hassid in lactating mammary glands of cows and guinea pigs (16) and later in bovine milk (17).

The first clue to α-lactalbumin's role as a constituent of the lactose synthetase system came in 1966 when Brodbeck and Ebner (1) resolved the soluble lactose synthetase from bovine milk into two proteins by chromatography on Bio Gel P-30. They designated the two proteins A and B according to their elution on the gel filtration column. When assayed separately neither A nor B exhibited lactose synthetase activity but when the two proteins were combined lactose synthesis was detected. Subsequently Ebner et al. (2, 18) showed that the B protein was identical to α-lactalbumin, the common whey protein. Hill and his coworkers (3, 19) have made the important observation that the A protein by itself can catalyze the transfer of galactose to N-acetylglucosamine to form N-acetyllactosamine. In the presence of α-lactalbumin the formation of N-acetyllactosamine is inhibited and lactose synthesis will occur in the presence of glucose.

Hill (3) suggested that α-lactalbumin should be considered as a "specifier protein" since it changes the activity of an existing protein. However Fitzgerald, et al. (20) found that the A protein can catalyze the formation of lactose in the absence of α-lactalbumin when the glucose concentration is high (K_m = 1.4 M). α-Lactalbumin lowers the apparent K_m of glucose (mM) so that it becomes a good substrate. Klee and Klee (21) have shown that α-lactalbumin lowers the K_m for both glucose and N-acetylglucosamine but the magnitude of the reduction for glucose is much greater, molar to millimolar.

Morrison and Ebner (4, 22, 23) analyzed the steady state kinetics of the lactose synthetase reaction and demonstrated that α-lactalbumin does participate in the reaction.

The reaction has an ordered mechanism with the reactants adding in the order: Mn^{++}, UDP-galactose, the carbohydrate acceptor, and α-lactalbumin. Mn^{++} reacts with the free enzyme under conditions of thermodynamic equilibrium and does not dissociate after each catalytic cycle. At high concentrations, carbohydrate can add randomly to all enzyme forms but an active complex is not formed unless Mn^{++} and UDP-galactose have added previously. They reached the general conclusion that α-lactalbumin is a special type of modifier which combined with the enzyme only after the addition of a carbohydrate reactant and displaces an already established equilibrium. Morrison and Ebner (4) proposed that the effect of α-lactalbumin on the kinetics of the reactions catalyzed by galactosyltransferase can be explained by the mechanism illustrated in Figure 1. With N-acetylglucosamine as the carbohydrate substrate, reaction occurs readily in the absence of α-lactalbumin and proceeds via the linear pathway of Figure 1. However, increasing concentrations of α-lactalbumin force more and more of the reaction flux to proceed via the branched pathway until at an infinite concentration of α-lactalbumin, a lower limiting maximum velocity is reached. With glucose as the carbohydrate substrate the reaction can occur along the linear sequence in the absence of α-lactalbumin only when glucose is in the 1 to 2 molar range. When α-lactalbumin is present the reaction could theoretically occur along either pathway, however because of the marked effect of α-lactalbumin reducing the apparent K_m value for glucose virtually all of the reaction flux would occur along the branched

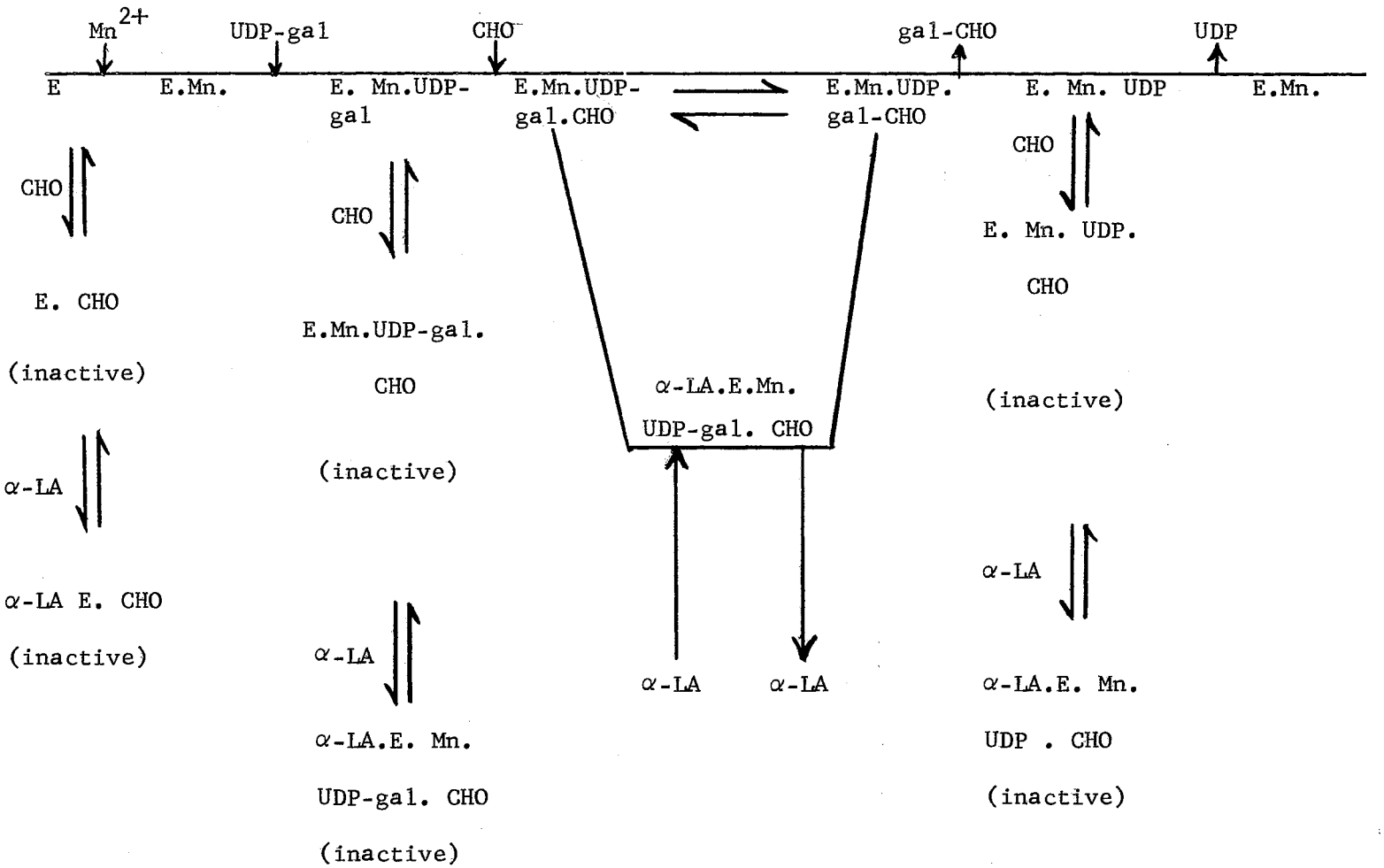

Figure 1. Schematic Representation of Order of Addition of Substrated and Release of Products of the Galactosyltransferase Reaction.

α-Lactalbumin is represented by α-LA, UDP-galactose by UDP-gal, and carbohydrate by CHO.

pathway. This proposal also offers an explanation for the failure of Schanbacher and Ebner (24) to demonstrate the formation of an α-lactalbumin-galactosyltransferase complex under the conditions of maximum product formation by the use of sucrose density centrifugation, equilibrium dialysis, fluorescence quenching, and gel filtration techniques. Since then it has been demonstrated that galactosyltransferase from bovine (25) and human (26) milk can be purified in the presence of glucose or N-acetylglucosamine by affinity chromatography using a column of Sepharose to which α-lactalbumin is covalently bound. This led Mawal, et al. (5) to use the affinity chromatography column for the detection of enzyme-reactant complexes as predicted by the steady state mechanism. α-Lactalbumin bound to Sepharose is capable of functioning as a modifier in the same manner as does free α-lactalbumin and that reaction of any enzyme form with bound α-lactalbumin causes retardation of the passage of the enzyme through the Sepharose-α-lactalbumin columns. By varying the reaction components in the elution buffer they found evidence for the postulated deadend complexes responsible for substrate inhibition. Due to the high concentration of α-lactalbumin present they also detected complexes which are not of importance in the reaction mechanism.

Recently Klee and Klee (27) have presented evidence for the interaction of α-lactalbumin and galactosyltransferase by another technique. In their procedure the band forming cell of Vinograd is used and the migration of a band of A protein through solutions of α-lactalbumin is followed in the analytical ultracentrifuge equipped with a photoelectric scanner. Their experiments showed that a complex is formed between galactosyltransferase and α-lactalbumin which contains one mole-

cule of each protein. The complex is stable enough to be observed only in the presence of one of the substrates. By using yellow, nitrated α-lactalbumin they also demonstrated binding by conventional sedimentation velocity techniques using absorption optics at 430 nm. From their studies (27) it is difficult to tell if the complex observed represents a catalytically active complex or a dead-end complex. The fact that they observed a complex with nitrated α-lactalbumin is hard to explain since nitrated α-lactalbumin appears to be inactive (6).

Since a complex has been definitely demonstrated the next area for study is the molecular interactions between the two proteins. To investigate the binding areas of the two proteins it is first necessary to know the general characteristics of the two proteins. The soluble bovine milk galactosyltransferase has been characterized by Klee and Klee (27) and Trayer and Hill (8). Klee and Klee (27) reported that the galactosyltransferase is a single chain glycoprotein of molecular weight 44,000 with a sedimentation coefficient of 3.25. The enzyme behaves on disc gel electrophoresis as a family of closely related components all of which are enzymically active and contain carbohydrate. The complete amino acid analysis showed that the proline content was very high. They stained disc gels to demonstrate the presence of carbohydrate and estimated that the enzyme contained about 5% reducing sugar. Trayer and Hill (28) reported that the enzyme is a glycoprotein with a molecular weight between 40,000 and 44,000. They found no evidence for subunits. They performed both amino acid and carbohydrate analyses. Their results on amino acid analysis differed considerably with those of Klee and Klee (27) but they did agree on the high proline content. From the carbohydrate composition it was estimated that

there were 28 residues of carbohydrate per enzyme molecule or about 12% by weight. Lehman, et al. (29) found 10.2% carbohydrate, of which the neutral sugars account for 4.1%, the hexosamines 4.0% and the sialic acid constitutes 2.1%. They have found that the protein contains both asparagine-glucosamine and serine/threonine--galactosamine glycopeptide linkages. Recently, Magee et al. (30) have demonstrated the presence of multiple forms, one with a molecular weight of 44,000 and the other with a molecular weight of 55,000. Both forms are enzymatically active and contain carbohydrate.

It is interesting to note that Klee and Klee (27) treated the enzyme with neuraminidase which removed the terminal sialic acid but did not lose any activity, thus the sialic acid is not directly involved in the functional role of the enzyme. Since the galactasyltransferase is available only in small amounts the bulk of the research attempted to date regarding the binding sites has been on α-lactalbumin. A later section in this chapter reviews the α-lactalbumin data.

Protein-Protein Interactions

Frieden (31) divides multienzyme complexes into two classes: those in which the binding of proteins having different activities is quite tight and not easily disrupted; and those in which the binding may be weaker and the specificity of at least one of the interacting enzymes is altered by the others. He lists lactose synthetase as an example of the second class of enzymes with the reservation that the extent of interaction may change in vivo. Other examples of this class of enzymes are tryptophan synthetase (of some organisms) and cysteine synthetase.

A careful comparison of the similarities of these enzymes might lead to an understanding of the basis for this loose, freely reversible, binding which causes large, obvious changes in activity.

Fortunately a great deal is known about the molecular architecture of tryptophan synthetase. The E. coli tryptophan synthetase complex is composed of two easily dissociable (32) subunits (α and β_2). The subunits are completely different proteins having quite different physical and catalytic properties. Separately the α and β_2 subunits have distinct, but only partial activities on the enzymatic half reactions:

$$\text{Indole glycerol-P} \overset{\alpha}{\rightleftharpoons} \text{indole} + \text{glyceraldehyde 3-P} \qquad (2.4)$$

$$\text{Indole} + \text{L-Serine} \overset{\beta_2;\ \text{pyridoxal-P}}{\rightleftharpoons} \text{L-tryptophan} \qquad (2.5)$$

When the α and β_2 subunits are physically associated to reconstitute the $\alpha_2\beta_2$ complex, the rates of the partial reactions are 30-100 fold greater than with the individual subunits, but indole is not liberated from the complex. Thus the functionally significant reaction is:

$$\text{Indole glycerol-P} + \text{L-serine} \xrightarrow{\text{Pyridoxal-P}} \text{L-tryptophan} + \text{glyceraldehyde-3-P} \qquad (2.6)$$

With pyridoxal-P, the β_2 subunit also catalyzes a serine deamination reaction (33) and a thiol-dependent transamination reaction (34) which are completely inhibited by the normally present α-subunit. In contrast, the combination of the α and β_2 subunits slightly stimulates a thiol addition reaction in the presence of mercaptoethanol, L-serine, and pyridoxal-P (35) and greatly enhances the indole addition of reaction 2.5. Crawford and coworkers (34) have evidence that phos-

phopyridoxylaminoacrylic acid is a common intermediate in all reactions of tryptophan synthetase. The α subunit is not required for formation of this intermediate but it does regulate its fate once formed and thereby directs the further reaction of the β_2 intermediate toward tryptophan synthesis instead of other alternate pathways. Presumably these effects are a consequence of conformational changes induced in both types of subunits as a result of the interactions between them.

Yanofsky and co-workers (36) have purified, crystallized and determined the amino acid sequence of the α subunit. The α polypeptide chain has a molecular weight of 30,000 and contains three cysteinyl residues. The three cysteinyl residues vary in their reactivity toward N-ethylmaleimide, a sulfhydryl specific reagent (37). Labeling the α protein with radioactive N-ethylmaleimide produces (a) unlabeled protein, (b) singly labeled protein at cysteine, 80, 117, and 153 and (c) doubly labeled protein at both cysteine 117 and 153. Cysteine 80 appears to be protected in the $\alpha_2\beta_2$ complex from reaction with low concentrations of N-ethylmaleimide. However the alkylation of cysteine 80 had little effect on the association of the chemically modified α subunit with the β_2 subunit or with a specific antibody. The alkylated derivative is about 50% active in reactions 2.4, 2.5 and 2.6 when in combination with the β_2 subunit but is inactive by itself. Labeling at either cysteine 117 or 153 produces major structural and functional changes in the α subunit. The doubly labeled derivative loses all enzymatic activity and antibody recognition, and associates with the β_2 protein to form a large aggregate that is three to four times larger than that of the normal $\alpha_2\beta_2$ complex. Two other sulfhydryl reagents,

p-hydroxymercuribenzoate and 5, 5' dithiobis (-2 nitrobenzoic acid), react in a similar manner.

In comparison to the lactose synthetase, we find in both systems a small protein, the α protein and α-lactalbumin, interacting in a freely dissociable manner with a larger protein to specify the reaction product. In both cases chemical modification has been used to determine the amino acid residues necessary for biological activity (37, 6). Although the X-ray crystallographic analysis of both α-lactalbumin and the α protein of tryptophan synthetase are nearing completion, the knowledge of the three dimensional structure will be of limited value in determining the critical amino acids in the binding domain. The large size of the complexes prevents the rapid analysis of the binding domain by X-ray crystallography directly. Hence chemical and enzymatic modification remain the preferred techniques at the present time.

Another technique for determining binding sites between proteins which shows promise has been developed by Arnon et al. (38). They demonstrated that a peptide serves as an antigentic determinant on the lysozyme molecule. An isolated fragment of lysozyme (residues 60-83) when coupled to a synthetic polymer was capable of eliciting antibodies that react specifically with the native lysozyme molecule. A peptide was synthesized by the solid phase technique of Merrifield (39) replacing cysteine 76 with alanine. This replacement allowed the specific formation of a disulfide bond between cysteine 60 and cysteine 84. The formation of this loop peptide allowed for testing of the conformation dependancy of the antibody. This conjugate of synthetic loop peptide and synthetic polymer was capable of eliciting the formation of antibodies which in turn were capable of reacting with both lysozyme and

loop peptide prepared from lysozyme. These immunological interactions can be inhibited by either lysozyme or the loop peptide, but not by a performic acid-oxidized open-chain peptide. Thus the antibodies elicited recognize a conformation-dependent determinant in the native protein.

This technique would adapt well to the lactose synthetase system since α-lactalbumin is a small protein with several loop areas which could be synthesized with a reasonable effort. While it would be possible to do some of the preliminary experiments with purified peptic digests of α-lactalbumin the synthesis of peptides would give an added dimension to the variety of experiments possible.

Another protein upon which considerable research has been done on the molecular structure of protein-protein interactions is hemoglobin. It was the first protein containing more than one subunit for which X-ray analysis has reached a resolution down to 2.8 A (40, 41). Hemoglobin consists of four subunits, two α subunits and two β subunits. The packing of the polypeptide chains into the hemoglobin molecule is such that there is close, interlocking contact of side groups between unlike chains, but virtually no contact between α and α, or β and β. There are two kinds of unlike-chain contacts: between chains with neighboring hemes and between chains with widely separated hemes. There are a few hydrogen bonds and charged-group interactions, but the great majority of contact interactions are hydrophobic. When the heme groups are widely separated the subunit interface contacts are more extensive than between chains with neighboring heme groups, about 34 side chains being involved as compared with 19. The contact interface with fewer residues involved is also smaller in area and smoother thus

allowing substantial movement during oxygenation as compared to the larger interface which is relatively unperturbed during oxygenation.

With this structural information at hand, Perutz (42) has rationalized some of the effects of mutations on the quaternary structure of hemoglobin. For example, in hemoglobin Philly, replacement of tyrosine 35 in the β chain by phenylalanine is accompanied by increased dissociation to monomers. This tyrosine projects into the interior of the tetramer and forms a hydrogen bond with aspartic acid 126 of the chain. Phenylalanine cannot form such a hydrogen bond, since it lacks the hydroxyl group and hence a contact in the interface is weakened. About 100 abnormal human hemoglobins arising from point mutations are now known, many of which are single residue substitutions.

The complex of trypsin with its basic pancreatic inhibitor represents another model which offers a chance for investigating the interaction of two biologically active proteins whose primary structures are known (43, 44). The basic pancreatic inhibitor interacts stoichiometrically with trypsin to give competitive inhibition. The complex is very stable in alkaline media and has a dissociation constant of 2×10^{-10} (45).

Many chemical modification experiments have been performed on both the inhibitor and the enzyme to determine the functional groups involved in the interaction. Since a stable complex is formed, exclusion-type experiments in which modifying reagents can be blocked from reacting with the active groups can be run. For example, Spande and Witkop (46) have observed that when trypsin-pancreas inhibitor complex is oxidized with N-bromosuccinimide at pH 4.0, one tryptophan residue out of the four that would be oxidized in the native enzyme is protected

from oxidation. Steiner (47) found two to three tyrosine residues were protected from iodination. Pudles and Bachellerie (48) found that in the native enzyme histidine-46 was modified upon reaction with both diisopropylfluorophosphate (DFP) and 1-chloro-3-tosylamide-7-amino-2-heptanone (TLCK) with resultant loss of activity. They could find no interaction between DFP treated enzyme and native inhibitor. They were also able to show that the enzyme was fully protected from inactivation by either DFP or TLCK when bound to the inhibitor.

The sequence, position, and function of the disulfide bonds in the bovine pancreatic inhibitor have been investigated by Kress and Laskowski (49). One of the three disulfide bonds (between residues 14 and 38) can be reduced without damage to the other two, and with no loss of activity. However if the new sulfhydryl groups are carboxymethylated, activity is lost and the protein becomes prone to hydrolysis by trypsin. This experiment plus the elegant work on the antigentic loop in lysozyme leads to the conclusion that not only is there an essential group of amino acids involved in the binding domain but there is also an essential conformation. Thus, the critical point arises, are the changes in activity upon chemical modification due to the modification of the amino acid or are they due to secondary changes which result in a shift from the active conformation? Efforts to answer this question are presented in this dissertation.

