

A Label Selection Procedure for Determining the Location of Protein-Protein Interaction Sites by Cross-linking with Bisimidoesters

APPLICATION TO LACTOSE SYNTHASE*

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A procedure is described that is designed to identify the primary site of cross-linking by bisimidoesters of a component of an interacting protein system. It is based on the mutually exclusive nature of acetylation and amidination. The procedure has been applied to the regulatory protein of lactose synthase, α -lactalbumin.

A sample of bovine α -lactalbumin was acetylated with a trace amount of high specific activity [^3H]acetic anhydride to produce a population of protein molecules essentially all of which contain 0 or 1 acetyl group; partial labeling of all 13 amino groups was obtained. This material was mixed with bovine colostrum galactosyltransferase in the presence of Mn^{2+} , UDP-glucose, and *N*-acetylglucosamine, at pH 8.0, to promote complex formation and was cross-linked with dimethyl 3,3'-dithiobispropionimidate. Covalently cross-linked α -lactalbumin-galactosyltransferase (1:1) complex with characteristic enzymic and other properties was purified from the reaction mixture, and the distribution of [^3H]acetyl label on each amino group of the α -lactalbumin component was determined, using procedures similar to those described in previous differential labeling studies (Richardson, R., and Brew, K. (1980) *J. Biol. Chem.* 255, 3377-3385). In comparison with the original labeled sample used for cross-linking, the specific activity of tritium label in 10 amino groups showed little change, whereas the labeling of three groups was changed markedly. The acetyl moiety on the ϵ -amino groups of lysines 5 and 108 showed major decreases in specific activity while that of lysine 114 was greatly increased. Similar results were obtained when the cross-linking was performed under different conditions of temperature, and cross-linker concentration. As the changes in lysines 5 and 114 are similar to those observed in differential labeling, they are attributed to alterations in the affinity for galactosyltransferase resulting from acetylation of these groups. In contrast, lysine 108, which is not sufficiently close to the interaction site to be perturbed in differential labeling studies but is greatly decreased in tritium content in the cross-linked complex, appears to represent the major site through which α -lactalbumin is cross-linked to galactosyltransferase as a result of the exclusion of protein molecules acetylated in this position from covalent cross-linking. Studies with a homologous series of bis-

imidoesters indicate that lysine 108 is situated 6.1 to 7.3 Å from an amino group on galactosyltransferase in the cross-linked complex. The general utility of the procedure and the nature of the interaction site in lactose synthase are discussed.

Bifunctional imidoesters have proved to be valuable reagents for probing protein-protein interactions and have aided in studies of the oligomeric structures of enzymes and the topology of protein associations in such complex structures as ribosomes and biological membranes (see Refs. 1 and 2). In reversibly associating systems of proteins, such cross-linking reagents have been used to obtain information about the functional properties of the system when "frozen" in an associated state. Because of the nature of such systems, there are considerable theoretical objections to many approaches to determining the location of the association sites on the surfaces of the interacting components. Conventional chemical modification may give misleading information because of the ease of perturbation of the association by even subtle conformational alterations, while the protective labeling approach requires conditions where a high proportion of the proteins under study are in the associated state, a situation that may not be readily attainable.

Identification of major sites of cross-linking in covalently cross-linked complexes in such systems can give information about the location of the interaction site and the distances between specific groups on the interacting proteins and can form an important corollary to functional studies.

Lactose synthase consists of two protein components which associate in a reversible, ligand-dependant manner to form 1:1 complexes during the catalytic cycle. The catalytic component, galactosyltransferase, is an enzyme that in isolation catalyzes the transfer of galactose from UDP-galactose to GlcNAc¹ or nonreducing terminal GlcNAc in the oligosaccharide moieties of *N*-glycosidically linked glycoproteins but does not catalyze lactose synthesis at significant rates under normal conditions because of a high K_m for glucose (about 2 M). The regulatory protein of lactose synthase, α -lactalbumin, promotes the binding of glucose to galactosyltransferase through a series of synergistic equilibria, so that the K_m is lowered by 10^3 , and lactose synthesis is catalyzed effectively at physiological concentrations of glucose. Galactosyltransferase is found in many tissues where it functions in catalyzing the transfer of galactose to GlcNAc in glycoproteins; the production of α -lactalbumin in the mammary gland during lactation directs the production of lactose only in this tissue. α -Lactalbumin is

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¹ The abbreviations used are: GlcNAc, *N*-acetylglucosamine; SDS, sodium dodecyl sulfate.

homologous in primary structure and similar in three-dimensional structure to lysozymes of the chicken egg-white group, and so the identification of functional areas in α -lactalbumin is of interest in the elucidation of the structural basis of its evolutionary functional divergence from these lysozymes, as well as in relation to the molecular basis of regulation in lactose synthase (see Refs. 3 and 4).