Structural and Physical Properties of Various α-Lactalbumin

Research efforts led by Martin Kronman and Robert Hill have established bovine α-lactalbumin as the standard of comparison among

the various α-lactalbumins. Hill and coworkers (50, 51, 52) have determined the complete amino acid sequence of bovine α-lactalbumin by characterization of the tryptic, chymotryptic and peptic peptides isolated from enzymatic hydrolysates of the two unique fragments obtained on cleavage of S-aminoethyl α-lactalbumin with cyanogen bromide. There are 123 residues with amino-terminal glutamic acid and carboxyl-terminal leucine. The four disulfide bonds were determined by sequence analysis of disulfide-containing peptides after chromatography. One peptic peptide contained four half cysteinyl residues and required further degradation with thermolysin. The four disulfide bonds are formed through linkages between residues six and 120, residues 28 and 111, residues 61 and 77, and residues 73 and 91. Browne et al. (53) have proposed a possible three-dimensional structure of bovine α-lactalbumin based on main chain conformation of lysozyme. They cite similarities in molecular weights, amino acid compositions and sequences, amino and carboxyl terminal groups, and corresponding disulfide linkages to justify using lysozyme as a model for α-lactalbumin. They constructed a wire model of lysozyme and then modified it to accomodate the α-lactalbumin sequence by changing the side chains that differ in the two molecules and by rearranging the main chain to suit the proposed deletions. In general, homologous side chains were kept in the same orientation in the two models and, when different side chains were placed in the interior of the molecule, care was taken to orient the new side chain in such a manner that it occupied nearly the same position as the corresponding side chain in lysozyme.

Their conclusions were that the differences between the two molecules are compatible with their having similar conformations. They

saw mostly hydrophobic replacements for hydrophobic internal side chains and compensating changes in neighboring residues. In contrast, the replacements of external side chains appear generally to be uncoordinated. There are, however, a number of changes that cause the substrate binding cleft to be considerably shortened. This would be consistant for interaction with a disaccharide substrate although present evidence indicates α-lactalbumin acts by modifying the galactosyltransferase protein.

Bovine α-lactalbumin is a tightly folded globular protein, and is very stable to denaturation by heat (54, 55). The molecular weight calculated from the amino acid sequence (56) is 14,437. Determinations by sedimentation velocity gave molecular weights of 15,400 (57) and 14,900 (58) while light scattering indicated 16,500 (54).

Kronman and coworkers (59, 60, 61, 62, 63) have investigated structural changes at acid and alkaline pH. The most drastic conformational change occurs below pH 4.0 and is referred to as acid denaturation. Both ultracentrifuge (59) and titration curve experiments (60) indicate that acid denaturation involves molecular swelling and a tendency to associate and aggregate. They define association as a rapidly reversible formation of low molecular weight polymers such as dimers and trimers. Aggregation is a fully reversible, time dependent process which forms a much higher molecular weight product, greater than 300,000. At pH 5.24 and 6.0 there is a very weak association reaction and the aggregation reaction is not detectable except at very high protein concentrations. Above pH 10 another conformational change was found which also leads to an expansion of the protein but appears to be less drastic than that occurring at acid pH (61, 62, 63). No

aggregation reaction was observed but association was demonstrated occurring above pH 9.5 at protein concentration of 30 mg/ml. Thus the molecular sites for aggregation and association are not identical. Efforts have been made to correlate the degree of tryptophan exposure with the molecular swelling (64, 65) by use of the solvent perturbation method of difference spectrophotometry. They found no increase in exposure upon acid denaturation but there was an alteration in the environment of the tryptophan residues as evidenced by changes in emission and absorption spectra (66, 60, 64). Such measurements of tryptophan exposure cannot be made at high pH due to the interference of the tyrosine ionization.

Inman (67) has shown by X-ray diffraction spectroscopy that α-lactalbumin is an elongated parallelepiped which may occasionally be arranged in rosettes. The densities of the wet crystals are 1.213 and 1.210 g/cm^3. The partial specific volume determined by Gordon and Semmett (57) was 0.735 cm^3/g and that determined from the amino acid sequence by Gordon and Ziegler (68) was 0.729.

Kronman has reported an absorptivity of ($E^{1\%}_{280}$) of 20.1 for bovine α-lactalbumin in phosphate buffer (66).

Barman (69) has reported the presence of a minor component in highly purified α-lactalbumin which has the same amino acid composition but contains 11 to 12 sugar residues per molecule of protein. This glyco-α-lactalbumin was equally active as a lactose synthetase specifier protein.

Schmidt and Ebner (70, 71) have isolated and characterized α-lactalbumin from goats, sheep, pigs and humans. The ultraviolet spectra and amino acid composition of the proteins were similar, and the

molecular

molecular weights were all within the range of 14,500 $\pm$ 500. Variations were found in the electrophoretic mobilities on starch gel at both pH 3.3 and pH 8.6. N-terminal amino acids of the ruminant α-lactalbumins were glutamic acid and the nonruminant α-lactalbumins were lysines. The C-terminal amino acids were leucine for bovine, goat, sheep, and humans; methionine for pig, and glutamine for guinea pigs. Peptide maps of the various α-lactalbumins showed a general similarity, but there were definite differences in peptide sequence. The various human α-lactalbumin peptide maps were almost identical and the sheep and goat also showed a great similarity. Multiple forms were observed in the goat, pig, and sheep α-lactalbumins (71).

Blumberg and Tombs (72) have shown by paper electrophoresis that two distinct forms of α-lactalbumin are present in Fulani cattle. The fast moving band has been designated α-lactalbumin A and the slower moving band α-lactalbumin B. The α-lactalbumin B is typical of American and English cattle. These two types of α-lactalbumin have been found in a number of other cattle and are believed to correspond to two genetic variants A and B (73).

Mawal (74) found slight differences in the peptide maps of Indian bovine α-lactalbumin A, bovine α-lactalbumin B and buffalo α-lactalbumin. Gordon et al. (75) have since reported that α-A and α-B are identical in amino acid composition except that α-B has an arginine at position 10 where α-A has glutamic acid or glutamine.

Tanahashi et al. (76, 2) have shown that purified α-lactalbumin from eight different species have similar specific activities in the lactose synthetase system. However Ley and Jenness (77) reported different dissociation constants for six α-lactalbumins of different spe-

cies. They found that bovine galactosyltranferase had the highest affinity for bovine α-lactalbumin. Less affinity was found for other ruminants, goat and deer, with nonruminants, pig, rat, and human respectively less.

Very recently human α-lactalbumin has been the object of intensive studies. Preliminary studies on the purification and the sedimentation properties of human α-lactalbumin were reported by Johanson (78) and Maeno and Kiyosawa (79). Phillips and Jenness (80) reported an improved purification procedure, amino acid compositon, and a molecular weight of about 14,000 as determined from the sedimentation velocity. Barel et al. (81) have studied the physiochemical characteristics of human α-lactalbumin and compared it to human lysozyme. A molecular weight of 14,900 $\pm$ 1500 was estimated from the sedimentation velocity while a molecular weight of 14,580 was calculated from the amino acid composition.

Findlay and Brew (82) have reported the complete amino acid for human α-lactalbumin. Comparison with bovine α-lactalbumin shows an identity in 72% of the residues with a further 6% being chemically similar amino acids. The N-terminal residue of human α-lactalbumin is lysine, thus differing from bovine α-lactalbumin which has an N-terminal glutamyl residue. The C-terminal residues are identical with both being leucine. The amino acid sequence for bovine, human and guinea pig are presented in Table I (83).

The percent helical structure of human α-lactalbumin was less than bovine α-lactalbumin (42% compared to 50%) when calculated from the optical rotation (81). The circular dichroism spectra of human α-lactalbumin is in general accord with that reported by Kronman (84) and

TABLE I

AMINO ACID SEQUENCES OF α-LACTALBUMINS FROM THREE DIFFERENT SPECIES

	10 20
BαLA[1]	Glu-Gln-Leu-Thr-Lys-Cys-Glu-Val-Phe-Arg-Glu-Leu-Lys-Asp-Leu-Lys-Gly-Tyr-Gly-Gly-Val-Ser-Leu-Pro-Glu
HαLA[2]	Lys-Gln-Phe-Thr-Lys-Cys-Glu-Leu-Ser-Gln-Leu-Leu-Lys-Asp-Ile-Asp-Gly-Tyr-Gly-Gly-Ile-Ala-Leu-Pro-Glu
GPαLA[3]	Lys-Gln-Leu-Thr-Lys-Cys-Ala-Leu-Ser-His-Glu-Leu-Asn-Asp-Leu-Ala-Gly-Tyr-Arg-Asp-Ile-Thr-Leu-Pro-Glu
	30 40 50
BαLA	Trp-Val-Cys-Thr-Thr-Phe-His-Thr-Ser-Gly-Tyr-Asp-Thr-Glu-Ala-Ile-Val-Glu-Asn-Asn-Gln-Ser-Thr-Asp-Tyr
HαLA	Leu-Ile-Cys-Thr-Met-Phe-His-Thr-Ser-Gly-Tyr-Asp-Thr-Gln-Ala-Ile-Val-Glu-Asn-Asp-Gln-Ser-Thr-Glu-Tyr
GPαLA	Trp-Leu-Cys-Ile-Ile-Phe-His-Ile-Ser-Gly-Tyr-Asp-Thr-Gln-Ala-Ile-Val-Lys-Asn-Ser-Asn-His-Lys-Glu-Tyr
	60 70
BαLA	Gly-Leu-Phe-Gln-Ile-Asn-Asn-Lys-Ile-Trp-Cys-Lys-Asn-Asp-Gln-Asp-Pro-His-Ser-Ser-Asn-Ile-Cys-Asn-Ile
HαLA	Gly-Leu-Phe-Gln-Ile-Ser-Asn-Lys-Leu-Trp-Cys-Lys-Ser-Ser-Gln-Val-Pro-Gln-Ser-Arg-Asn-Ile-Cys-Asp-Ile
GPαLA	Gly-Leu-Phe-Gln-Ile-Asn-Asn-Lys-Asp-Phe-Cys-Glu-Ser-Ser-Thr-Thr-Val-Gln-Ser-Arg-Asp-Ile-Cys-Asp-Ile
	80 90 100
BαLA	Ser-Cys-Asp-Lys-Phe-Leu-Asn-Asn-Asp-Leu-Thr-Asn-Asn-Ile-Met-Cys-Val-Lys-Lys-Ile-Leu-Asp-Lys-Val-Gly
HαLA	Ser-Cys-Asp-Lys-Phe-Leu-Asn-Asp-Asn-Ile-Thr-Asn-Asn-Ile-Met-Cys-Ala-Lys-Lys-Ile-Leu-Asp-Ile-Lys-Gly
GPαLA	Ser-Cys-Asp-Lys-Leu-Leu-Asn-Asp-Asn-Leu-Thr-Asn-Asn-Ile-Met-Cys-Val-Lys-Kys-Ile-Leu-Asp-Ile-Lys-Gly
	110 120
BαLA	Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys-Ala-Leu-Cys-Ser-Glu-Lys-Leu-Asp-Gln-Trp-Leu-Cys-Glu-Lys-Leu
HαLA	Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys-Ala-Leu-Cys-Thr-Glu-Lys-Leu-Glu-Gln-Trp-Leu-Cys-Glu-Lys-Leu
GPαLA	Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys-Pro-Leu-Cys-Ser-Asp-Lys-Leu-Glu-Gln-Trp-Tyr-Cys-Glu-Ala-Gln

[1] Bovine α-lactalbumin

[2] Human α-lactalbumin

[3] Guinea pig α-lactalbumin

Robbins and Holmes (85) for bovine α-lactalbumin. Exact comparisons between the two circular dichroism spectra of human α-lactalbumin published by Cowburn et al. (83) and Barel et al. (81) are difficult to make due to the small scale of the figures but they are qualitatively the same. Cowburn et al. (83) analyzed the circular dichroism of four α-lactalbumins and four lysozymes for which the amino acid sequence is known. All have multiple cotton effects in the near-ultraviolet region, associated with the absorption bands of the aromatic amino acids. They conclude that the large negative Cotton effect arises largely from tyrosine residues. All the proteins show evidence of a positive feature at about 295 nm, which must arise from tryptophan residues. Calculations have been made from the circular dichroism spectra using the method of Greenfield and Fasman (86) to determine the percent of α-helix as reported by Robbins and Holmes (85). Upon swelling at both acid and basic pH the percent α-helix increased to 36%. They found the β pleated sheet decreased from 14% to 4% upon swelling and that unordered structure remained constant at 60%. Differences between the values for percent α-helix from the Moffitt equation and from the method of Greenfield and Fasman (86) utilizing circular dichroism spectra are most likely due to the extrapolation necessary to evaluate the B_o term in the Moffitt equation. Thus 26% α-helix is probably the more accurate estimate; however, because of uncertainties in the dichroic properties of model conformations which can exist in proteins, and the empirical nature of the methods used for estimating their contribution, the final answer resides in the X-ray crystallographic analysis.

Immunological Properties of Various α-Lactalbumins

Evidence from immunological studies and lactose synthetase activity measurements indicate that the immunological determinant is a different site from the binding domain which is responsible for the modifier activity of α-lactalbumin. Tanahashi et al. (76) found no cross reaction between bovine α-lactalbumin antisera and α-lactalbumin isolated from pig, guinea pig, and humans. The ruminant α-lactalbumins (bovine, buffalo, sheep, and goat) all react with antibodies to bovine α-lactalbumin.

Both ruminant and nonruminant α-lactalbumin are active in the lactose synthesis reaction.

McFarland and Ebner (87) have found that α-lactalbumin isolated from mouse and rat milk does not react with bovine antisera. They found one preparation of α-lactalbumin isolated from rat mammary tissue which did react with bovine and human antisera. Efforts are now underway to confirm these results and to investigate the mechanism by which the differences between the rat milk and rat tumor α-lactalbumin arise. They also found that bovine α-lactalbumin when modified with N-acetylimidazole or tetranitromethane was still reactive with the bovine antisera. Thus neither the tyrosine residues or the lysine residues are antigentic determinants.

Modification of α-Lactalbumin as Related to Structure and Function

Gorbunoff (88) was the first investigator to make an indepth study of α-lactalbumin using chemical modification techniques. She attempted to measure the reactivity of the tyrosine residues with cyanuric fluoride, a tyrosine specific reagent. Cyanuric fluoride is a very reactive aryl halide. Cyanuration proceeds by a nucleophilic displacement, in which the tyrosine oxygen is the nucleophile (89). The product is an ether linkage between the two aromatic rings.

In theory the spatial requirements of the transition state complex will be such that only tyrosines on the surface of the protein will react. Upon raising the pH the α-lactalbumin molecule swells and more tyrosine residues become reactive to cyanuric fluoride. Thus the number of tyrosine residues on the surface of the protein can be compared with the number of residues buried within the protein. At the time this work was done (1967) it was thought that the α-lactalbumin contained five tyrosines groups, thus her interpretation of the data must be reviewed. She reported four residues reactive to cyanuric fluoride and one unreactive residue. Of the four reactive residues, three were accessible in the native state, and the fourth accessible only upon treatment with high pH. No change in reactivity was found at 3°. Therefore in α-lactalbumin a change in temperature does not induce any significant changes in the protein conformation neighboring to the tyrosine residues. The buffer for the reactions is 1M $KHCO_3$ containing 10% dioxane, which may cause the protein to differ in conformation from its native state. No activity measurements were made on the reaction products.

The nitration of α-lactalbumin with tetranitromethane was first reported by Attassi et al. (90). Sokolovsky et al. (91) have shown that the nitration of tyrosine residues occurs readily under mild conditions, with only a few important side reactions. Treatment of proteins with tetranitromethane can result in: a) conversion of tyrosine to 3-nitrotyrosine (91); b) conversion of tryptophan to 7-nitrotryptophan (92); c) oxidation of sulfhydryl groups to disulfides (91); and d) formation of intramolecular crosslinkages between tyrosine residues (93).

Habeeb and Atassi (94) report the nitration of 2.51 $\pm$ 0.03 tyrosine residues and 1.4 tryptophan residues. They report 1.50 tyrosine residues remaining after nitration for a total of four tyrosine residues. Thus they found no evidence for crosslinked α-lactalbumins. In contrast Robbins et al. (95) nitrated α-lactalbumin with tetranitromethane and reported that both tyrosine and tryptophan are modified with loss of activity. All four tyrosines could be modified, but two were more reactive than the others. Denton and Ebner (6) confirmed that both tyrosine and tryptophan were modified with loss of activity. They correlated the loss of activity with the loss of tyrosine on a time basis and established that inactive dimers and other polymers of α-lactalbumin were formed during the course of the reaction. Tryptic peptide mapping shows that all four tyrosines are randomly modified (96).

Klee and Klee (27) also report the nitration of α-lactalbumin with tetranitromethane in which spectral determinations showed three to four tyrosine residues had been nitrated. Amino acid analysis showed the balance of the four tyrosines as free tyrosine, and no evidence

for a crosslinked product. They reported a lowered affinity for the nitrated α-lactalbumin but found it could substitute for native α-lactalbumin in enzyme assays.

Denton and Ebner (6) have investigated the effect of iodination on the activity of α-lactalbumin. Covelli and Wolff (97) have shown that both tyrosine and histidine are highly reactive to I_3^-, with mono- and diiodinated products resulting. Tryptophan has also been reported to be reactive (98). All three amino acids were iodinated in α-lactalbumin with the loss of activity again corresponding to the loss of tyrosine. Iodination of α-lactalbumin at an I_2/α-lactalbumin molar ratio of 40/1 results in the modification of 1.1 of the tryptophan residues, 1.0 histidine residue, and 3.6 of the tyrosine residues. As in nitration, the iodination of α-lactalbumin also results in the formation of inactive polymers. In both nitration and iodination it is suggested that after the modification of one tyrosine residues, α-lactalbumin undergoes a change in shape which increases the apparent molecular weight as determined by gel filtration.

Lin (99) modified the carboxyl groups of α-lactalbumin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and glycinamide. There was a rapid loss of lactose synthetase activity and an average of 20 carboxyl groups were modified in 400 minutes. No unique carboxyl group was found, although free carboxyl groups in α-lactalbumin may be essential for maintaining its biological activity. Partial protection could be achieved by binding with the A protein.

Castellino and Hill (100) have examined the reaction of iodoacetate with bovine α-lactalbumin. Iodoacetate is a widely used alkylating reagent. It reacts very rapidly with free sulfhydryls and more

slowly with histidine and methionine, and with amino groups at very high pH (7). The reaction with α-lactalbumin was found to carboxymethylate a single methionine and three histidine residues. Examination of tryptic peptides indicated that methionine 90 was the most reactive residue with histidine 68, histidine 32, and histidine 107 reacting at slower rates respectively. The relative rates of reaction were predicted on the basis of their exposure to reagent.

Carboxymethylation of methionine 90 has little effect on the activity of α-lactalbumin, but there is a progressive loss in activity as the histidine residues are carboxymethylated. When all three histidine residues are modified about 40% of the activity of α-lactalbumin remained.

Barman (101, 102) has investigated the reaction of 2-hydroxy-5-nitro-benzyl bromide with α-lactalbumin. He had earlier reported that the reagent was specific for tryptophan but other investigators have reported reaction with cysteine, tyrosine and α amino groups (103, 104). Mono- and disubstituted tryptophan was found depending on the molar ratio of reagent to protein. Investigation of the reaction was made at pH 2.7 and at pH 6.0 at low molar ratios of reagent to protein. At pH 2.7, three of the four tryptophan residues reacted, namely tryptophans 26, 104 and 118 and each gave rise to monosubstituted isomers and a limited yield of disubstituted isomers. No evidence was found for cross-linking.

At pH 6.0, the reaction was more complex. The three tryptophans were labeled but in addition, histidine 32 was modified and about one third of the incorporated reagent could be eliminated by incubation under mild conditions. It was proposed that the modification of his-

tidine 32 was due to special properties conferred by neighboring groups in the α-lactalbumin molecule since no reaction could be observed on free histidine. A yet to be published paper will report the effect of modification with 2-hydroxy-5-nitrobenzyl bromide on the modifier activity of α-lactalbumin.

Kronman et al. (105) have studied the acetylation of α-lactalbumin with N-acetylimidazole, a reagent selective for tyrosine and amino groups (7). They found that all four tyrosyl residues were acetylated, two were acetylated at a more rapid rate than the third, which was more reactive than the fourth. Determination of free amino groups after acetylation showed that the number of amino groups acetylated varied with the molar ratio of N-acetylimidazole to protein. A plateau was observed after about four residues and then increased rapidly to eight to nine residues. They interpreted this as a conformational change which renders the ε-amino groups more susceptible to reaction. They found all amino groups were acetylated at molar ratios in excess of 160 to 1, but they found no alterations of the structure by solvent perturbation measurements. They found a sedimentation constant comparable to that of acid-treated protein, indicating swelling of the molecule had occurred. Measurement of the circular dichroism spectra of acetylated α-lactalbumin revealed small changes from the native α-lactalbumin spectrum. The spectrum was shifted in the same manner as the spectrum of the acid-denatured α-lactalbumin, but the shift was not as great in magnitude. No activity measurements were made on the acetylated protein.

Tamburro et al. (106) have reported the effects of various modifications on the conformation of bovine α-lactalbumin. The changes in

conformation were monitored by circular dichroism and light absorption spectroscopy. Oxidation of the unique methionyl residue by irradiation in the presence of hematoporphyrin resulted in flexibility of the three-dimensional structure. Irradiation of α-lactalbumin in the presence of proflavin resulted in the selective oxidation of three of the four tryptophyl residues as shown by amino acid analysis. Two of the three reactive tryptophyl residues were oxidized at a greater rate than the third. A time dependance decrease in the rotational strength of the near-ultraviolet dichroic spectrum was observed which resembled that of denatured α-lactalbumin. It was postulated therefore that the difference in spectrum was due to a limited conformational rearrangement rather than the degradation of the tryptophyl residues. Reduction of the disulfide bonds leads to a complete loss of the peak present at 252 nm, but reoxidation of the disulfide bridges leads to a recovery of the original spectrum.

Enzymatic Modification of Proteins

In theory the use of an enzyme to catalyze the modification of proteins is an ideal system. An enzymatic system has the advantages of (a) very mild conditions with respect to temperature, pH and solvent system; (b) specificity of reaction, although not always true; (c) the inability to penetrate the protein surface (thus residues in crevices, or in the inner core of the protein are not modified); and (d) the rate of reaction can be easily controlled.

Hill et al. (50, 51, 52) have used the enzymes, trypsin, chymotrypsin, and thermolysis to cleave α-lactalbumin into specific peptides

for the determination of the amino acid sequence. This use illustrates the value of a well characterized enzyme modification system.

In 1957 Yasunobu and Dandliker (107) reported the oxidation of α-lactalbumin by tyrosinase and that about 75% of the tyrosines were oxidized as determined by amino acid analysis. Denton and Ebner (6) have found conflicting results with the use of tyrosinase on α-lactalbumin. They found no loss of tyrosine after tyrosinase treatement for 300 minutes at 37°. They did find that tyrosinase oxidizes 1.1 to 1.5 residues of tryptophan in α-lactalbumin. The effect on the activity of α-lactalbumin was very slight. Yasunobu and Dandliker (107) may have had an impure preparation of tyrosinase which contained a proteolytic enzyme, thus the tyrosines may have been oxidized after partial degradation of the α-lactalbumin.

Lactoperoxidase has been used by Phillips and Morrison (108) to study the vectorial arrangement ofproteins in the intact human erythrocyte. Lactoperoxidase catalyzes iodide incorporation into the exposed tyrosine and histidine groups on a protein. The predominant reaction is the iodination of the aromatic ring of tyrosine in either or both positions ortho to the hydroxyl group. The reaction involves three substrates; peroxide, iodide, and the phenolic compound which is iodinated. Kinetic studies (109) have established that a ping-pong mechanism is involved.

The use of lactoperoxidase for modifying α-lactalbumin will be discussed in this thesis.

CHAPTER III

MODIFICATION OF α-LACTALBUMIN

Experimental Procedure

Materials and Reagents

TPCK treated trypsin, pyruvate kinase (Type I from rabbit muscle, crystalline ammonium sulfate suspension containing lactic dehydrogenase), glyclyglycine, phosphoenolpyruvate (PEP), NADH, Tris (tris-hydroxymethylaminomethane), bovine serum albumin and N-acetylimidazole were purchased from Sigma Chemical Company. Lactoperoxidase (grade B), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and Pronase (grade A) were purchased from Calbiochem. Trinitrobenzene sulfonic acid (picryl sulfonic acid) and tetranitromethane were purchased from Aldrich Chemical Company. Precoated TLC plates 0.1 mm thickness were purchased from Brinkman Instruments, Inc. Disc gel electrophoresis reagents were obtained from Canalco. UDP-galactose was purchased from California Biochemicals. Size 18 cellulose casing was purchased from Union Carbide and boiled and rinsed before use. Bio-Gel P was purchased from Bio-Rad Laboratories and Sephadex G from Pharmacia. Ultrapure urea was purchased from Mann Chemical Company. DEAE-cellulose was purchased from Whatman (DE-32). N-acetylimidazole and ethylene amine were obtained from Pierce Chemical Company. Disodium ethylenidiamine tetraacetate (EDTA) was purchased from Fisher Scientific Company. Hydrogen peroxide

was purchased from Mallinckrodt and hydroxylamine from Baker Chemical. Special Agar-Noble was purchased from Difco Labs and antibodies were purchased from Antibodies Incorporated. ^{125}I was obtained fresh as a carrier free solution of $Na^{125}I$ from New England Nuclear. Standard p-nitrophenyl acetate for the acetyl group determination was prepared from the sodium salt of p-nitrophenoxide, by acidifying and refluxing with excess acetic anhydride for one hour. It was recrystallized twice from 50% ethanol.

Fresh skim milk was obtained from the Oklahoma State University Dairy and α-lactalbumin was isolated by acid precipitation (109) and chromatography on Bio-Gel P-30 and DEAE cellulose (2). The final product exhibits a single band (100 μg) on disc gel electrophoresis.

Bovine galactosyltransferase was prepared according to the procedure described by Fitzgerald et al. (20) and the product was obtained as an ammonium sulfate solution of partially purified galactosyltransferase from the HA_I step of the purification.

Spectrophotometric Assay for α-Lactalbumin

α-Lactalbumin was assayed in the presence of saturating amounts of galactosyltransferase (20). One unit of enzymatic activity is defined as that amount of enzyme which catalyzes the formation of one nanomole of UDP per minute and is equivalent to a change of 0.0062/min/ml at A_{340}. Enzymatic activity was assayed spectrophotometrically at 340 nm by coupling UDP formation to NADH oxidation by adding PEP and pyruvate kinase. Reaction rates were measured on a Cary Model 14 spectrophotometer. Assays were prepared in a final volume of 1.0 ml and contained

0.15 mM NADH, 1.0 mM PEP, 0.05 ml of 1 to 10 dilution of pyruvate kinase (Sigma, Type I, 25 mg protein/ml, with 2.4 IU pyruvate kinase/mg protein), 5 mM glyclyglycine, pH 8.5, 5 mM $MnCl_2$, 0.25 mM UDP-galactose, 20 mM glucose, and approximately 25 units of galactosyltransferase. An endogenous rate with only galactosyltransferase was determined for each assay. Standard curves for α-lactalbumin were determined for each group of assays.

Gel Filtration Methods

Glass tubes of the desired length and diameter were fitted with glass frits and Luer tips and silanized with a one percent solution of dichlorodimethyl silane in benzene prior to packing. Gels were swollen, deaerated and packed in the columns according to the BioRad gel filtration manual (110). Sucrose was added to samples (10% by weight) to permit layering of the sample on top of the gel just below the eluant. Blue dextran (10 mg/ml) was used to determine the void volume and the uniformity of packing.

Gel Electrophoresis Method

Standard seven percent, pH 9.5, separating gel solution was prepared according to Canalco specifications (111). Glass tubes (0.5 cm x 6 cm) were filled with gel solution, layered with distilled water and allowed to polymerize in the dark for 30 minutes at room temperature. Gels were preelectrophoresced for 30 minutes at a constant current of five milliamps per gel. Protein solutions were made 10% in sucrose and layered directly on the separating gel. Sample size did not exceed 60 μl, and sample concentration was not less than 0.5 mg/ml. Gels were

electrophoresed in a Canalco electrophoretic apparatus at five milliamps per gel until the tracking dye (0.005% Bromophenol blue in water) reached the end of the gel (about 22 minutes).

The gels were stained for four hours or overnight with 0.5% Aniline Blue Black in 7% acetic acid. They were electrophoretically destained at 0.5 amperes until the background color was gone.

Method for Acetylation with N-Acetylimidazole

Acetylation was performed according to the procedure of Riordan and Vallee (112). Acetylation was performed at room temperature by addition of a weighed amount of N-acetylimidazole to a solution of α-lactalbumin in buffer at pH 7.5 while stirring. Identical results were obtained with 20 mM Tris or 50 mM sodium borate buffer.

N-acetylimidazole is very hygroscopic and must be stored over a desicant at 4° in a tightly sealed container. It is very unstable at extremes of pH, and readily undergoes spontaneous decomposition at neutral pH in water solutions. For spectral and circular dichroism measurements the excess reagent was removed by dialysis or gel filtration.

De-O-acetylation was accomplished by making an aliquot of the acetylated protein 0.2 M in hydroxylamine at pH 7.5 for varying lengths of time at ambient temperature. Other procedures gave unsatisfactory results.

Method for Acetylation with Acetic Anhydride

Acetylation with acetic anhydride was performed by the method of Riordan and Vallee (112). A solution of α-lactalbumin in 0.15 M KCl

was adjusted to pH 7.5 and maintained on a pH stat at 4°. Acetic anhydride was added with a syringe at a slow rate, while stirring. The pH was maintained constant by the automatic addition of 1.0 N NaOH. The reaction was judged to be complete when the alkali uptake ceased, approximately 30 minutes. Excess reagent was removed by dialysis or gel filtration. De-O-acetylation was accomplished as stated earlier.

Determination of O-Acetyltyrosine

The number of acetylated tyrosines was determined by the method of Balls and Wood (113). Deacetylation of the acetylated α-lactalbumin was carried out at room temperature by mixing equal volumes of the protein solution and 2 M hydroxylamine at pH 7.5 for 10 minutes. The liberated acethydroxamate is determined spectrophotometrically after reaction with 10% ferric chloride. Filtration is necessary before reading the absorbance at 540 nm. A standard curve was prepared using p-nitrophenolacetate over a concentration range of 0.25 to 3.0 μmoles.

Determination of Acetylated Amino Groups

Unacetylated amino groups in α-lactalbumin were determined by reaction with trinitrobenzene sulfonic acid (TNBS) by the method of Kakade and Leiner (114). One mg of lyophilized acetylated α-lactalbumin was dissolved in 1 ml of 4% ammonium bicarbonate pH 8.5. One ml of freshly prepared 0.1% TNBS in water was added and allowed to react for two hours in a shaking water bath at 40 . Three mls of concentrated HCl was then added and the samples were autoclaved for one hour. After extracting twice with ether the absorbance was measured at 346 nm. The number of amino groups was calculated from an extinction coefficient of

1.46×10^4. The number of acetylated (blocked) amino groups was determined by the difference between control α-lactalbumin and acetylated α-lactalbumin. Nine free amino groups were found per molecule of control α-lactalbumin.

Determination of Sulfhydryl Groups

Free sulfhydryl groups were measured by the method of Ellman (115) using 5, 5'dithiobis(2-nitrobenzoic acid) (DTNB). Three mls of α-lactalbumin, 1 mg/ml, in 0.1 M phosphate buffer pH 8.0 was treated with 0.02 ml of stock DTNB solution. After 10 minutes the absorbance at 412 nm was recorded. The number of sulfhydryl groups was calculated from an extinction coefficient of 11,400 (123). The stock solution of DTNB contained 39.6 mg of DTNB in 10 ml of 0.1 M phosphate buffer pH 7.0.

Peptide Mapping Procedure

The method of Jones (116) was used to prepare α-lactalbumin for peptide mapping. The protein was reduced in urea with β-mercaptoethanol and amino ethylated with ethylenimine. De-salting was performed on a Sephadex G-25 column (35 x 2.2 cm) using 0.2 M acetic acid as the eluent. The sample was then evaporated to dryness, dissolved in 0.2 M ammonium bicarbonate pH 8.5 and digested with trypsin using a 1 to 75 enzyme to protein ratio.

The digested solution was spotted directly on the TLC chromatograms. Commercial thin layer chromatograms, 20 x 20 cm, having a 300 MN cellulose thickness of 0.10 mm were used. Whatman 3 mm wicks were washed with one percent acetic acid and then with the electrophoresis

buffer. This was to remove any ninhydrin positive impurities (117). An aliquot of the digested solution was spotted at a corner of the TLC plate (118). The electrophoresis buffer containing pyridine, acetic acid, and water (100:30:3000), pH 5.5 was sprayed onto the plate until the surface was damp. The plate was immersed in the Buchler Universal Electrophoresis Cell, 24.5 x 25 cm. The cell, maintained at 4^{o}, contained the electrophoresis buffer with an over-layer of varsol, used as a coolant. Paper wicks, extending into the buffer, were placed on each end of the plate. Glass rods, ground to give a flat surface, were placed on the wicks to insure intimate contact with the thin-layer plate. After electrophoresis for two hours at 300 volts the plate was dried with warm air not exceeding 30^{o}. This was followed by equilibration in the chromatography tank, and then chromatography in the second dimension chromatography buffer, n-butanol, pyridine, glacial acetic acid, and water (150:100:30:120). The plate was removed when the buffer reached the top of the plate, which takes approximately 10 hours and 30 minutes. The peptides were detected with spray consisting of ninhydrin, 50 mg; ethanol, 30 ml; glacial acetic acid, 10 ml; and collidine, 4 ml (118). The spots were developed in an 80^{o} oven for 5-10 minutes and then were fully developed at room temperatures for approximately 12 hours (117, 118). Tracings were made of the peptide maps which were then wrapped in saran wrap and stored in the refrigerator. ^{125}I labelled peptides were detected by exposing the plates to Supreme MonoPak X-ray film for two weeks.

Immunological Titration

The Oudin technique as described by Larson and Hageman (119) was used to quantitate α-lactalbumin after incubation at high pH. A one percent solution of agar was melted and mixed with antibodies to α-lactalbumin at 47° and placed in glass tubes (0.2 x 10 cm) to solidify. The antibodies were diluted 1-10 with Phosphate buffer pH 7.0. A standard curve was prepared from a stock solution of α-lactalbumin (10--100 mg/ml). The precipitin band was measured with a Vernier caliper at 25 and 36 hour intervals.

The band width (mm) was divided by the square root of the number of hours and plotted versus the protein concentration on semi-logrithmetic paper.

Iodination Procedure Using Lactoperoxidase

Lactoperoxidase was used to iodinate α-lactalbumin according to the procedure used by Morrison and Bayse (8). α-Lactalbumin solutions were made in 0.05 M phosphate buffer, pH 7.4 containing 90 μM potassium iodide and 1.0 mM EDTA. Hydrogen peroxide and lactoperoxidase were always added separately and control experiments were made by determining the ratio in the absence of either hydrogen peroxide or lactoperoxidase. Hydrogen peroxide concentrations were determined from the absorbance at 230 nm using a molar extinction coefficient of 72.4. Hydrogen peroxide was added from a 11 mM solution to give a final concentration of 100 μM in the reaction mixture.

Lactoperoxidase concentration was determined from a millimolar extraction coefficient of 114 at 412 nm. Stock solutions of lactoperoxidase (1 mg/ml) were kept at 4° for periods of up to one month without

inactivation. Dilute solutions are less stable (8). One μl of the stock solution was normally used per ml of reaction volume to give a final lactoperoxidase concentration of 13.0 nM. The reaction was stopped and the free iodide was separated from the protein on a Sephadex G-25 column (2.2 x 35 cm). The elution buffer was 0.1 M ammonium bicarbonate. Two radioactive peaks were observed, one peak corresponding to α-lactalbumin and the other corresponding to sodium chloride.

Circular Dichroism Measurements

Circular dichroism measurements were made at ambient temperatures on a Cary model 61 spectropolarimeter with one and 10 mm cells. Protein concentrations and path lengths were chosen to yield absorbances no greater than 1.4 at any wavelength. The solvent used for all spectra was 0.15 M KCl pH 7.0. Baseline spectra were run before and after each group of spectra and appeared very stable. Repetive spectra of control α-lactalbumin were run with each group of spectra to insure reproducibility.

The instrument was calibrated according to section four of the Cary Model 61 CD Spectropolarimeter Instruction Manual (120) using an aqueous solution (0.1%) of d-10-camphorsulfonic acid (121). The operating instructions of the manual were followed closely for all spectra. The maximum scan speed was calculated by dividing the spectra bandwidth by the period. The scan speed was never greater than the value of one spectral bandwidth per period.

The data is expressed as mean residue molar ellipticity, $[\theta]$. A mean residue weight of 118 was used.

Results

Modification of α-Lactalbumin with N-Acetylimidazole

α-Lactalbumin was acetylated to assess the functional roles of tyrosine and lysine in the lactose synthetase reaction. Modification with N-acetylimidazole or acetic anhydride results in the addition of an acetyl group to the hydroxyl group of tyrosines and an N-acetyl group to the ε-amino group of lysines. Since the O-acetyl group of tyrosine readily undergoes hydrolysis to regenerate tyrosine, it is possible to assess the functional role of tyrosine and lysine separately, by selective deacetylation. Deacetylation of the O-acetyl group is catalyzed by hydroxylamine at pH 7.5 or by hydroxyl ions at high pH.

The effect of acetylation on the activity of α-lactalbumin in the lactose synthetase reaction was monitored as a function of time at two ratios of N-acetylimidazole to protein (Figure 2). The activity of α-lactalbumin in the lactose synthetase system decreases with time as more residues are acetylated. At 500-1 reagent to protein ratio the loss of activity is more rapid than at the 40-1 ratio. Upon treatment with hydroxylamine at low concentration (0.2 M) the activity of α-lactalbumin slowly returns as shown in Figure 3. Treatment of acetylated α-lactalbumin with 2 M hydroxylamine for 30 minutes causes a loss of activity. Control experiments in which native α-lactalbumin (1 mg/ml) was treated with 2 M hydroxylamine for 30 minutes at pH 7.5 showed a loss of 38% of the enzymatic activity (Table II).

Incubation of acetylated α-lactalbumin at pH 12 to deacetylate the tyrosines resulted in a small regain of activity that did not increase with time. α-Lactalbumin incubated for 30 and 60 minutes at

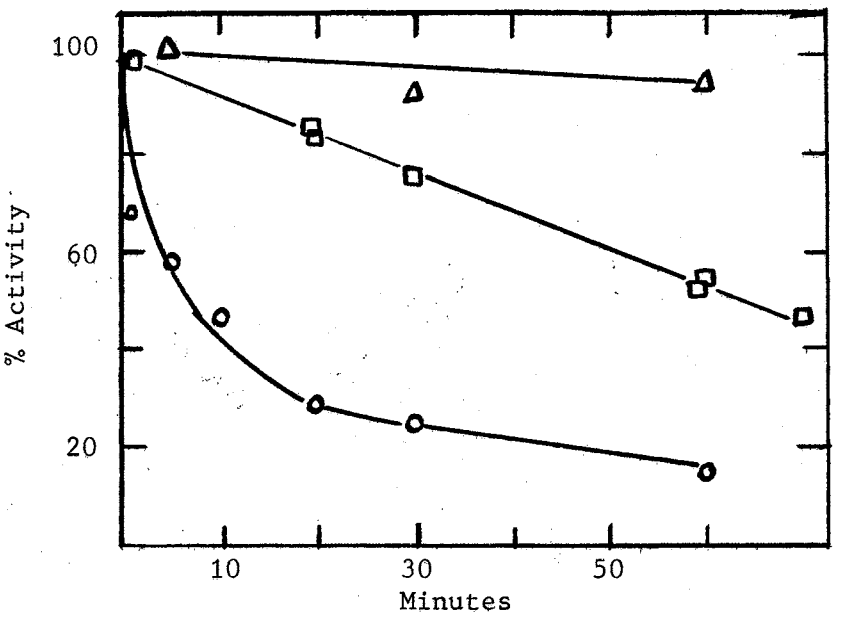

Figure 2. Loss of α-lactalbumin Activity Upon Reaction with N-Acetylimidazole

Time course of acetylation of α-lactalbumin as measured by the effect on lactose synthetase activity. 100% Activity equals 820 nmoles of UDP formed per minute per mg of α-lactalbumin. (o) Acetylation at 500:1, reagent to protein. (□) Acetylation at 40:1, reagent to protein. (△) Reaction control, no N-acetylimidazole.