Previously, cross-linking with dimethyl pimelimidate (5) and dimethyl suberimidate (6) has been used to produce stable, enzymically active 1:1 complexes of α -lactalbumin and galactosyltransferase, which have proved useful in kinetic (5, 7) and other studies (6) and show maximal regulatory effects of α -lactalbumin. However, attempts to chemically identify the site of cross-linking, using radiochemically labeled dimethyl pimelimidate met with failure owing to the distribution of radioactive label into many peptide pools after cleavage, possibly as a result of nonspecific incorporation of reagent and a partial lability of the amidine linkage.² We report here the results of a different approach to identify the site of cross-linking on α -lactalbumin which has potential applicability to other systems.

The procedure utilizes a preparation of one interacting component (α -lactalbumin in the present case) which has been labeled by acetylation with a trace amount of high specific activity [³H]acetic anhydride. At low levels of labeling, this produces a population of protein molecules of which the major proportion bears zero or one acetyl group (see "Results"). All amino groups are to some extent labeled under these conditions (8). As partial acetylation does not inactivate the protein, on cross-linking such a labeled preparation with the other interacting component (galactosyltransferase) under conditions that favor complex formation, all species of α -lactalbumin should be able to form covalently cross-linked complexes except those molecules bearing a [³H]acetyl group on the amino group that represents the site of cross-linking. As imidoesters are highly specific for amino groups (1), and acetylation precludes this reaction, molecules labeled at the site of cross-linking will be excluded from forming covalent complexes. Consequently, the α -lactalbumin component of the purified cross-linked complex will contain no tritium label at this position. In a situation where there is a major site of cross-linking, together with additional minor sites, α -lactalbumin molecules that are incorporated into complexes should show a major drop in labeling at the major site of cross-linking.

The possibility also exists that, although acetylation does not inactivate α -lactalbumin, acetylation at specific positions may inhibit or enhance interaction with galactosyltransferase. Under circumstances where there is competition between these molecules for binding (*i.e.* α -lactalbumin excess), this could lead to discrimination against or in favor of molecular species that are acetylated in such positions. It should be noted that the labeling pattern in the cross-linked material will not be affected by reactions with the cross-linking reagent that are not essential for the formation of cross-linked complexes, *i.e.* internal cross-linking and monofunctional modification.

In this study we have used the disulfide containing reagent dimethyl 3,3'-dithiobispropionimidate so that, after purification of the α -lactalbumin-galactosyltransferase complex, cross-links can be cleaved and blocked by reduction and carboxymethylation, to facilitate our strategy for cleaving the molecule during the analysis of labeling patterns. Using procedures closely similar to that previously described for differential labeling studies (8), the specific activity of ³H in each of the 13 amino groups was determined as a ³H/¹⁴C ratio in

samples of the protein prior to cross-linking, and in the α -lactalbumin component of the cross-linked complex. Ten of these amino groups showed little change in reactivity, while three showed marked changes. The ϵ -NH₂ groups of lysines 5 and 108 showed distinctly lowered labeling in the cross-linked preparation, whereas that of lysine 114 was considerably increased. Similar results were obtained in two separate experiments where the cross-linking was performed under different conditions of temperature and cross-linker concentration. From a comparison with the results of previous differential labeling studies (8), it appears that lysines 5 and 114 are close to the association site for galactosyltransferase; acetylation of the former inhibits interaction, but that of the latter favors interaction by negating charge repulsion between the ϵ -NH₂ group of this residue and a positively charged group on galactosyltransferase. In contrast, the lowered specific activity of lysine 108, a residue that was not perturbed under differential labeling conditions, suggests that it is the major site of cross-linking in the lactose synthase complex. Other studies which define the distance of this residue from an amino group in galactosyltransferase are reported.