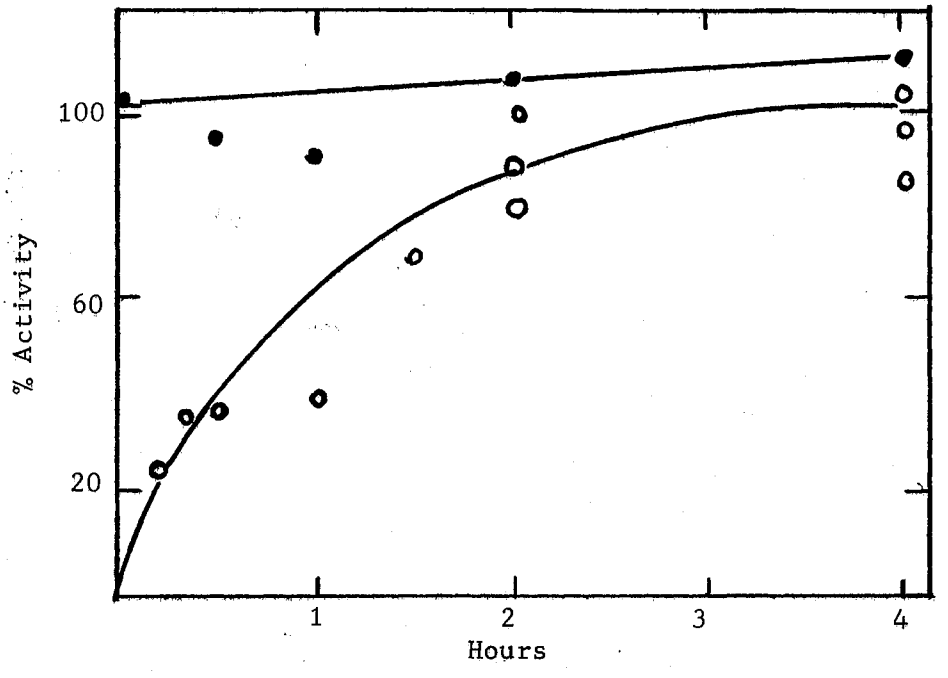

Figure 3. Effect of Deacetylation on α-Lactalbumin Activity

The effect of 0.2 M hydroxylamine pH 7.5 at 25 on the activity of acetylated α-lactalbumin, o-o, and native α-lactalbumin, ●-●. 100% Activity equals 820 nmoles of UDP formed per minute per mg of α-lactalbumin.

pH 12 lost activity. These undesirable complications were eliminated by deacetylating the tyrosyl residues with 0.2 M hydroxylamine at pH 7.5, which caused no detectable changes in the activity of control α-lactalbumin.

TABLE II

EFFECT OF DEACETYLATION METHODS ON THE ACTIVITY AND RE-ACTIVATION OF α-LACTALBUMIN

Treatment	% Activity Lost by Control	% Activity of Acetyl-ated α-LA	% Activity After De-O-Acetylation	% of Activity Regained
2M NH_2OH[1] for 30 min. at pH 7.5	38	26	20	-6
pH 12 for[2] 30 min.	39	34	47	13
pH 12 for[3] 60 min.	60	42	47	5
0.2M NH_2OH[4] for 30 min. at pH 7.5	0	14	65	51

[1] 100% Activity equals 940 nmoles of UDP formed per minute per mg of α-lactalbumin.

[2] 100% Activity equals 565 nmoles of UDP formed per minute per mg of α-lactalbumin.

[3] 100% Activity equals 670 nmoles of UDP formed per minute per mg of α-lactalbumin.

[4] 100% Activity equals 820 nmoles of UDP formed per minute per mg of α-lactalbumin.

At this level of hydroxylamine the deacetylation reaction should be complete at about 150 minutes (112). The experimental data shown in Figure 3 agreed well with this value.

Table III shows the results of acetylation and deacetylation of the tyrosyl residues as measured by the colorimetric hydroxamate procedure of Balls and Wood (113). This method is superior to the spectral procedure utilizing the change in absorbance at 280 nm (105). Also shown in Table III is the number of amino groups acetylated at the two different ratios of N-acetylimidazole to protein. The number of unacetylated amino groups was determined from the absorbance at 345 nm after reaction with trinitrobenzene sulfonic acid (TNBS). Calculation of the number of reactive amino groups in native α-lactalbumin after reaction with TNBS using an extinction coefficient of 1.46×10^4 (114) gave a value of 9.1 ε-amino groups. The number of amino groups acetylated at a 40-1 ratio of N-acetylimidazole to α-lactalbumin agrees exactly with value found by Kronman et al. (105). Unfortunately their maximum reagent to protein ratio was 160-1, so no comparison can be made at the 500-1 level.

In order to show a direct correlation between the loss of activity of α-lactalbumin and the number of tyrosyl residues acetylated, appropriate ratios of N-acetylimidazole to protein were chosen to give 1, 2, 3, and 4 residues acetylated in one hour. The results of this study are shown in Table IV. The loss of activity corresponds closely with number of acetylated tyrosine residues. It was not possible to completely inactivate α-lactalbumin by acetylation at 500-1, reagent to protein ratios. Riordan and Vallee (112) suggest that the spontaneous hydrolysis of the N-acetylimidazole in water effectively lowers the

concentration of the reagent. If this were the case it would be expected that the reaction would slow down with time and this appears to happen in the 500-1, reagent to protein ratio as shown in Figure 2. There would also be a reduction in rate as the number of reactive groups on the protein are depleted.

TABLE III

NUMBER OF AMINO AND TYROSYL GROUPS OF α-LACTALBUMIN ACETYLATED WITH N-ACETYLIMIDAZOLE

	ε-Amino Groups Acetylated	Tyrosines Acetylated
Control	0	0
Acetylated 40-1 ratio	3.5	2.1 ± 0.2
Acetylated 500-1 ratio	9.1	4.15 ± 0.35
Deacetylated 500-1 ratio	---	0

Gel electrophoresis was run on the acetylated and deacetylated protein to determine the effects of acetylation on electrophoretic mobility. A large change in the rate of migration was noted upon acetylation which was probably due to the removal of positive charges when the amino groups are acetylated. Little difference in the migration was noted upon deacetylation at both 40-1 and 500-1 ratios (Figure 4).

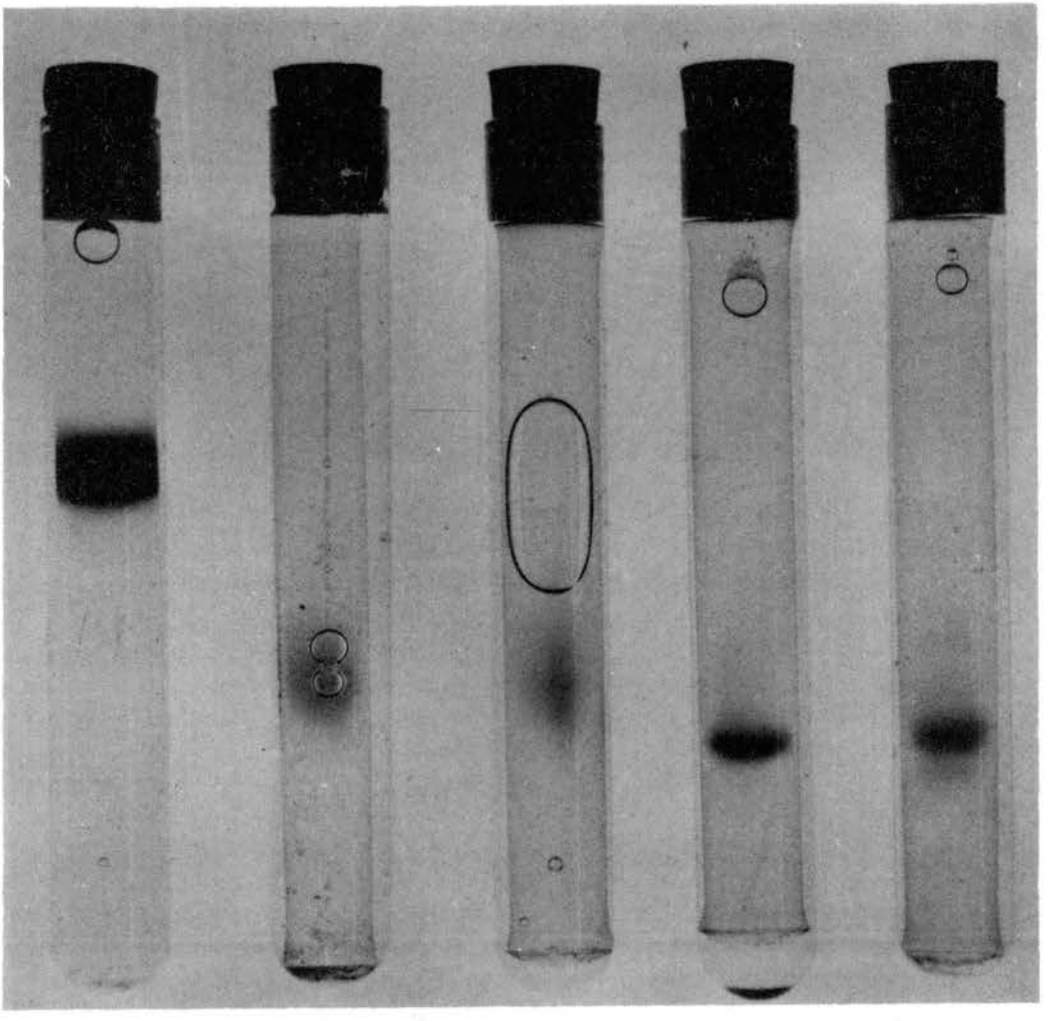

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Figure 4. Gel Electrophoresis of α-Lactalbumin Treated With N-Acetylimidazole

The effect of acetylation and de-O-acetylation on the electrophoretic mobility of α-lactalbumin. Tube 1, native α-lactalbumin; Tube 2, α-lactalbumin acetylated at at a 40:1 ratio; Tube 3, α-lactalbumin acetylated at a 40:1 ratio and then deacetylated; Tube 4, α-lactalbumin acetylated at a 500:1 ratio; Tube 5, α-lactalbumin acetylated at a 500:1 ratio and then deacetylated. Total protein on each gel was 30 μg.

TABLE IV

CORRELATION OF LOSS OF α-LACTALBUMIN ACTIVITY WITH NUMBER OF TYROSYL RESIDUES ACETYLATED

Acetylation Ratio	Tyrosines Acetylated	% Activity Lost
10-1	1*	26
40-1	2.1 ± 0.02	47
160-1	3*	59
500-1	4.1 ± 0.3	86

* Literature value (105)

Denton and Ebner (6) observed that nitration and iodination of α-lactalbumin produced inactive dimers and polymers; this prompted experiments to determine if acetylation also gave similar side reactions. A sample of α-lactalbumin treated for one hour with N-acetylimidazole, 500-1 ratio was applied to a previously calibrated P-100 column. One symmetrical peak was found which eluted in the same position as native α-lactalbumin (Figure 5). Since this technique successfully demonstrated the presence of dimers after nitration and iodination, polymerization was not a significant side reaction during acetylation.

The circular dichroism spectra of native bovine α-lactalbumin is quite complex. The elipticity bands are all negative. In the near ultraviolet region (250 to 310 nm) the spectrum is characterized by a small peak centered near 296 nm, a trough near 252 nm and a broad spectral envelope extending from about 255 nm to 290 nm with a maximum

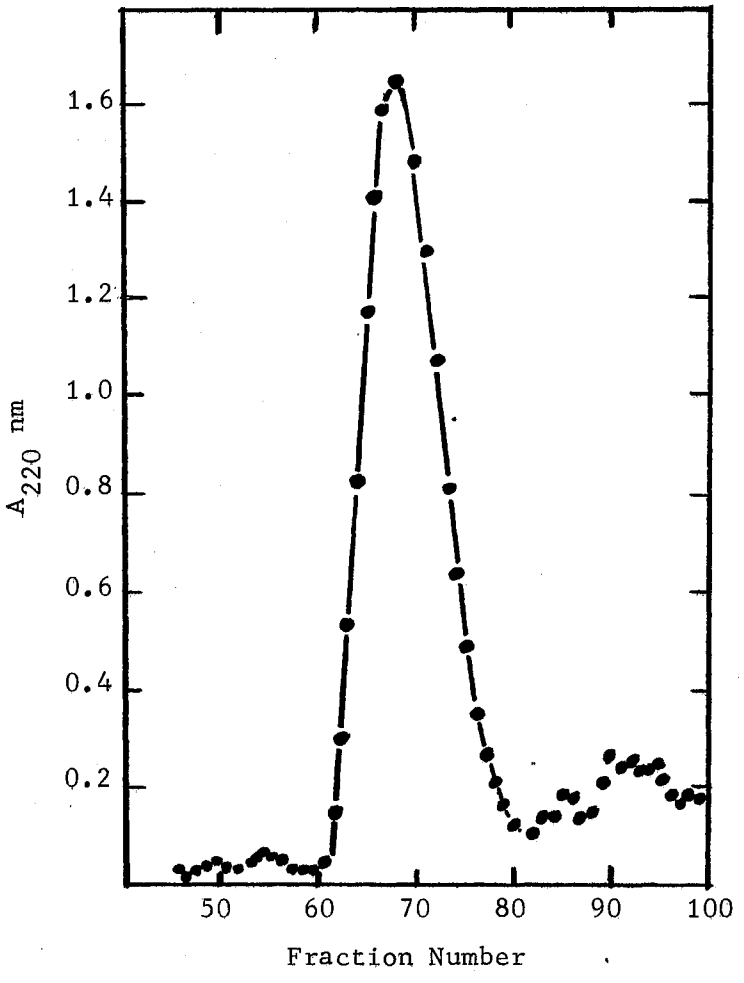

Figure 5. Chromatography of Acetylated α-Lactalbumin on Bio-Gel P-100

α-Lactalbumin treated with a 500-1 ratio of N-acetylimidazole to protein as eluted from a Bio Gel P-100 column (0.6 cm x 110 cm) at 25 with 0.5 M Tris, pH 8.0, 0.1 M KCl. Fractions of 0.2 ml were collected and diluted to 1 ml with water before reading the absorbance at 220 nm.

near 272 nm. This spectrum is consistant with the presence of optically active cystine and aromatic chromatophores, tyrosine and tryptophan (85).

In the far ultraviolet region (200-250 nm) the spectra is characterized by a negative peak with a maximum near 208 nm and a plateau from 212 to 225 nm.

Acetylation at a 500-1 ratio of N-acetylimidazole to α-lactalbumin resulted in several changes in the circular dichroism spectra as shown in Figures 6 and 7.

There is a general reduction of the amplitude of bands from 270 to 310 nm. The spectra show almost no differences from 230 nm to 270 nm. The plateau found with native protein from 212 to 225 nm disappears and is replaced by a monotonic decrease to the maximum at 208 nm. The peak at 208 nm becomes more prominent, increasing from a value of -10,800 to -13,400 degrees cm^2/decimole.

Robbins and Holmes (85) have concluded that the small peak at 296 nm is due to an asymetric tryptophan in native α-lactalbumin. Upon acetylation there is a decrease in the elipticity of this band and also the general tryptophan area (270-300 nm). This is probably due to an increased freedom of movement in the modified protein. Supportive evidence for this explanation is the similarity of circular dichroism spectrum of acid-denatured α-lactalbumin and the spectrum for acetylated α-lactalbumin (105).

The change in elipticity at 252 nm could be due to either cystine or tyrosine residues since both exhibit elipticity bands in this wavelength region. Neither explanation can be confirmed at this time and the change may be a combination of two effects, acetylation of the ty-

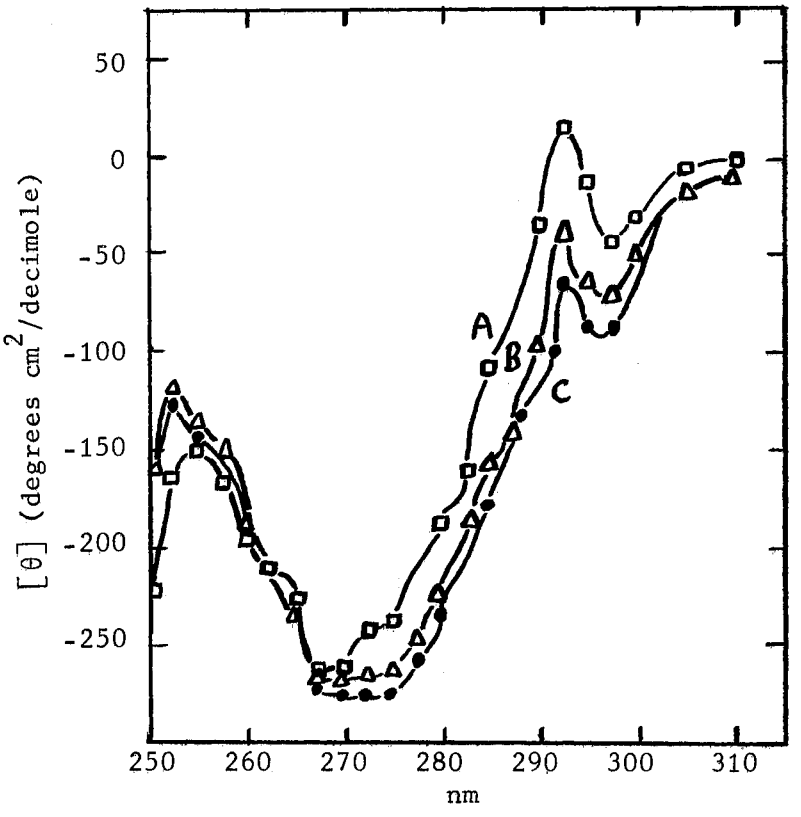

Figure 6. Near Ultraviolet Circular Dichroism Spectra of Acetylated and De-O-Acetylated α-Lactalbumin

α-Lactalbumin was acetylated at 500-1 reagent to protein ratio with N-acetylimidazole. De-O-acetylation was accomplished by treating with 0.2 M hydroxylamine at pH 7.5 for one hour. Curve A, (□) acetylated α-lactalbumin, Curve B, (△) de-O-acetylated α-lactalbumin, Curve C, (●) control α-lactalbumin. [θ] is the mean residue ellicipticity (mean residue weight was 118).

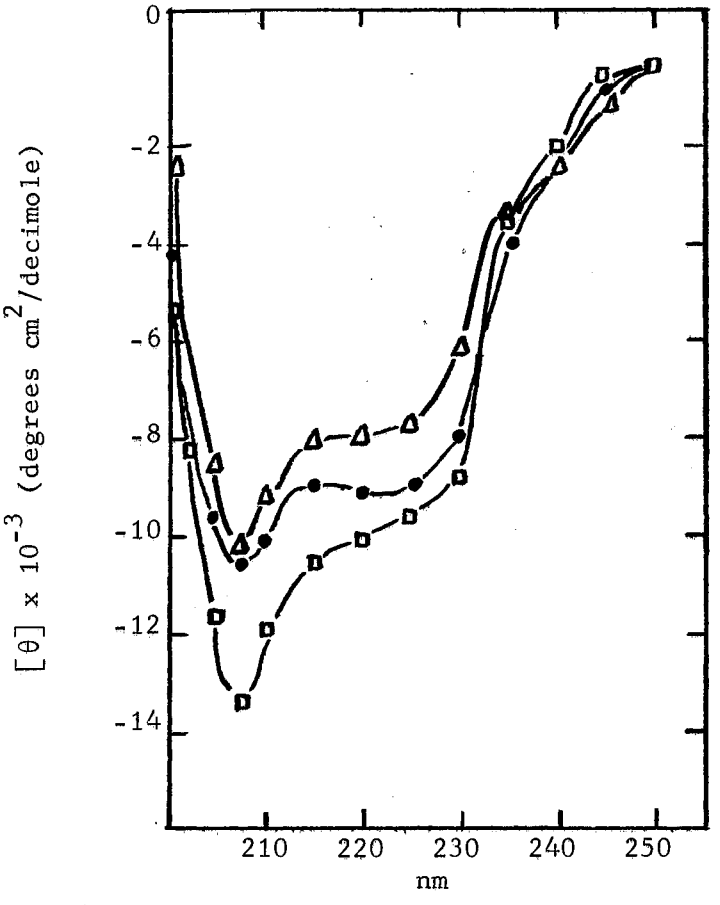

Figure 7. Far Ultraviolet Circular Dichroism Spectra of Acetylated and De-O-Acetylated α-Lactalbumin

α-Lactalbumin was acetylated at 500-1 reagent to protein ratio with N-acetylimidazole. De-O-acetylation was accomplished by treating with 0.2 M hydroxylamine at pH 7.5 for one hour. Curve A, (□) acetylated α-lactalbumin, Curve B, (△) de-O-acetylated α-lactalbumin, Curve C, (●) control α-lactalbumin. [θ] is the mean residue ellicipticity (mean residue weight was 118).

rosyl residues and a change in the dihedral angle of the sulfur atoms of the disulfide bridge after a conformational change.

Interpretation of the changes occurring in the far ultraviolet spectra is based on the work of Greenfield and Fasman (86) and Chen and Yang (119). Greenfield and Fasman (86) have examined the circular dichroism spectra of poly-L-lysine in various conformations and have proposed an empirical formula for calculating the percent α-helix.

$$\% \ \alpha\text{-helix} = \frac{[\theta]_{208 \text{ nm}} - 4000}{3300 - 4000} \times 100 \qquad (3.1)$$

The formula requires only the mean residue elipticity at 208 nm and has given reasonable results when applied to several protein whose structures were known from X-ray crystallographic analysis. Chen and Yang (119) have criticized the use of synthetic peptides for reference standards because (a) globular protein molecules contain short helical or β-segments, or both, whose spectra should differ in magnitude from the spectra of polypeptides and (b) the tertiary structures of most protein molecules are compact and rigid unlike the "random coils" of synthetic polypeptides. They have proposed the use of proteins of known structure for a model system. From the proteins, myoglobin, lysozyme, ribonuclease, papain and lactic dehydrogenase, they have proposed three equations based on three different wavelengths.

$$[\theta]_{220} = -27700 \ f_H - 3380 \qquad (3.2)$$

$$[\theta]_{221} = -28500 \ f_H - 3020 \qquad (3.3)$$

$$[\theta]_{222} = -30300\ f_H - 2340 \quad \text{where } f_H \text{ is the fraction of } \alpha\text{-helical structure of the protein.} \qquad (3.4)$$

The selection of the equation to be utilized is dictated by the elipticity maximum in the region 220 - 222 nm. The percent α-helix for native, acetylated, and de-O-acetylated α-lactalbumin was calculated by both methods and the results are shown in Table V.

TABLE V

EFFECT OF N-ACETYLIMIDAZOLE ON THE α-HELICAL CONTENT OF α-LACTALBUMIN

	Native α-Lactalbumin	Acetylated α-Lactalbumin	De-O-Acetylated α-Lactalbumin
α-Helix Content Based on $[\theta]_{208}$	22.7%	33.0%	21.4%
α-Helix Content Based on $[\theta]_{220}$	21.0%	24.0%	16.7%

The values found for native α-lactalbumin are in good agreement with the value reported by Robbins and Holmes (85). Their calculations were based on the mean residue elipticity at 208 nm. They reported an increase of 10% in the α-helical content upon denaturation at high pH. This is very close to the increase in helical content upon acetylation. Deacetylation of the tyrosyl residues resulted in an apparent loss of helical structure back to near the value of native α-lactalbumin.

Other changes upon de-O-acetylation were minor resulting in a spectra that more closely resembled the spectra of native α-lactalbumin. There was an apparent decrease in the magnitude of elipticity in the far ultraviolet region but the shape of the spectrum was very close to that of native α-lactalbumin.

Modification of α-Lactalbumin with Acetic Anhydride

Acetic anhydride is an effective tyrosyl modifying reagent when conditions are favorable for the stability of the resulting ester (112). Acetylation of amino and sulfhydryl groups also occurs. Since α-lactalbumin contains no free sulfhydryl groups, acetylation with acetic anhydride should give the same results as acetylation with N-acetylimidazole. However significant differences were found. The major difference was that deactivation was not appreciably reversed under conditions which were successfully used to reactivate N-acetylimidazole-inactivated α-lactalbumin.

Table VI lists the results obtained upon treatment of α-lactalbumin at 50-1 and 10-1 ratios of acetic anhydride to protein. Acetylation at 50-1 results in almost complete inactivation, and no significant activity returns upon treatment with hydroxylamine, 0.2 M at pH 7.5. Incomplete acetylation (10-1) results in the loss of over half of the control activity and only a small activation occurs upon treatment with hydroxylamine. Estimation of the number of acetylated amino groups is also shown in Table VI. Free amino groups were determined by reaction with trinitrobenzene sulfonic acid.

TABLE VI

EFFECT OF ACETIC ANHYDRIDE ON THE ACTIVITY OF α-LACTALBUMIN

	% Loss of Activity	% Regained After Treatment with NH_2OH	Number of Amino Groups Acetylated
Acetylated 50-1 Ratio	94	2	9
Acetylated 10-1 Ratio	63	7	3

Electrophoresis of α-lactalbumin treated at 50-1 with acetic anhydride yields one fast moving band analogous to the N-acetylimidazole treated α-lactalbumin. At a ratio of 10-1 reagent to protein there is insufficient reagent to acetylate all of the four tyrosyl residues and the nine available lysyl residues. A series of seven fast-moving bands, one of which corresponded to native α-lactalbumin was obtained upon electrophoresis on standard 7% acrylamide gels. Treatment of both the 50-1 and 10-1 acetylated proteins with hydroxylamine did not change migration distances. This corresponded to results obtained with N-acetylimidazole. To verify that the multiplicity of bands was due to incomplete acetylation, N-acetylimidazole was used in a 10-1 ratio to acetylate α-lactalbumin. The results are shown in Figure 8 and are compared with acetic anhydride treated α-lactalbumin.

Since there was an irreversible loss of activity upon treatment with acetic anhydride, the acetylated α-lactalbumin was checked to see if polymerization had occurred. The acetylated α-lactalbumin was chro-

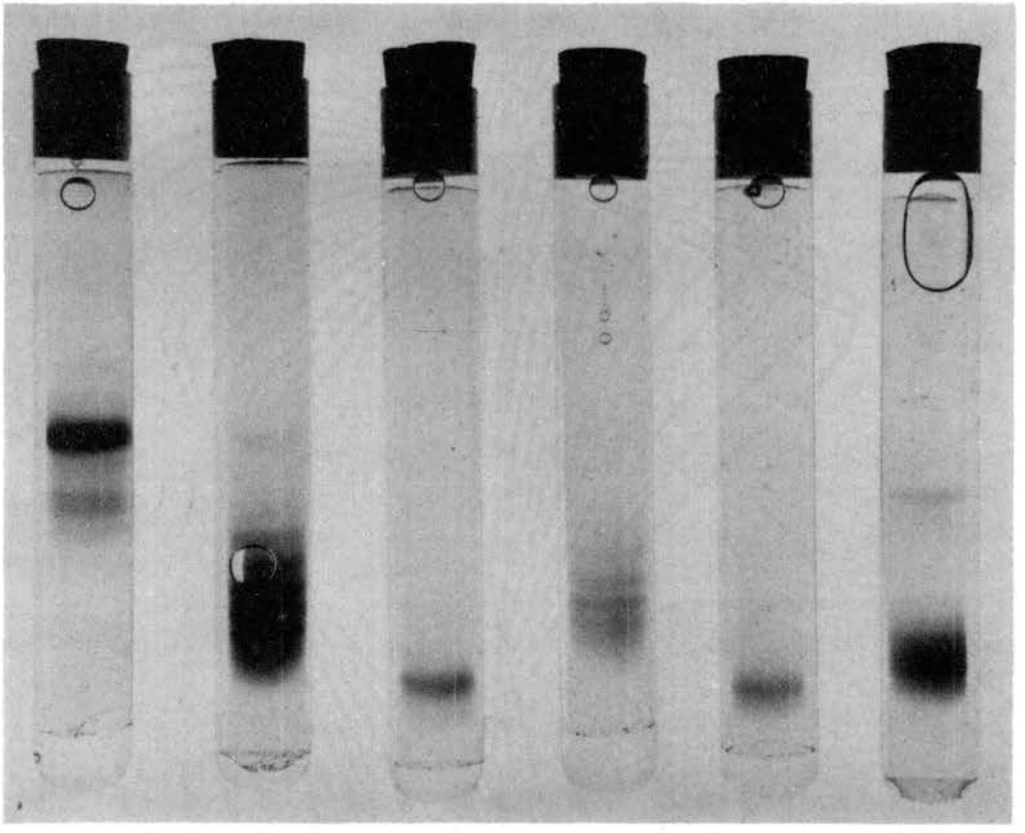

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Figure 8. Gel Electrophoresis Patterns of Acetylated α-Lactalbumin

The effect of acetylation on the electrophoretic mobility of α-lactalbumin. Tube 1, native α-lactalbumin; Tube 2, α-lactalbumin acetylated at a 10:1 ratio with acetic anhydride; Tube 3, α-lactalbumin acetylated at a 50:1 ratio with acetic anhydride; Tube 4, α-lactalbumin acetylated at a 10:1 ratio and then de-O-acetylated; Tube 5, α-lactalbumin acetylated at a 50:1 ratio and then de-O-acetylated; Tube 6, α-lactalbumin acetylated at a 10:1 ratio with N-acetylimidazole. Total protein on each gel was 40 μg.

matographed on a Bio-Gel P-100 column and the results are shown in Figure 9. Activity measurements were made on each fraction and a constant specific activity was found. No evidence for polymers was found.

Summary of Acetylation

Acetylation of α-lactalbumin causes loss of activity. De-O-acetylation of the tyrosyl residues under mild conditions results in reactivation thus establishing the tyrosyl residues as necessary for biological activity. Since the exposed lysyl groups are irreversibly acetylated during this procedure it was concluded that the surface lysyl groups are not necessary for biological activity. The degree of acetylation of the tyrosyl residues was correlated with the loss of enzymatic activity; however, no differences were found between the four tyrosyl residues. Results obtained from the circular dichroism spectra indicate that changes in the conformation of the protein have occurred upon acetylation which are largely but not totally reversible. The use of acetic anhydride for acetylation also caused an inactivation of α-lactalbumin. In contrast to the acetylation with N-acetylimidazole the inactivation was not reversible. Presumably there is an undetected side reaction or a change in specificity.

Effects of High pH on α-Lactalbumin

Experiments in which incubation at high pH was used to catalyze deacetylation of the tyrosyl residues of modified α-lactalbumin led to large losses of activity in the control as shown in Table II. The loss in activity was time dependant and over 50% of the activity was lost in one hour, when incubated at pH 12 at room temperature.

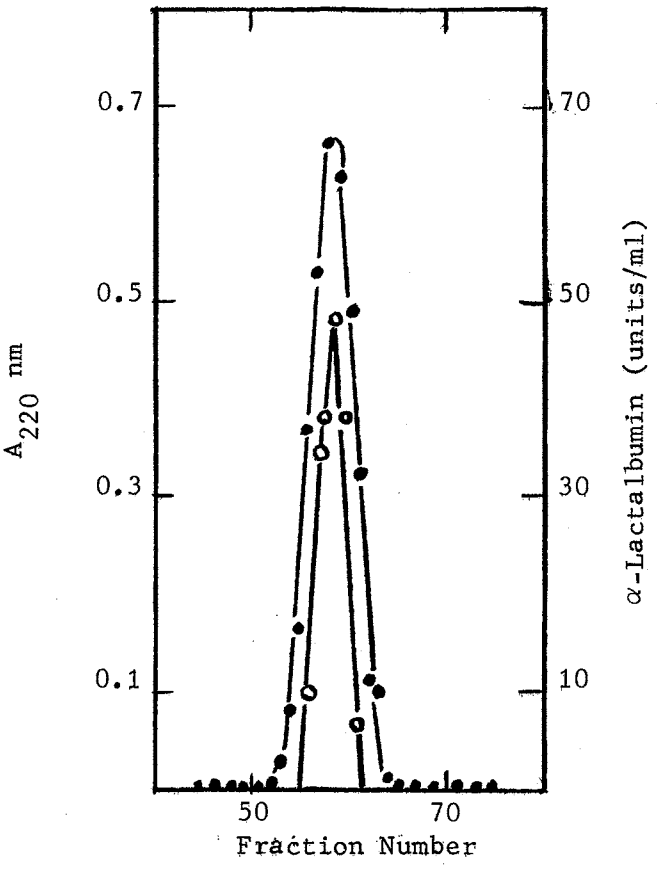


Figure 9. Bio-Gel P-100 Chromatography of α-Lactalbumin Treated with Acetic Anhydride

α-Lactalbumin treated with a 10-1 ratio of acetic anhydride to protein as eluted from a Bio-Gel P-100 column (0.6 cm x 110 cm) at 25 C with 0.05 M Tris, 0.1 M KCl pH 8.0. Fractions of 0.2 ml were collected and diluted to 1 ml with water before reading the absorbance at 220 nm. The specific activities in units/mg for tubes 36 through 41 are 55, 139, 118, 150, 156 and 42. (•), A_{220} nm. (o) α-lactalbumin, lactose synthetase activity (units/ml).

Kronman et al. (62) reported that α-lactalbumin undergoes a structural change to an expanded conformation above pH 9.5. This finding was based on ultracentrifuge measurements of the sedimentation coefficient at pH 8.55, 9.5, 10.0, and 12.0. The frictional ratios and molecular radii were calculated from the sedimentation constants and were found to increase with the pH. The expanded structure led to a difference spectra which was attributed to changes in tryptophan absorption. The expansion of the structure occurs very quickly and completely. The changes in the fluorescence properties (66) were complete in one minute and there were no further changes for periods up to two hours. The changes in fluorescence were fully reversible when a solution of α-lactalbumin was brought from higher pH (pH 12) back to pH 6.0.

Intermolecular interactions were also found above pH 9.5. An association of low molecular weight species (dimers and trimers) was detected in ultracentrifuge measurements of the concentration dependancy of the sedimentation constant. This association was rapidly reversed upon return to pH 6.0. Exposure of α-lactalbumin to pH 10 and above for periods of 24 hours led to the formation of a heavy component molecular weight greater than 300,000). The formation of this heavy component increased with time and readjustment of the pH to 8.55 did not reverse the aggregation. It was also not reversed by the addition of 4 M urea. Kronman et al. (62) concluded that there was some destruction of disulfide bonds as well as formation of intermolecular disulfide bonds. The following experiments were undertaken to see if the loss of biological activity corresponded to any of the aforementioned processes.

Figure 10 shows the loss of activity as a result of incubation at pH 7.5, 9.0, 10.5 and 11.6 for periods of time up to two hours. The loss of activity increases with time and with increasing pH.

To determine if molecular oxygen was participating in the reaction a solution of α-lactalbumin was purged with nitrogen and then the pH was raised to 11.4 and incubated for the four hours under a nitrogen atmosphere. The activity loss at two hours corresponded to that shown in Figure 10 after two hours of incubation.

Figure 11 shows the effect of lowering the pH back to 7.3 after incubation at high pH. A solution of α-lactalbumin, 0.1 mg/ml in 0.02 M Tris buffer was raised to pH 11.4 and incubated at room temperature for 2.5 hours. The pH was then adjusted down to 7.3 and incubated at room temperature for varying lengths of time before assaying. About 25 percent of the activity returned but at a very slow rate.

Since the loss of activity was largely irreversible, the same type experiment was run in the presence of low levels of mercaptoethanol, to observe if protection of the easily oxidized groups would prevent loss of activity. Slightly different conditions were used to inactivate the α-lactalbumin. α-Lactalbumin was dissolved, 0.5 mg/ml in 0.15 M KCl, and incubated for six hours at pH 10.5 at room temperature with 0.014 M mercaptoethanol present. The pH of the solution was then lowered by dialysis against 0.02 M Tris pH 8.0 at 4°C. Dialysis for 24 hours resulted in a gain of only 2% activity. The control α-lactalbumin which was treated with 0.014 M mercaptoethanol at pH 7.5 lost 9% activity in six hours as compared to a 95% loss in activity at pH 10.5 The 95% loss was a much greater loss of activity than anticipated and probably indicates that mercaptoethanol acts as a catalyst in the deactivation

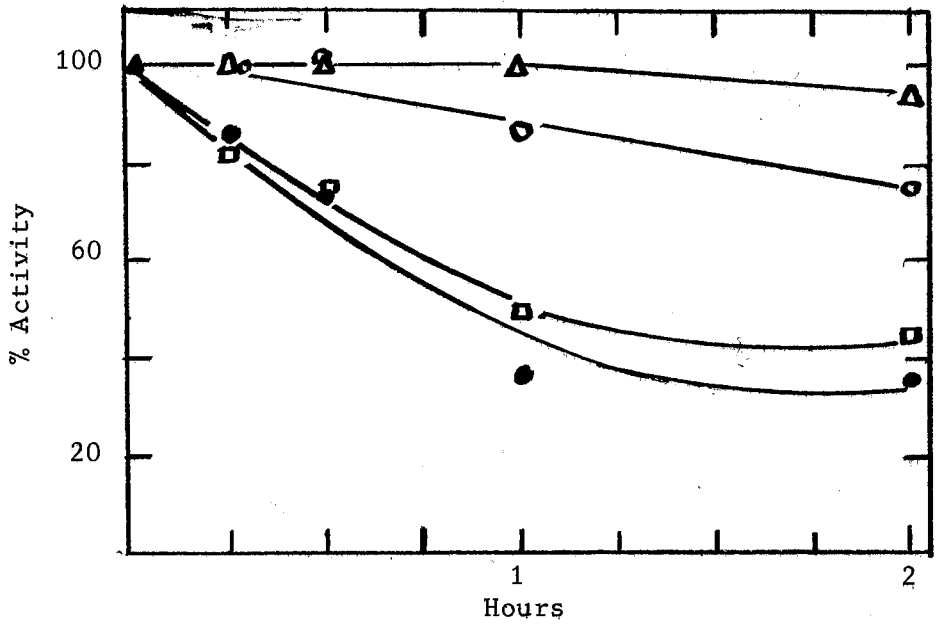


Figure 10. The Effect of High pH on the Activity of α-Lactalbumin

α-Lactalbumin solutions of 0.2 mg/ml were adjusted to pH 7.5 (Δ), pH 9.0 (o), pH 10.5 (□), and pH 11.6 (●). The lactose synthetase activity of each sample was monitored for two hours. The buffer was 0.02 M Tris at room temperature. 100% Activity equals 650 nmoles of UDP formed per minute per mg of α-lactalbumin.

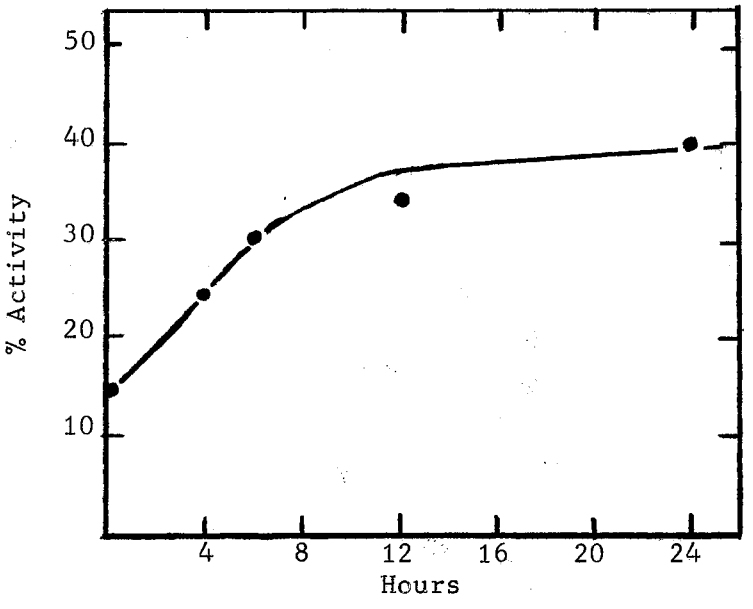


Figure 11. Effect of Incubation at pH 7.3 on Base Inactivated α-Lactalbumin

α-Lactalbumin previously inactivated by incubation at pH 11.4 at room temperature for 2.5 hours was incubated at pH 7.3 and the activity was measured as a function of time. Protein concentration was 0.1 mg/ml in 0.02 M Tris buffer. 100% activity equals 650 nmoles of UDP formed per minute per mg of α-lactalbumin.

process. The most likely role for mercaptoethanol is in promoting disulfide interchange. This could especially be the case if the disulfide bridges are strained when the α-lactalbumin molecule expands at high pH.