EXPERIMENTAL PROCEDURES³

Materials—Bovine colostrum galactosyltransferase and bovine α -lactalbumin were purified as described previously (8). The concentrations of solutions of α -lactalbumin were determined from the absorbance at 280 nm assuming a value for $E_{280}^{1\%}$ of 20.0. Galactosyltransferase concentrations were determined from activity measurements under standard conditions as described in previous publications (8).

The hydrochlorides of dimethyl 3,3'-dithiobispropionimidate and dimethyl pimelimidate were purchased from Pierce. Dimethyl malonimidate, dimethyl succinimidate, dimethyl glutarimidate, and dimethyl adipimidate were gifts from Dr. J. Hajdu and Dr. V. Dombradi, Institute of Enzymology, Hungarian Academy of Sciences, Budapest and Institute of Medical Chemistry, University of Medicine, Debrecen, Hungary. [³H]Acetic anhydride (6.0 Ci/mmol) and [¹⁴C]acetic anhydride (118.6 nCi/mmol) were obtained from Amersham. DEAE-cellulose (DE52) was purchased from Whatman and Spherisorb 5 μ ODS was from Laboratory Data Control. All other reagents were from the same sources as in previous studies (8).

Determination of Radioactivity—³H/¹⁴C ratios in individual *N*-acetyl amino groups were determined by drying suitable aliquots from column fractions or pools, redissolving by the addition of 0.75 ml of water and 7.5 ml of scintillation fluid and counting in the standard ³H and ¹⁴C channels of a Beckman scintillation counter. ³H counts were corrected for a 42% overlap of ¹⁴C counts; there was no overlap of ³H counts into the ¹⁴C channel.

Cross-linking with Dimethyl 3,3'-Dithiobispropionimidate—Cross-linked α -lactalbumin-galactosyltransferase (1:1) complex was prepared and purified by a slight modification of procedures used in previous work with dimethyl pimelimidate (5). Galactosyltransferase (*M_r* = 50,000) purified from several different batches of bovine colostrum was pooled and concentrated by ultrafiltration at 4 °C to a protein concentration of 0.3–0.6 mg/ml. Trace *N*-[³H]acetylated bovine α -lactalbumin was added to a concentration of 1–2 mg/ml and the mixture was subjected to gel filtration with columns of Sephadex G-25 (2.2 × 32 cm) equilibrated and eluted with 0.1 M Tris hydrochloride buffer containing 5 mM *N*-acetylglucosamine, pH 8.0, at 4 °C. MnCl₂ (1 M solution), *N*-acetylglucosamine, and UDP-glucose were added so as to bring their final concentrations to 2, 25, and 2 mM, respectively. Finally a solution of dimethyl 3,3'-dithiobispropionimidate hydrochloride (20 or 40 mg/ml) in 0.1 M Tris-HCl, adjusted to pH 8.0 with NaOH, was added to bring its final concentration to 2 or 4 mg/ml. The cross-linking reaction was allowed to proceed for 3 h at 15 or 25 °C before termination by application to a column of Sephadex

³ Portions of this paper (including parts of "Experimental Procedures" and "Results," Figs. 3 to 14, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 80M-2422, cite author(s), and include a check or money order for \$7.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

² K. Brew, unpublished results.

G-75 (superfine) equilibrated with 0.02 M Tris hydrochloride buffer, pH 7.4, at 4 °C. The column, which was used for the initial purification of the cross-linked complex, was eluted with the same buffer. Fractions were analyzed for radioactivity content, *N*-acetylglucosamine synthase activity, and lactose synthase activity at 5 mM glucose in the absence of α -lactalbumin (5). Details of the further purification and characterization of the cross-linked complex are given under "Results."