Since native α-lactalbumin contains no free sulfhydryl groups (52) it was possible to determine if any of the four disulfide bridges were broken during incubation at high pH by merely assaying for free sulfhydryl groups. A stock solution of α-lactalbumin in 0.15 M KCl was incubated at pH 12.0 and aliquots were removed, the pH adjusted to 8.0, and assayed by reaction with Ellman's reagent (115). The number of moles of free sulfhydryl groups was calculated using the extinction coefficient (ϵ_{412} = 11,400/cm) based on 3-carboxylate-4-nitrothiophenolate (123). Figure 12 shows the results obtained. The number of free sulfhydryl groups increased with incubation time. An aliquot was removed after six hours of incubation and assayred for lactose synthetase activity. The sample had lost 53% of the activity compared to the control which was incubated at pH 6.0.

Electrophoresis of α-lactalbumin incubated at high pH is shown in Figure 13. α-Lactalbumin was incubated at pH 11.4 for 2.5 hours and layered directly on the disc gel without adjustment of pH. An aliquot of the same solution was removed before the pH was raised for a control and allowed to incubate at pH 7.0. A third aliquot was allowed to incubate at pH 11.4 for 1.5 hours and then the pH was adjusted to 7.0 and further incubation of one hour was allowed before electrophoresis. The major band of all three samples migrated at the same rate, but the two samples which had been incubated at pH 11.4 showed a very broad faint band just preceding the major band.

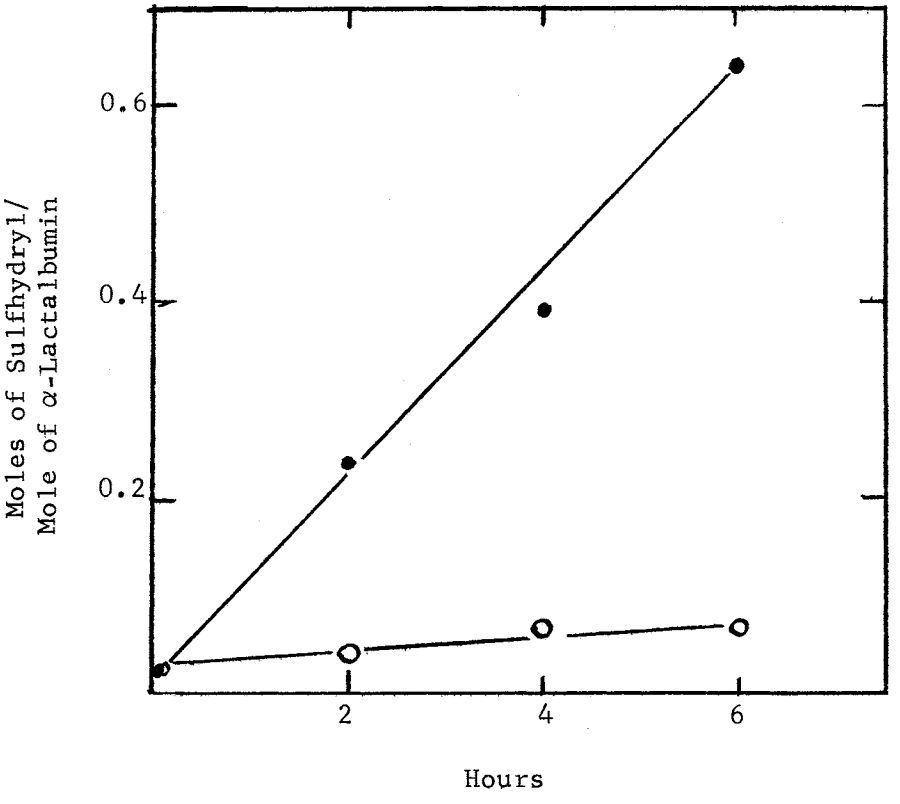
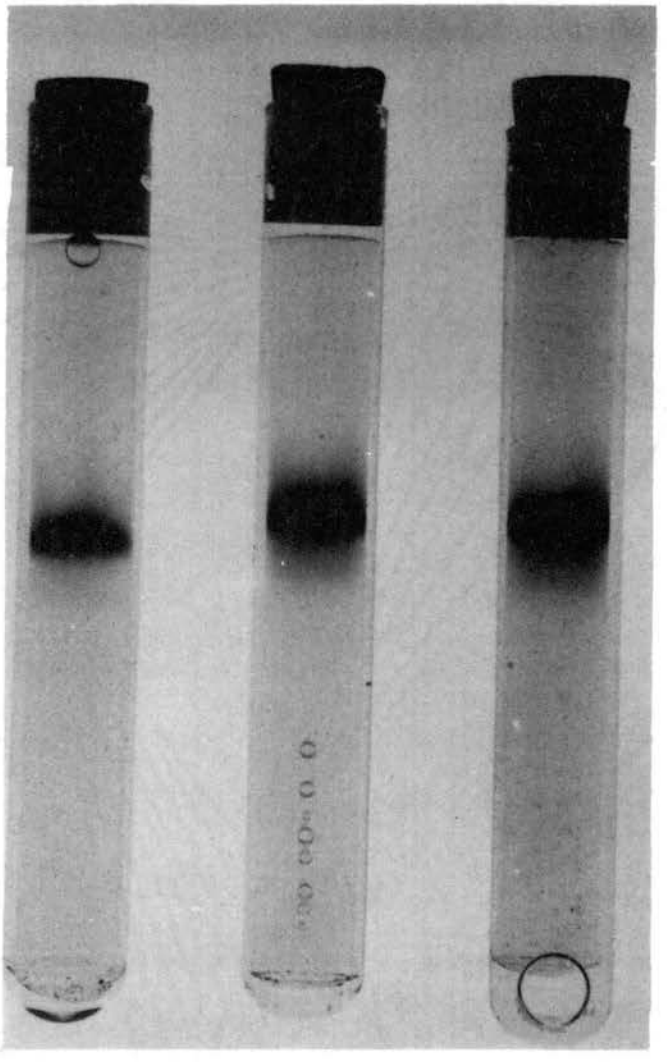


Figure 12. Production of Sulfhydryl Groups During Incubation of α-Lactalbumin at pH 12

α-Lactalbumin was incubated at pH 12.0 at room temperature in 0.15 M KCl (●). Aliquots were removed at two hour intervals and assayed for free sulfhydryl groups by the method of Ellman (115). α-Lactalbumin was also incubated at pH 6.0 and treated in the same manner (o).



1 2 3

Figure 13. Effect of Incubation of α-Lactalbumin at High pH on the Migration in Gel Electrophoresis

Control α-lactalbumin (1) is compared to α-lactalbumin incubated at pH 11.4 for 2.5 hours (2), and α-lactalbumin at pH 11.4 for 1.5 hours and then incubated at pH 7.0 for one hour (7.0).

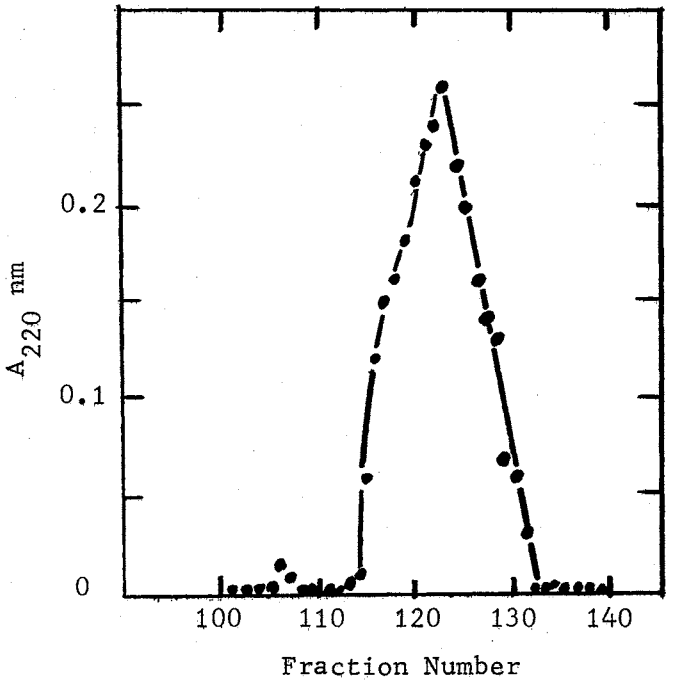


Figure 14. Chromatography of Base Inactivated α-Lactalbumin on Bio-Gel P-100

α-Lactalbumin was incubated at pH 11.4 for 2.5 hours and then lyophilized and chromatographed on a Bio-Gel P-100 column (0.6 x 110 cm). Elution buffer was 0.05 M Tris, 0.1 M KCl at pH 8.0. One tenth ml fractions were collected and diluted with 0.9 ml of H_2O before reading the absorbance at 220 nm.

An aliquot of the α-lactalbumin solution was lyophilized and chromatographed on a Bio-Gel P-100 column to determine if aggregation had occurred. Figure 14 shows the presence of a single peak corresponding in position to native α-lactalbumin. Thus it appeared that no irreversible aggregation had occurred.

In order to obtain a more sensitive measure of the effect of high pH on the molecular structure of α-lactalbumin the modified protein was compared to native α-lactalbumin by the Oudin quantitative diffusion test. α-Lactalbumin was incubated at pH 12.0 in 0.15 M KCl for six hours. The pH was then adjusted to 7.0 and a solution containing 100 μg/ml of protein was tested for reaction with antibodies to α-lactalbumin. The standard curve (see Methods) was prepared from α-lactalbumin incubated six hours at pH 6. The results obtained are shown in Figure 15. Approximately 60% of the α-lactalbumin did not react with the antibody. The same sample of α-lactalbumin had 0.64 moles of free sulfhydryl groups per mole of α-lactalbumin, and had lost 53% of its activity in the lactose synthetase reaction.

The circular dichroism spectra of base treated α-lactalbumin are compared to native α-lactalbumin in Figures 16 and 17. The near ultraviolet spectrum shows only one difference, a decrease in elipticity at 252 nm. This area reflects changes in tyrosine ionization and optically active cystine residues. Since both spectra were run at pH 6.4 no change in tyrosine ionization would be expected and the change is probably due to modification of the disulfide bridges. Changes were present in the far ultratiolet spectra and probably reflect an unfolding of the protein.

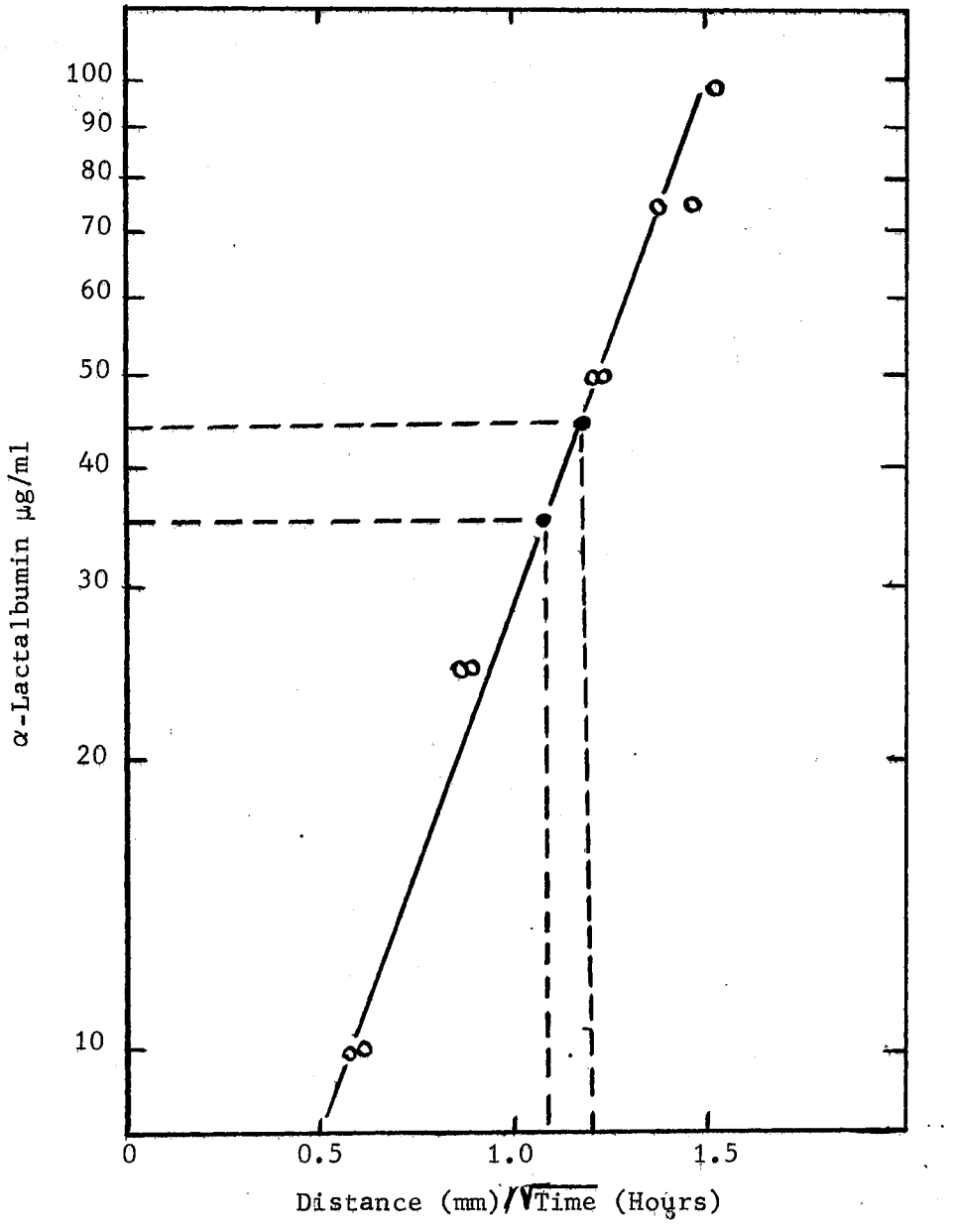


Figure 15. Standard Curve for Immunological Determination of α-Lactalbumin by the Oudin Technique

Control α-lactalbumin was incubated six hours at pH 6 in 0.15 M KCl and used for a standard curve for the Oudin immunological determination of α-lactalbumin (o). An aliquot of the same α-lactalbumin solution was incubated six hours at pH 12. The pH was adjusted to 7.0 and a solution containing 100 μg/ml was allowed to react with antibody for 36 hours (●).

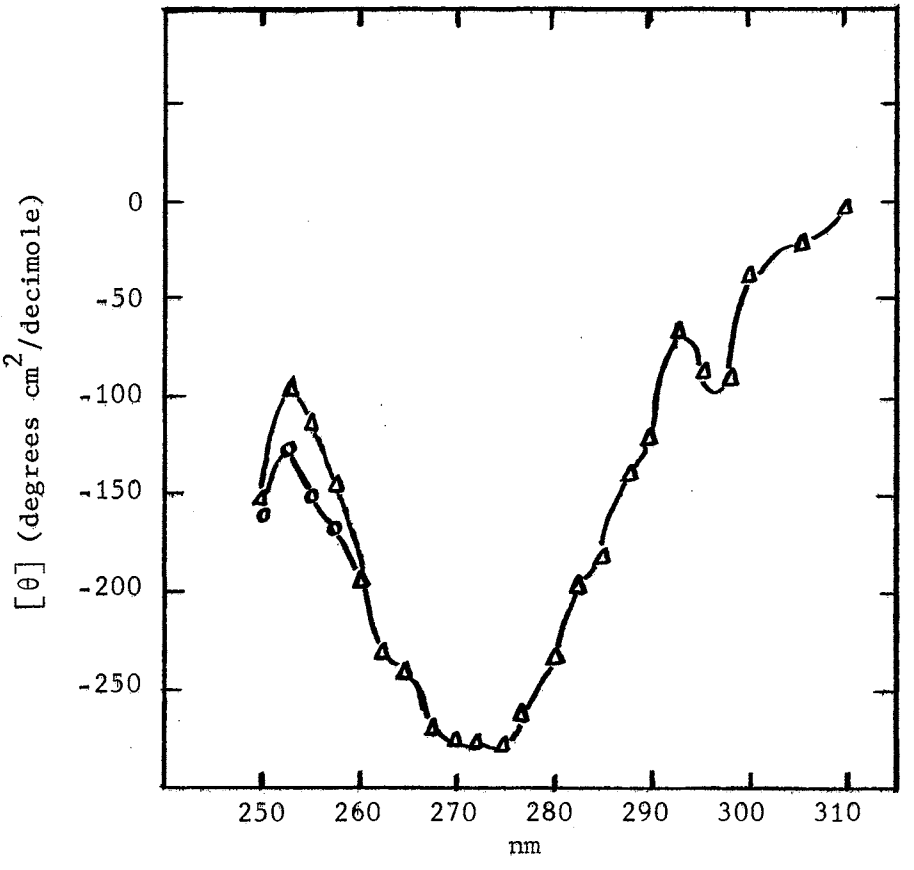


Figure 16. Near Ultraviolet Circular Dichroism Spectra of Base Treated and Native α-Lactalbumin

α-Lactalbumin was incubated at pH 10.5 for one hour in 0.15 M KCl, the pH was adjusted to 6.4 and the circular dichroism spectra was measured (Δ). Control α-lactalbumin was run before and after the one hour incubation at pH 6.4 in 0.15 M KCl (o). [θ] is the mean residue ellicipticity (mean residue weight was 118).

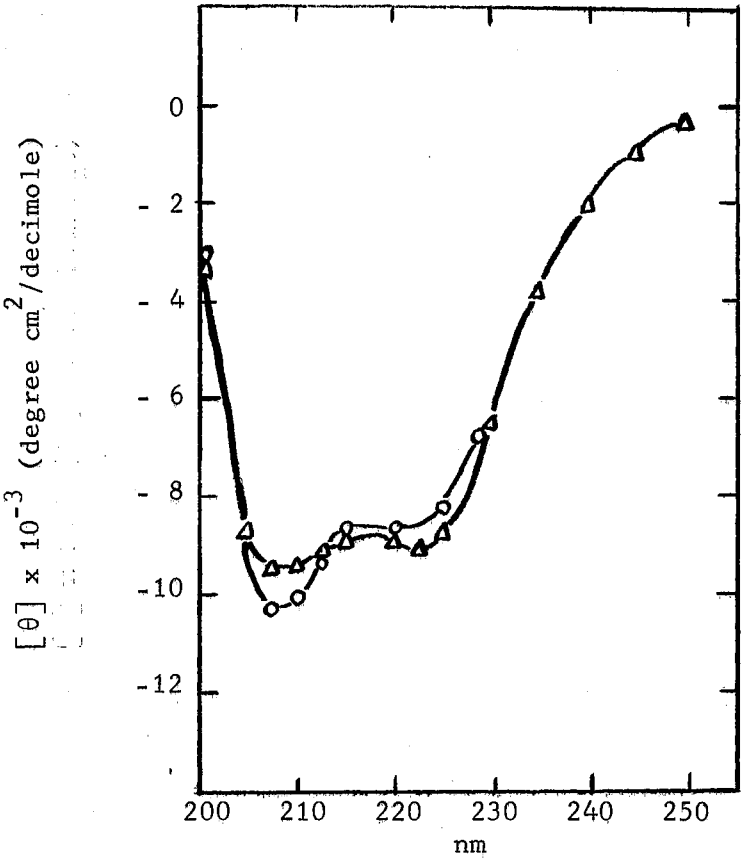


Figure 17. Far Ultraviolet Circular Dichroism Spectra of Base Treated and Native α-Lactalbumin

α-Lactalbumin was incubated at pH 11.1 for 45 minutes in 0.15 M KCl, the pH was adjusted to 6.4 and the circular dichroism spectra was measured (Δ). Control α-lactalbumin was run after 45 minutes incubation at pH 6.4 (o). [θ] is the mean residue ellicipticity (mean residue weight was 118).

Summary of the Effect of High pH on α-Lactalbumin

Incubation of α-lactalbumin at high pH resulted in a time dependent loss of activity. This loss of activity was largely irreversible under the conditions employed. No difference was found in the rate of inactivation when molecular oxygen was excluded from the reaction. The presence of low levels of mercaptoethanol increased the rate of deactivation. There was a time dependent cleavage of the disulfide bridges which corresponded to the loss of activity in the lactose synthetase reaction, and to a loss of antigenic activity. Electrophoresis of the modified material revealed the presence of an additional band. Circular dichroism spectra of the base treated material differed from native α-lactalbumin only in the area designated as the cystine absorption region. The effect of high pH on α-lactalbumin is most likely due to disulfide cleavage.