Cross-linking with Other Bisimidoesters—Cross-linking experiments with dimethyl malonimide, dimethyl succinimide, dimethyl glutarimide, dimethyl adipimide, and dimethyl pimelimide were performed under similar conditions. To solutions of (3–4 ml) galactosyltransferase (0.12 mg/ml) containing 1.5 mg/ml of α -lactalbumin, 2 mM $MnCl_2$, 2 mM UDP glucose, and 25 mM *N*-acetylglucosamine in 0.1 M Tris-HCl buffer, pH 8.0, a solution of cross-linker (100 mg/ml) in 2 M Tris-HCl, pH 8.0, was added to give a final cross-linker concentration of 4 mg/ml. The reaction was allowed to proceed for 3 h at 15 °C; then the reaction mixture was directly applied to a Sephadex G-75 (superfine) gel filtration column (1.6 \times 95 cm). The column was equilibrated and eluted with 0.02 M Tris-HCl, pH 7.4, at 4 °C at a flow rate of 5.7 ml/h and 1.9-ml fractions were collected. After locating the protein fractions by A_{280} measurement the fractions were assayed for *N*-acetylglucosamine synthase activity as well as for lactose synthase activity.

RESULTS

Acetylation of α -Lactalbumin—As the experimental procedure involved the binding of acetylated α -lactalbumin with galactosyltransferase, the effects of different levels of *N*-acetylation on the activity of α -lactalbumin was examined. For this purpose, α -lactalbumin was acetylated with different amounts of low specific activity [^{14}C]acetic anhydride, the extent of incorporation being determined by radioactivity content. The activity of the modified protein was determined by relating the lactose synthase activity at two different concentrations, in the presence of a fixed concentration of galactosyltransferase, to a standard curve constructed with different concentrations of unmodified α -lactalbumin with the same amount of galactosyltransferase under the same conditions.

These results (data not shown) showed that at low levels of modification (up to two acetyl groups/molecule) α -lactalbumin showed an activity decrease of about 10%, but higher degrees of acetylation produced a progressive loss of activity down to about 30% when 10 groups/molecule were modified. From this, it can be deduced that low levels of acetylation do not produce any major disruption in the conformation and that there is no specific amino group in α -lactalbumin where acetylation produces complete inactivation.

The *N*-[3H]acetylated α -lactalbumin used for cross-linking, prepared by reaction with high specific activity (6 Ci/mmol) [3H]acetic anhydride (see miniprint supplement), was modified to the extent of 0.082 groups/molecule, calculated on the basis of the specific activity of the acetic anhydride, and contained 1.33×10^7 cpm/mg as measured under our counting conditions. Based on this level of modification, a simple calculation of the probability that any single molecule will be modified in two amino groups out of the 13 is approximately 0.3%. Hence, of the labeled α -lactalbumin molecules in this preparation (1 out of 12), 96% will be *N*-acetylated at only one position in the polypeptide chain.

Conditions of Cross-linking—Cross-linking of galactosyltransferase and *N*-[3H]acetyl- α -lactalbumin was performed in the presence of appropriate ligands to promote complex formation (Mn^{2+} , UDP-glucose, and GlcNAc; see Ref. 8) at pH 8.0. Two separate cross-linking experiments were carried out using somewhat different conditions to establish the reliability of the procedure as an indicator of cross-linking sites; the primary site of cross-linking should be independent of the extent of cross-linking and strength of interaction of the

component proteins. In the first experiment, 7.35 mg (0.334 mg/ml) of galactosyltransferase and 19.0 mg of [3H]- α -lactalbumin (0.86 mg/ml), in a total volume of 22.0 ml buffer, were treated with 44 mg of cross-linking reagent (2 mg/ml) for 3 h at 15 °C under the conditions described under "Experimental Procedures." In the second experiment 4.2 mg (0.32 mg/ml) of galactosyltransferase and 17.0 mg (1.3 mg/ml) of α -lactalbumin in 13.0 ml of buffer were treated with 52.0 mg (4 mg/ml) of reagent for 3 h at 25 °C. Thus, the second experiment was performed under conditions designed to produce a greater extent of modification by the cross-linking reagent (higher concentration and higher temperature).

Isolation and Characterization of the Cross-linked Complex—After cross-linking, samples were subjected to the same separation procedure and the purified complex in each case was analyzed for distribution of radioactivity by a closely similar method. As the results are (qualitatively) in good agreement, we present here a set of complete results from the first experiment (cross-linking performed at 15 °C) together with the final results from the second experiment.

The initial separation of the cross-linked reaction mixture is shown in Fig. 1. It can be seen that a considerable amount of oligomeric material is formed under the reaction conditions. This material was excluded from the Sephadex G-75 column (4.3 \times 84 cm) and showed little enzymic activity. Although this material contains some α -lactalbumin, as indicated by tritium content, it was not examined further in this study. A similar peak was obtained with the second cross-linked preparation. The presence of specifically cross-linked lactose synthase complex is shown by the peak of lactose synthase activity (fractions 68–80), emerging at the leading edge of the uncross-linked galactosyltransferase peak (*N*-acetylglucosamine synthase activity). The presence of α -lactalbumin is indicated by the presence of tritium counts. Two further peaks of tritium-labeled material were obtained (fractions 88–105 and 110–130) which, on the basis of previous studies, were

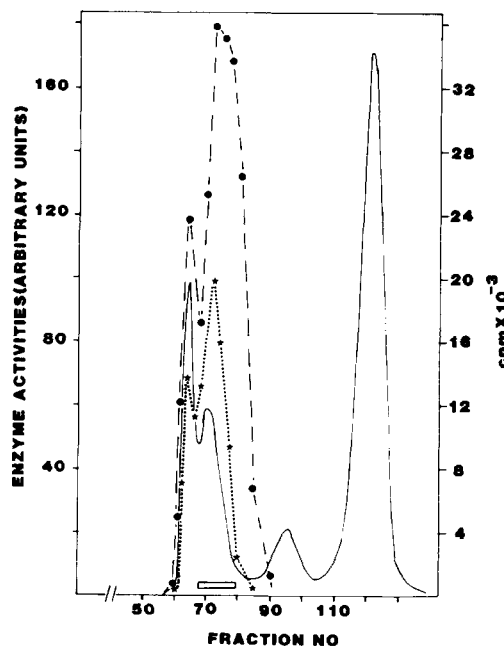


FIG. 1. Isolation of cross-linked complex by gel filtration with a column (4.3 \times 84 cm) of Sephadex G-75 (superfine). Fractions, 5.4 ml, were collected and 20- μ l aliquots were taken for tritium determination by scintillation counting. —, distribution of tritium counts in the fractions; —●—, *N*-acetylglucosamine synthase activities; ★····, lactose synthase activities. The enzyme activities are in arbitrary units. The fractions which contained cross-linked complex are marked by the bar and were combined.

judged to be α -lactalbumin dimer and monomer (5). The fractions with lactose synthase activity were pooled as indicated in Fig. 1, brought to 20 mM in GlcNAc and 10 mM in MnCl_2 , and applied to a column (1.3×25 cm) of α -lactalbumin-Sepharose equilibrated with 0.02 M Tris hydrochloride, pH 7.4, containing 20 mM GlcNAc and 10 mM MnCl_2 at 4 °C. Free galactosyltransferase is absorbed by this column under these conditions, whereas the cross-linked complex is not (5). The applied radioactive material was washed from the column with Tris buffer containing GlcNAc and MnCl_2 in a volume of 59 ml, which was concentrated to 15 ml by ultrafiltration at 4 °C. Unlabeled α -lactalbumin (5 mg) was added as a precautionary measure to displace any noncovalently bound α -lactalbumin that might be associated with the preparation, and the cross-linked enzyme was subjected to a final purification by reseparation with a column (4.3×84 cm) of Sephadex G-75 (superfine) under the same conditions as before. Fractions were again assayed for tritium content, lactose synthase activity in the absence of exogenous α -lactalbumin, and *N*-acetyl-lactosamine synthase activity. A small proportion of radioactivity emerged in the void volume, but the bulk was retarded in a single peak which coincided with both lactose synthase and *N*-acetyl-lactosamine synthase activity, as shown in Fig. 2. In keeping with the previously observed properties of the complex, the lactose synthase activity exceeded *N*-acetyl-lactosamine synthase activity by a factor of 2–3 (5). Although the removal of uncross-linked galactosyltransferase is not essential in these experiments, as the procedure is untried we chose to work with purified and characterized cross-linked complex. The fractions containing this complex were combined, and from the radioactivity content it was calculated that the total α -lactalbumin present was 0.43 mg. The preparation was dialyzed extensively against 1 mM ammonium bicarbonate at 4 °C, freeze-dried, and stored at –20 °C.