Modification of α-Lactalbumin by Iodination with Lactoperoxidase

The acetylation of α-lactalbumin with N-acetylimidazole causes a large loss in activity which closely parallels the acetylation of tyrosyl residues. This observation in addition to the work of Denton and Ebner (6) suggests that the tyrosyl residues of α-lactalbumin are critical for α-lactalbumin activity in the lactose synthetase reaction. Since there are four widely separated tyrosyl residues in bovine α-lactalbumin Tyr 18, 36, 50 and 103, it is likely that not all are essential for binding the galactosyltransferase molecule. Schmidt and Ebner (70) have found α-lactalbumin isolated from goat, pig, sheep and human have only three tyrosyl residues which would indicate that at

least one tyrosyl residue is not essential. Attempts were made to find a method which would chemically alter fewer than four tyrosyl residues upon reaction with α-lactalbumin.

Chemical methods of modification were largely ruled out because all tyrosines react readily with tyrosyl reagents thus no selectivity was possible. One exception has been reported by Gorbunoff (88), who found that cyanuration of three tyrosyl residues occurs readily and that reaction of the fourth residue occurs only at high pH.

Enzymatic modification of the tyrosyl residues should possibly offer some selectivity of reaction, especially if any of the four residues are located in a crevice, or in the inner core of the protein and thus inaccessible to the enzyme. Lactoperoxidase has been used by Phillips and Morrison (108) to incorporate iodide into the exposed tyrosyl and histidyl groups on the surface of the intact human erythrocyte. Lactoperoxidase is well suited for this type of experiment due to its large size (M.W. = 78,000) and stability under the reaction conditions. The conditions of the iodination reaction are very gentle with respect to pH, solvent, ionic strength, and temperature. The rate of reaction can be varied by the amount of lactoperoxidase used to catalyze the reaction. Reaction condition control experiments on α-lactalbumin show no detectable losses in lactose synthetase activity for periods up to 30 minutes.

The iodination reaction can be monitored in several ways. The most direct method is to observe the increase in absorbance at 290 nm due to the formation of monoiodotyrosine (MIT). Table VII lists the results of treatment of α-lactalbumin with lactoperoxidase at different weight to weight ratios. The time required for complete reaction is

shown. The moles of MIT formed per mole of α-lactalbumin were calculated from a molar extinction coefficient of 2,340 for pH 7.4 (8).

TABLE VII

IODIDE INCORPORATION AS MEASURED SPECTROPHOTOMETRICALLY

α-Lactalbumin to Lactoperoxidase Ratio	Minutes Required for Complete Reaction	Moles of MIT Formed/ Mole of α-Lactalbumin
10,000 - 1	12	0.54
10,000 - 1	13	0.45
1,000 - 1	3	0.42
200 - 1	1	0.60

The extent of the iodination reaction can also be determined by measuring the incorporation of I^{125} into α-lactalbumin. The free iodide was removed by gel filtration, as described in Methods, after completion of the reaction.

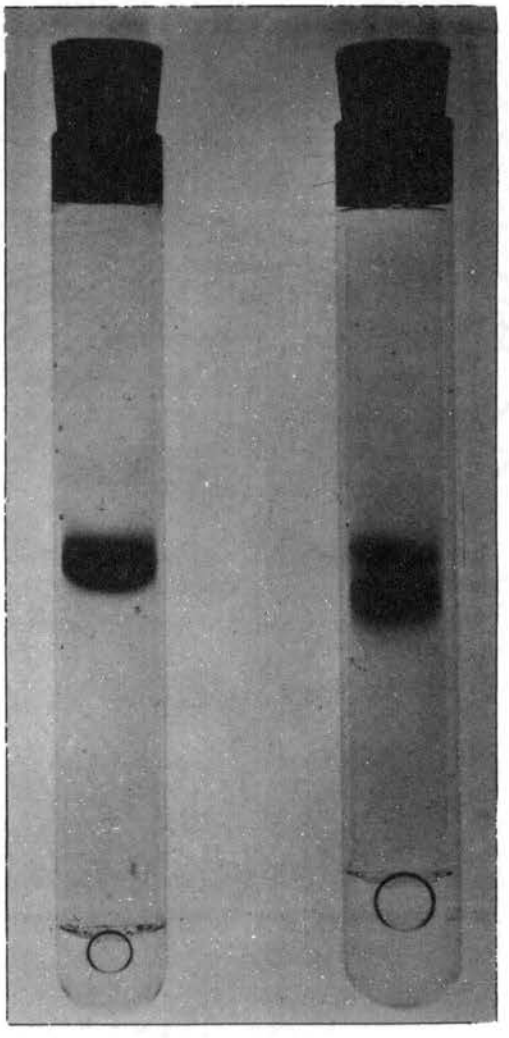
Aliquots of the iodinated α-lactalbumin were counted and the number of moles of iodide incorporated was calculated. Table VIII shows the results of varying conditions on the incorporation of iodide.

Approximately 0.5 moles of iodide per mole of α-lactalbumin were incorporated. This is in good agreement with the results found by the increase in absorbance at 290 nm as shown in Table V. When α-lactalbumin was reduced and aminoethylated before iodination, solubility prob-

lems are encountered. This is probably the reason for the low incorporation. Varying the reaction conditions did not produce a superior method for iodination. High concentration of iodide are reported to inhibit lactoperoxidase (8) and this was verified by running the reaction at 10 fold higher iodide concentration (0.9 mM KI). Increasing the length of incubation before separating the reaction products did not appreciably change the incorporation. High concentrations of hydrogen peroxide are also reported to inhibit lactoperoxidase, although the optimum concentration range is wide (8). Doubling the hydrogen peroxide concentration from 100 μM to 200 μM produced no change in in incorporation. Lowering the hydrogen peroxide concentration to 8 μM caused a decrease in incorporation.

Although the incorporation was very low, the effect on activity was determined on two separate preparations (Table IX). No significant loss of activity was noted in either preparation, although the expected loss of activity due to iodination of one half mole of tyrosine would be 12.5% provided iodination was random. This value is very close to the minimum detectable change in activity when using the lactose synthetase assay for α-lactalbumin.

Gel electrophoresis of α-lactalbumin after iodination with lactoperoxidase yielded two bands, one corresponding to native α-lactalbumin and one slightly faster moving band. Calculations from the increase in absorbance at 290 nm indicated that 0.42 moles of iodide were incorporated per moles of α-lactalbumin. Thus approximately half of the α-lactalbumin should be mono-iodinated and this would provide an explanation for the two bands shown in Figure 18.



C I

Figure 18. Disc Gel Electrophoresis of Iodinated α-Lactalbumin

Gel electrophoresis of α-lactalbumin iodinated with lactoperoxidase (I) is compared to control α-lactalbumin (C). Standard 7% gels were used as described in Methods.

TABLE VIII

EFFECT OF DIFFERENT REACTION CONDITIONS UPON THE INCORPORATION OF IODIDE INTO α-LACTALBUMIN

Sample	Moles of Iodide Incorporated per Moles of α-Lactalbumin
α-Lactalbumin Treated with Lactoperoxidase (see Methods)	0.56
α-Lactalbumin Reduced and Aminoethylated Before Treatment with Lactoperoxidase	0.13
α-Lactalbumin Treated with Lactoperoxidase at 0.9 mM KI	0.41
α-Lactalbumin Treated with Lactoperoxidase at 8 μM H_2O_2	0.008
α-Lactalbumin Treated with Lactoperoxidase for 2 hours	0.56
α-Lactalbumin Treated with Lactoperoxidase for 8 hours	0.52
α-Lactalbumin Treated with Lactoperoxidase for 24 hours	0.54

Peptide maps of iodinated α-lactalbumin provide an assessment of the number of groups which have been labeled with ^{125}I in the lactoperoxidase catalyzed reaction. Morrison and Bayse (8) report that tyrosine, monoiodotyrosine, and histidine are iodinated with lactoperoxidase. Thus possible reaction products would be monoiodotyrosine, diiodotyrosine, and iodohistidine. However, there are three histidyl residues and four tyrosyl residues and each of these groups are in tryptic peptides of different size and charge and therefore, the number of pos-

sible radioactive spots is very large. Morrison and Bayse (8) also report that the rate of iodination of tyrosine is much greater than the rate of iodination of monoiodotyrosine. This is a direct contrast to the chemical iodination reaction, in which the second iodide molecule is incorporated more easily than the first iodide molecule.

TABLE IX

EFFECT OF IODINATION ON THE ACTIVITY OF α-LACTALBUMIN

	Moles of Iodide/ Moles of α-Lactalbumin	% Activity of Control α-Lactalbumin
Control α-Lactalbumin	0	100*
Reaction Control α-Lactalbumin	0	101
Iodinated α-Lactalbumin	0.6	96
Iodinated α-Lactalbumin	0.54	89

* 100% = 820 nmoles of UDP formed per minute per mg of α-lactalbumin.

α-Lactalbumin was iodinated with lactoperoxidase and then reduced and aminoethylated as reported in Methods. The protein was digested with trypsin for four hours and the resulting tryptic peptides were separated by electrophoresis and chromatography on a cellulose thin-

layer plate. The plate was sprayed with ninhydrin to detect peptides, although some peptides are in such low yield that they were not visible. This was evidently the case with the iodinated peptides becuase they appeared as spots upon autoradiography of the TLC plate even though no ninhydrin spots were visible. Figure 19 shows a typical peptide map of iodinated α-lactalbumin. The ninhydrin spots correspond closely to the peptide map of native α-lactalbumin. The radioactive spots (represented as cross-hatched areas) migrated at a much faster rate during chromatography than the nonlabeled peptides. Two of the iodinated spots (numbers 1 and 2 in Figure 19) contain most of the radioactivity, approximately 50% in number 1 and approximately 25% in number 2.

Iodinated α-lactalbumin was also hydrolyzed with Pronase to yield individual amino acids. A 16-1 ratio of Pronase to α-lactalbumin was used to insure complete hydrolysis within the 24 hour incubation period. The amino acids were separated by descending chromatography on cellulose thin-layer plates. The amino acids were identified by staining with ninhydrin and comparing migration rates to standards which were run on the same plate. As Figure 20 shows, the major product was monoiodotyrosine but traces of diiodotyrosine were also found.

The circular dichorism spectra of iodinated α-lactalbumin are compared to native α-lactalbumin in Figures 21 and 22. There is an apparent loss of amplitude in both near and far ultraviolet spectra of iodinated α-lactalbumin. However it is doubtful if iodination would cause this type of effect. A more probable explanation would be that

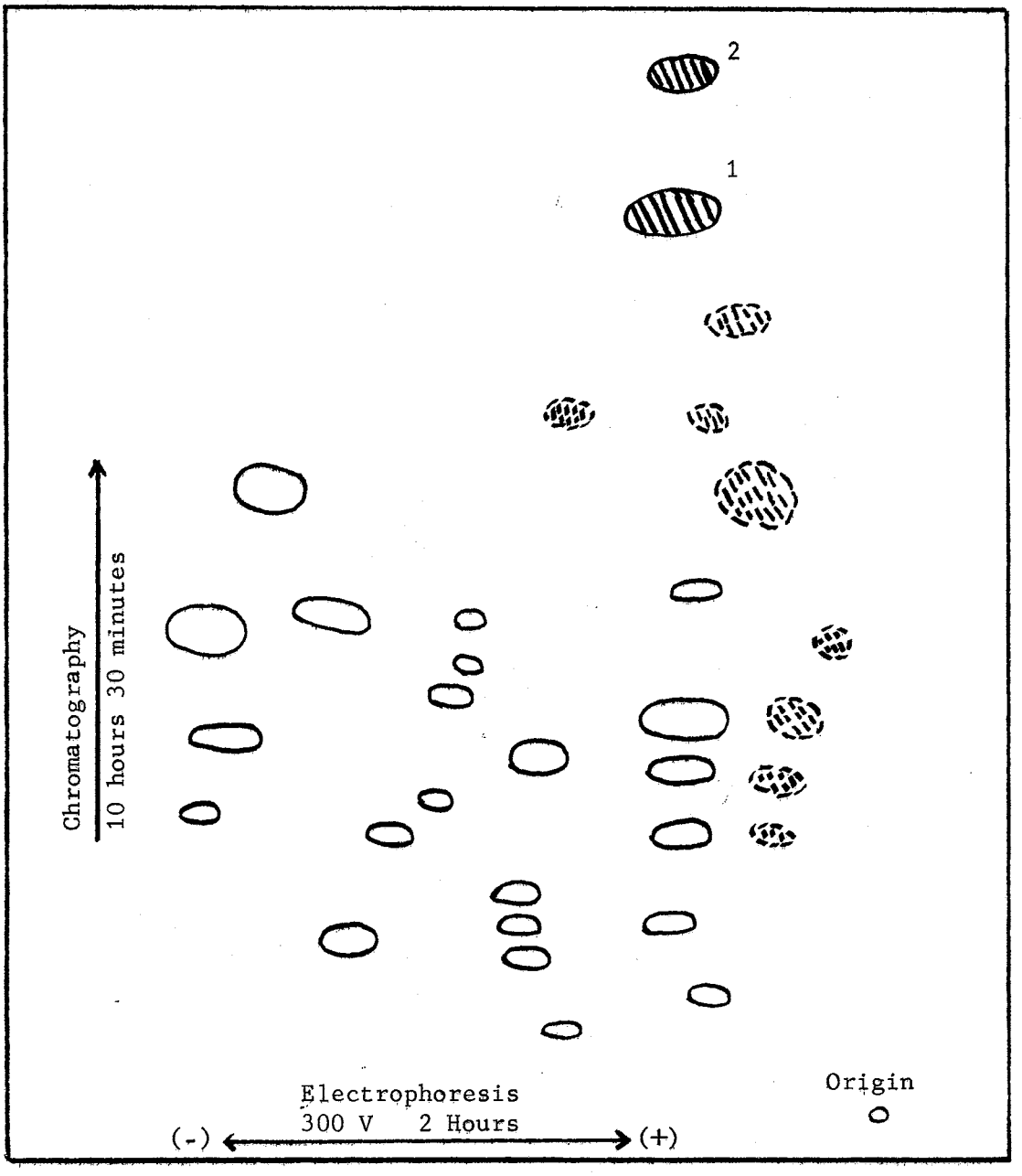


Figure 19. Peptide Map of a Tryptic Digest of Iodinated α-Lactalbumin

The peptides were separated in the first dimension by thin layer electrophoresis (pH 5.5, 2 hours, 300 V, 4) and in the second dimension by ascending chromatography in n-butanol-pyridine-glacial acetic acid-water, 150:100:30:120 for 10.5 hours at 25 . Cross-hatched areas represent spots on X-ray film exposed for two weeks. Number 1 is the major radioactive spot containing approximately 50% of the radioactivity, spot number 2 contains approximately 25% of the radioactivity. Very faint spots appear as broken circles. All other spots were detected with ninhydrin spray.

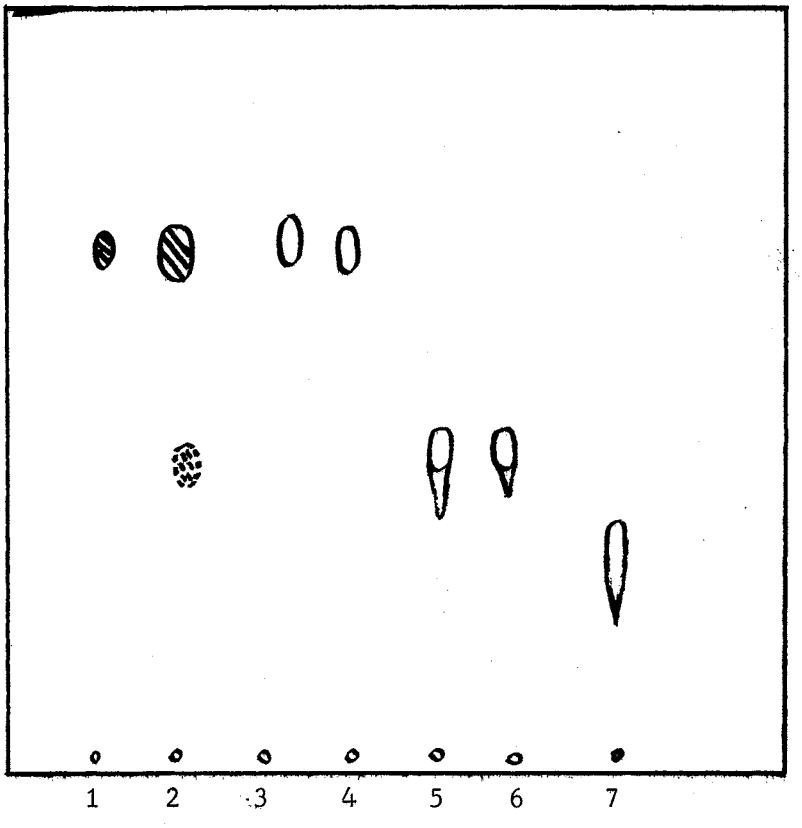


Figure 20. Thin Layer Chromatography of a Pronase Digest of Iodinated α-Lactalbumin

Iodinated α-lactalbumin was hydrolyzed with Pronase and chromatographed on a cellulose thin layer plate. Descending chromatography was run for 13 hours in n-butanol:0.5 N NH_2OH:absolute ethanol (5:2:1) at room temperature. Cross-hatched areas represent spots on X-ray film exposed to the TLC plate for 72 hours. Faint spots appear as broken circles. Origin (1) was spotted with 2 µl of Pronase digest of iodinated α-lactalbumin, (2) 4 µl of the same digest, (3) 5 µg of MIT, (4) 10 µg of MIT, (5) 12.5 µg of DIT, (6) 5 µg of DIT, (7) 10 µg of tyrosine. Control spots (3-7) were developed with ninhydrin spray.

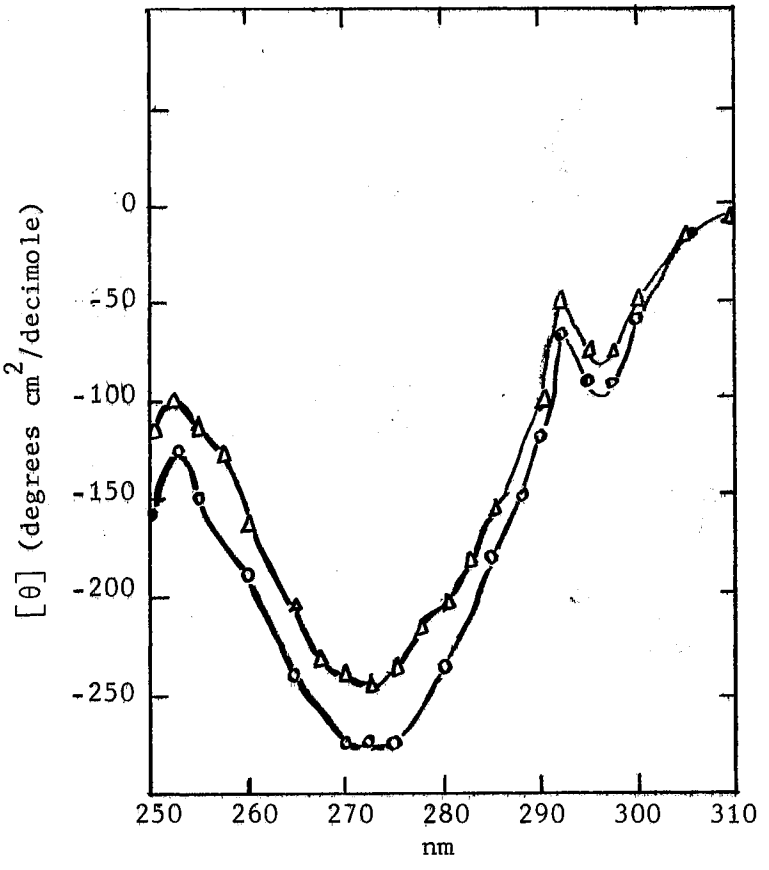


Figure 21. Near Ultraviolet Circular Dichroism Spectra of Iodinated and Native α-Lactalbumin

α-Lactalbumin was iodinated by the method of Morrison and Bayse (8) using lactoperoxidase (see Methods). The sample contained 0.56 moles of Iodine/mole of α-lactalbumin. Iodinated α-lactalbumin (Δ) and native α-lactalbumin (o). [θ] is the mean residue ellicipticity (mean residue weight was 118).