The material was further characterized by polyacrylamide gel electrophoresis in the presence of SDS (9), taking advantage of the cleavable nature of the cross-link. Samples were

subjected to electrophoresis before and after treatment with 1% mercaptoethanol for 4 h at 37 °C. After slicing, gels were solubilized and labeled bands were located by scintillation counting (10). The mobilities were compared to those of standard proteins electrophoresed under the same conditions. In the unreduced sample the radioactivity migrated as a single band with a mobility corresponding to a molecular weight of 65,000, closely similar to that expected for a 1:1 complex of α -lactalbumin ($M_r = 14,000$) and colostrum galactosyltransferase ($M_r = 50,000$), while the reduced sample showed a single band with an apparent molecular weight of 15,000, similar to that of uncross-linked α -lactalbumin.

Determination of the Distribution of [^3H]Acetyl Label between Individual Amino Groups—As discussed above (see the introduction to the text) the rationale of this procedure for identifying the major site of cross-linking on the α -lactalbumin component of the covalent complex is that because acetylation and amidination are mutually exclusive any amino group that represents the major cross-linking site will contain zero or minimal ^3H label in the protein isolated as a component of the complex. As this effect could produce a major reduction in label in that position rather than a complete exclusion of label, it is necessary to compare the distribution of label in the α -lactalbumin component of the complex with that of the original [^3H]acetylated protein used to generate the complex. Selection for or against molecules acetylated at residues near the interaction site might also be expected to perturb the labeling pattern. To obtain accurate quantitative information on the ^3H label distribution, as in previous protective labeling studies (8), an aliquot of a preparation of α -lactalbumin that had been uniformly *N*-acetylated with an excess of [^{14}C]acetic anhydride was added as an internal standard together with unlabeled α -lactalbumin (40 mg) to ensure the production of adequate amounts of peptides for separation and characterization. In the case of the cross-linked complex, labeled α -lactalbumin was not separated from the unlabeled galactosyltransferase component after cleavage of the cross-links and disulfide bonds by reduction and carboxymethylation. However, the addition of excess carrier α -lactalbumin ensured that peptides generated from the galactosyltransferase component (approximately 1.5 mg) did not interfere with the identification of peptides derived from α -lactalbumin.

For each cross-linked preparation, a sample of [^3H]acetylated uncross-linked α -lactalbumin of similar radioactivity content was subjected to the same treatment in parallel. The two samples (designated XL and control (c), respectively) were mixed with [^{14}C]acetylated protein to the same $^3\text{H}/^{14}\text{C}$ ratio (5.4:1 in the first and 25:1 in the second experiment). Carrier α -lactalbumin (40 mg) was then added. Each sample was completely acetylated in 8 M urea at pH 8.0 with an excess of unlabeled acetic anhydride, reduced with 10 mM dithiothreitol, and carboxymethylated with iodoacetic acid to produce a chemically uniform sample. The two samples, XL and control, were simultaneously fragmented as indicated in Scheme 1 to give peptides containing individual amino groups. CNBr cleavage of reduced, carboxymethylated α -lactalbumin at the single residue of methionine gives two fragments: CN1 (residues 1–90) and CN2 (residues 91–123). Thermolysin cleavage of CN1 generates peptides containing individual labeled residues (the NH_2 -terminal glutamyl residue and lysines 5, 13, 16, 58, 62 and 79). Cleavage of CN2 with thermolysin gives one peptide containing two residues (lysines 93 and 94) and others containing single labeled residues (lysines 98, 108, 114, and 122). The labeling of lysines 93 and 94 were separately examined in the second experiment by scintillation counting of the residues released during manual Edman degradation of CN-2 (steps 3 and 4). All peptides were purified to a high