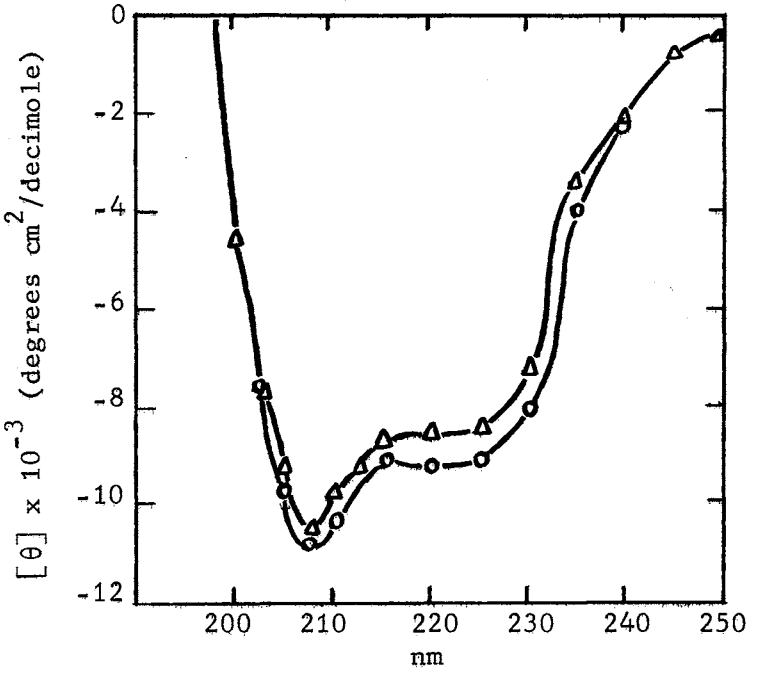


Figure 22. Far Ultraviolet Circular Dichroism Spectra of Iodinated and Native α-Lactalbumin

α-Lactalbumin was iodinated by the method of Morrison and Bayse (8) using lactoperoxidase (see Methods). The sample contained 0.56 moles of Iodine/mole of α-lactalbumin. Iodinated α-lactalbumin (Δ) and native α-lactalbumin (o). [θ] is the mean residue ellicipticity (mean residue weight was 118).

iodination causes an increase in the extinction coefficient of α-lactalbumin. This would result in the true concentration of α-lactalbumin being lower than the apparent concentration.

Summary of the Iodination of α-Lactalbumin with Lactoperoxidase

Approximately 0.5 moles of iodide per mole of α-lactalbumin was the maximum incorporation obtained using lactoperoxidase. Varying the reaction conditions did not produce a superior method for iodination. No significant loss of activity was noted. Peptide maps revealed the presence of two major iodinated peptides and eight minor iodinated peptides. Chromatography of a ronase digest of iodinated α-lactalbumin revealed the presence of both monoiodotyrosine and diiodotyrosine, however the labelling produced predominately monoiodotyrosine.

Thus the technique was not useful for determining if there exists a single critical tyrosyl residues.

CHAPTER IV

DISCUSSION

α-Lactalbumin has been the object of intensive investigation since its establishment as the B protein of lactose synthetase (2, 18). Much data has been accumulated on the physical structure of α-lactalbumin (50, 51, 52) and its properties in solution are well characterized (54, 55, 59, 60, 61, 62 and 63). However little is known about the molecular mechanism by which α-lactalbumin modifies the galactosyl transferase molecule. Kinetic studies by Morrison and Ebner (4) indicate that the apparent K_m for glucose is lowered in the presence of α-lactalbumin.

Mawal et al. (5) have shown by affinity chromatography that a complex can be formed between α-lactalbumin and the galactosyltransferase. This implies that there is a binding site or perhaps a large binding area on the surface of the α-lactalbumin molecule. Perhaps the best method for determining which of the 123 amino acids in α-lactalbumin are involved in this binding area is to modify specific amino acids and monitor the activity in the lactose synthetase reaction. If the reaction conditions are not too harsh (determined by running appropriate controls) any loss of activity should be due to blockage of a specific group or a resulting conformation change. The use of a variety of modification techniques should allow determination of critical amino acid residues and from this knowledge a better understanding of the function of α-lactalbumin should be obtained.

Several investigators have modified α-lactalbumin for various purposes (6, 27, 88, 90, 94, 95, 99, 100, 101, 102, 105, 106) but at the present few have reported the effect these modifications have on the activity of α-lactalbumin in the lactose synthetase reaction (6, 27, 95, 99, 100).

Lin (99) modifed the carboxyl groups of α-lactalbumin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and glycinamide. There was a rapid loss of lactose synthetase activity and an average of 20 carboxyl groups were modified in 400 minutes. No unique carboxyl group was found, although free carboxyl groups in α-lactalbumin may be essential for maintaining its biological activity. Partial protection was achieved in the presence of the galactosyltransferase.

Castellino and Hill (100) have examined the reaction of iodoacetate with bovine α-lactalbumin. They found a single methionine and three histidine residues were carboxymethylated. Examination of tryptic peptides indicated that methionine 90 was the most reactive residue with histidine 68, histidine 32 and histidine 107 reacting at slower rates respectively. Carboxymethylation of methionine 90 had little effect on the activity of α-lactalbumin, but there was a progressive loss in activity as the histidine residues are modified. When all three histidine residues are carboxymethylated about 40% of the activity of α-lactalbumin remained.

The nitration of α-lactalbumin has been reported by several investigators (6, 27, 94, 95). Habeeb and Atassi (94) found 2.5 tyrosyl residues and 1.4 tryptophyl residues modified upon nitration but they did not report any activity measurements. Robbins et al. (95) also found that tyrosines and tryptophans were modified with a loss of activ-

ity. Denton and Ebner (6) confirmed the modification of tyrosine and tryptophan and correlated the loss of activity with the loss of tyrosine on a time basis. They established that inactive polymers of α-lactalbumin were formed during the course of the reaction. Tryptic peptide mapping has shown that all four tyrosines are randomly modified (96). Klee and Klee (27) reported a lowered affinity for nitrated α-lactalbumin but found it could be substituted for native α-lactalbumin in enzyme assays if larger amounts of protein were used. It is unclear if the activity was due to nitrated α-lactalbumin or to unreacted native α-lactalbumin. This situation points out the need for careful quantitation of the extent of the modification reaction and for purification of the reaction products.

Denton and Ebner (6) have investigated the effect of iodination on the activity of α-lactalbumin. They found iodination of tyrosine, tryptophan, and histidine with the loss of activity again corresponding to the loss of tyrosine. Thus at the present there is only evidence for three different amino acids being involved in the binding site, tyrosine, histidine and possibly a carboxyl containing amino acid, glutamic or aspartic acid. Methionine 90 has been ruled out due to the fact that it can be carboxymethylated without loss of activity.

Although the evidence for tyrosine participation in the lactose synthetase reaction was already quite substantial, another modification was chosen to provide further evidence for the critical nature of the tyrosyl residues. α-Lactalbumin was acetylated with N-acetylimidazole which results in the formation of an acetyl group on the phenolic ring of tyrosine and an N-acetyl group on the ε-amino group of lysine. Since the O-acetyl group of tyrosine readily undergoes hydrolysis to

regenerate tyrosine, it is possible to assess the functional role of tyrosine and lysine separately by selective deacetylation.

The activity of α-lactalbumin in the lactose synthetase system was found to decrease with time as more residues are acetylated (Figure 2). Selective de-O-acetylation by treatment with hydroxylamine resulted in a regain of activity (Figure 3). This clearly establishes the tyrosines as the critical residues involved. The regain of activity excludes the possibility that inactivation was the result of the protein being denatured under the reaction conditions. It also allows the conclusion that the surface lysines (9 of the 12 total) are not critical for activity, since acetylation of the lysyl residues is irreversible under the conditions used to deacetylate the tyrosyl residues. This should not be taken to imply that all lysyl residues are unimportant. The buried residues may be very essential for preserving the structural integrity of the α-lactalbumin molecule.

To determine if a difference in reactivity existed between the four tyrosyl residues of α-lactalbumin, a correlation was made between the number of residues acetylated and the loss in activity. It appeared that the attack by N-acetylimidazole was random and complete. The loss of one mole of tyrosyl residue resulted in the loss of 26% of the activity, the loss of two moles of tyrosyl residue resulted in a 47% loss of activity etc. (Table IV). Total loss of activity was not observed and this was probably due to two factors; the spontaneous hydrolysis of N-acetylimidazole in water (effectively lowering the concentration of reagent) and the spontaneous de-O-acetylation of the tyrosyl residues.

Results obtained from the circular dichroism spectra indicate changes in the conformation of the protein occur upon acetylation (Figures 6 and 7). The expected change in elipticity at 252 nm (a tyrosyl absorption region) is present, but the results are complicated since there may also be contributions in this area from the disulfide bridges which are necessarily affected when there is a change in protein conformation. However, the spectra band at 252 nm returns to the same value as native α-lactalbumin upon de-O-acetylation, providing strong evidence that the change is due to the modification of the tyrosines. Evidence for a conformational change comes from the peptide band absorption area in the far ultraviolet region (Figure 7). There is an apparent increase in the α-helical content of α-lactalbumin upon acetylation. This apparent increase is reversible and disappears upon deacetylation.

Acetic anhydride also acetylates tyrosyl and lysyl residues but is reported to be a considerably more vigorous reagent (112) often leading to protein denaturation. Although acetic anhydride acetylates the same residues as N-acetylimidazole, a significant difference was found. Deactivation was not appreciably reversed under conditions which were successfully used to reactivate N-acetylimidazole inactivated α-lactalbumin. This could be due to a side reaction, perhaps another amino acid being modified, or a change in specificity among the amino groups modified.

Inactivation of α-lactalbumin was noticed when solutions were incubated at high pH to de-O-acetylate the tyrosyl residues. The loss in activity was time dependent and pH dependent (Figure 10) and involved very significant losses of activity. Attempts to reverse the inactiva-

tion were successful only to a small degree. Incubation of α-lactalbumin at high pH under a nitrogen atmosphere did not alter the rate of inactivation. Thus molecular oxygen was not participating in the reaction. The incubation was also run in the presence of low levels of mercaptoethanol, to observe if protection of the easily oxidized groups would prevent loss of activity. Rather than preventing loss of activity, the mercaptoethanol appeared to enhance the rate of inactivation, a 95% loss of activity was noted at pH 10.5 in six hours. This indicated that perhaps the mercaptoethanol acts as a catalyst in the deactivation process. The most likely role for mercaptoethanol is in promoting disulfide interchange. This could especially be the case if the disulfide bridges are strained when α-lactalbumin expands at high pH (62).

Since native α-lactalbumin contains no free sulfhydryl groups (52) it was possible to determine if any of the four disulfide bridges were broken during incubation at high pH by merely assaying for free sulfhydryl groups after readjustment of the pH. Reaction with Ellman's reagent (115) revealed that incubation at high pH did result in the cleavage of disulfide bridges into free sulfhydryl groups. The number of free sulfhydryl groups increased with incubation time (Figure 12). Assays on the incubated α-lactalbumin revealed that after six hours the sample had lost 53% of its activity and that 0.64 moles of sulfhydryl group were produced per mole of protein. The same sample was quantitatively assayed for reaction with antibodies to native α-lactalbumin and it was found that 60% of the antigentic activity was lost (Figure 15). This indicates that cleavage of a disulfide bond results in a change of conformation, to such an extent that the molecule has neither activity

in the lactose synthetase reaction, nor is it recognized by antibodies to native α-lactalbumin.

Kronman et al. (62) have reported that α-lactalbumin undergoes a structural change to an expanded conformation above pH 9.5. The expansion occurs very quickly and completely and results in changes in the fluorescence properties of the α-lactalbumin molecule. At high pH the emission maximum was shifted and the fluorescent intensity was decreased, but these changes were fully reversible when the pH was reduced (66). However the time of incubation at high pH was not reported.

Intermolecular interactions were also found above pH 9.5 (66). An association of low molecular weight species (dimers and trimers) was detected in ultracentrifuge measurements, which was rapidly reversed upon return to pH 6.0. Exposure of α-lactalbumin (30 mg/ml) to pH 10 and above fore periods of 24 hours led to the formation of a heavy component (molecular weight greater than 300,000). The formation of this heavy component increased with time and readjustment of the pH to 8.55 did not reverse the aggregation. It was also not reversed by the addition of 4 M urea. Kronman et al. (62) concluded that there was destruction of disulfide bonds as well as formation of intermolecular disulfide bonds. Although conditions used in this study to inactivate α-lactalbumin differ considerably in protein concentration and length of time from those which produced aggregation, a sample was chromatographed on an analytical gel filtration column to determine if large polymers were present (Figure 14). No evidence for significant polymerization was found.

Studies with low molecular weight disulfides have shown (124) that hydrolytic splitting occurs above pH 9.0 by the following mechanism:

$$RSSR + OH^- \quad RS^- + RSOH \qquad (4.1)$$

$$2RSOH \quad RSO_2H + RSH \qquad (4.2)$$

The equilibrium of reaction (4.1) is far to the left, and even in 0.1 M NaOH the equilibrium concentration of RS^- is very small. However if either of the components formed in reaction (4.1) is in some way removed from the equilibrium, the cleavage reaction will proceed to completion. Mercuric chloride (125) and p-hydroxymercuribenzoate (124) have been used to drive the reaction to completion in the studies on oxidized glutathione. Studies on proteins, insulin (126), ribonuclease (127), bovine serum albumin (128) and ovalbumin (128) indicated that splitting of disulfide bonds also occurs at pH 9 - 10 if the protein is denatured in urea solutions (128) or partially degraded as the result of proteolytic digestion (127). Anderson has reported the hydrolysis of bovine serum albumin dimer at pH 10 (129). Dimeric bovine serum albumin was prepared by oxidation of mercaptalbumin, and the rate of reversion back to the monomer was measured by gel filtration or centrifugation after stopping the exchange reaction by the addition of p-hydroxymercuribenzoate. One disulfide bond was split in approximately one hour and 40 minutes at pH 11, however it was pointed out that this was a composite ratio of several different disulfide bonds being split. The rate of hydrolysis of disulfide bonds appears to vary greatly, depending on several factors: the pH of the solution (124); the protein or disulfide bond concentration (127); the presence of catalytic amounts of metal ions (130); ionic strength (131) and steric strain (132). It is interesting to note that several investigators have found

that exclusion of molecular oxygen has no effect on the rate of disulfide bond hydrolysis (124, 128, 129, 131).

Cleavage of the disulfide bonds may proceed as illustrated in equation (4.1) and is the cause of the inactivation observed when α-lactalbumin is incubated at high pH. There is substantial evidence that the α-lactalbumin molecule exists in an expanded state at high pH (62). This expansion is the result of strong electrostatic forces generated by the increase in negative charge and may stain the disulfide bridges considerably, leading to a much more labile bond. Any condition which causes α-lactalbumin to assume a conformation other than the native conformation, (acid denaturation, chemical modification, high salt concentrations, denaturaing reagents such as urea or guanidine chloride) may cuase the disulfide bonds to be especially susceptible to cleavage and result in inactivation of the molecule.

Since there are four widely separated tyrosyl residues in bovine α-lactalbumin, it is likely that not all are essential for binding of the galactosyltransferase. Thus specific modification of the individual tyrosyl residues is needed to ascertain the number and location of the residues critical for lactose synthetase activity.

Enzymatic modification of the tyrosyl residues should possibly offer some selectivity of reaction, especially if any of the four residues are located in a crevice, or are partially buried in the surface of the protein and are thus inaccessible to the modifying enzyme. Since lactoperoxidase had been used by Phillips and Morrison (108) to incorporate iodide into the exposed tyrosyl and histidyl groups on the surface of the intact human erythrocyte, it was chosen for the iodination of α-lactalbumin. Lactoperoxidase is well suited for this type of

experiment due to its large size and stability under the reaction conditions. The required condition of the iodination reaction are very gentle with respect to pH, solvent, ionic strength, and temperature. The optimal concentrations of peroxide, iodide, and tyrosine for initial rates have been established by Morrison and Bayse (8). They also established the optimum pH and found evidence for a ping-pong type mechanism. Tyrosine was iodinated more rapidly than 3-Iodotyrosine. Histidine was also iodinated, but no evidence was found of iodination of other naturally occurring amino acids. The rate of the reaction can be varied by the amount of lactoperoxidase used to catalyze the reaction. Analysis of the number and position of the labelled tyrosyl residues can be made by separation of the peptides after digestion with trypsin. Characterization of the peptide is then performed to determine the location of the critical residue. It appeared probable that any residue in α-lactalbumin near enough to the surface to interact with the galactosyltransferase would also be available for enzymatic modification.

Approximately 0.5 moles of iodide per mole of α-lactalbumin were incorporated using lactoperoxidase. No reaction conditions were found which would give a significant increase in incorporation. No significant loss of activity was observed upon iodination. This could be random incorporation, which would be difficult to determine by the lactose synthetase assay for α-lactalbumin, or it may be that the critical residue is not available for labelling. Peptide maps revealed the presence of two major iodinated peptides and eight mino iodinated peptides. If monoiodination of the tyrosyl residues was the only product, the expected number of labelled peptides would be three (one peptide con-

tains two tyrosyl residues). However the presence of the eight minor labelled peptides proves that the reaction is not specifically labelling the tyrosines with a single iodine. Further evidence was provided by Pronase digestion of iodinated α-lactalbumin. Chromatography of the digest revealed that incorporation was largely monoiodotyrosine but significant amounts of diiodotyrosine were also present.

Thus the technique lacked the requied specificity to elucidate any essential tyrosyl residue. Exclusion experiments using the galactosyltransferase in a deadend complex to block the essential residues of α-lactalbumin would be the best way to determine their number and position. Unfortunately at this time the galactosyltransferase is not available in the quantities required.

SUMMARY

Acetylation of α-lactalbumin causes loss of activity. De-O-acetylation of the tyrosyl residues under mild conditions results in reactivation thus establishing the tyrosyl residues as necessary for biological activity. Since the exposed lysyl groups are irreversibly acetylated during this procedure, the surface lysyl groups are not necessary for biological activity. The degree of acetylation of the tyrosyl residues was correlated with the loss of enzymatic activity however, no differences were found between the four tyrosyl residues. Results obtained from the circular dichroism spectra indicate that changes in the conformation of the protein have occurred upon acetylation which are largely but not totally reversible. The use of acetic anhydride for acetylation also caused an inactivation of α-lactalbumin. In contrast to the acetylation with N-acetylimidazole the inactivation was not reversible. Presumably there is an undetected side reaction or a change in specificity.

Incubation of α-lactalbumin at high pH resulted in a time dependent loss of activity. This loss of activity was largely irreversible under the conditions employed. No difference was found in the rate of inactivation when molecular oxygen was excluded from the reaction. The presence of low levels of mercaptoethanol increased the rate of deactivation. There was a time dependent cleavage of the disulfide bridges which corresponded to the loss of activity in the lactose synthetase reaction, and to a loss of antigenic activity. Electrophoresis of the

modified material revealed the presence of an additional band. Circular dichroism spectra of the base treated material differed from native α-lactalbumin only in the area designated as the cystine absorption region. The effect of high pH on α-lactalbumin is most likely due to disulfide cleavage.

Approximately 0.5 moles of iodide per mole of α-lactalbumin was the maximum incorporation obtained using lactoperoxidase. Varying the reaction conditions did not produce a superior method for iodination. No significant loss of activity was noted. Peptide maps revealed the presence of two major iodinated peptides and eight minor iodinated peptides. Chromatography of a Pronase digest of iodinated α-lactalbumin revealed the presence of both monoiodotyrosine and diiodotyrosine, however the labelling was predominately monoiodotyrosine. Thus the technique was not useful for determining if there exists a single critical tyrosyl residue.

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VITA

Charles Richard Merriman

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE EFFECT OF TYROSYL MODIFICATION AND HIGH pH ON THE ACTIVITY OF α-LACTALBUMIN

Major Field: Biochemistry

Biographical:

Personal Data: Born in Woodward, Oklahoma, October 14, 1946, the son of Charles L. and Lolabelle Merriman.

Education: Graduated from Woodward High School, Woodward, Oklahoma in May, 1964; received the Bachelor of Science degree from Northwestern State College, Alva, Oklahoma with majors in Chemistry and Biology in August, 1968; completed requirements for Doctor of Philosophy degree in May, 1973.

Professional Experience: Recipient of a National Science Foundation Fellowship from September 1968 to September 1970. Served as a research assistant at Oklahoma State University from September 1970 to August 1972.