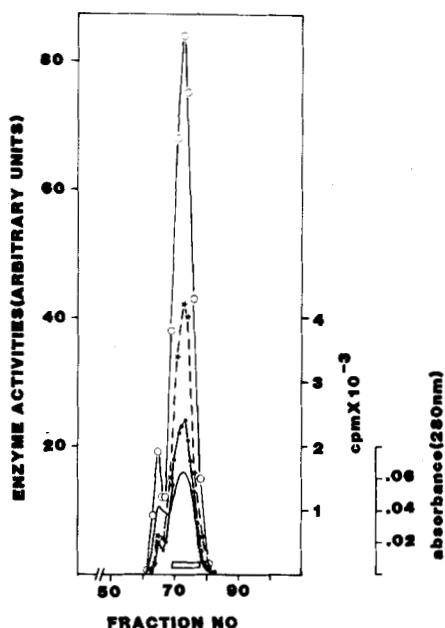
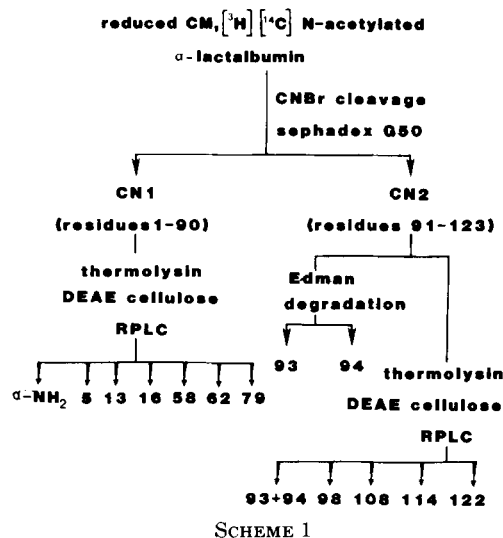


FIG. 2. Repurification of the cross-linked complex with Sephadex G-75 (superfine) column after removal of free galactosyltransferase with a column of α -lactalbumin-Sepharose. Fractions, 5.4-ml, were collected. —, A_{280} ; ●—●, tritium counts in 20- μl aliquots; ★—★, *N*-acetyl-lactosamine synthase activity; ○—○, lactose synthase activity. Both the enzyme activities are presented in arbitrary units.

degree by ion exchange chromatography and reverse phase liquid chromatography (RPLC). The details of these separations are given in the miniprint supplement (Figs. 3-14). The identity and purity of peptides was established by amino acid analysis, together with knowledge of their CNBr fragment of origin and the sequence of bovine α -lactalbumin (11). $^3\text{H}/^{14}\text{C}$ ratios were determined on peptides only after they had given satisfactory amino acid analyses. The amino acid compositions of peptides obtained in the first experiment are given in Tables I and II (miniprint supplement). Those obtained from the two samples in the second experiment are not given, but were of a similar high level of purity.

The $^3\text{H}/^{14}\text{C}$ ratios in the α -NH₂ group and individual lysine residues from the cross-linked and control sample in the first experiment are given in Table III. As in previous protective labeling experiments, where acetylation was performed under somewhat different conditions, a wide variation in ratio between individual residues can be seen. The general labeling pattern is similar to that previously obtained (e.g. high levels of labeling in lysines 98, 13, and 5). We have also defined a "selection factor," $R_s = r_{\text{XL}}/r_{\text{C}}$, where r_{XL} is the $^3\text{H}/^{14}\text{C}$ ratio in a peptide from the cross-linked sample and r_{C} is the ratio in the corresponding peptide from the control sample. Values for R_s are given in Table III for individual amino groups from both the first and second experiment.

It is evident from these data that 10 residues (α -NH₂ group,



SCHEME 1

lysines 13, 16, 58, 62, 79, 93, 94, 98, and 122) show little change in $^3\text{H}/^{14}\text{C}$ ratio between cross-linked and control samples, although it is possible that those with lower R_s values (e.g. Glu 1) and higher values (e.g. Lys 16) in both experiments may be the subject of selection to a minor extent. Three residues, lysines 5, 108, and 114, show major changes in labeling in both experiments, lysine 5 ($R_s = 0.31, 0.17$) and lysine 108 ($R_s = 0.35, 0.24$) being greatly decreased in ^3H content while lysine 114 ($R_s = 2.13, 4.30$) is considerably increased. The clear division of R_s values into unperturbed and perturbed groups can be seen in the graphical display of the same data in Fig. 15; the same pattern of selected and unselected labeled residues was obtained in the two experiments despite considerable differences in the conditions used for cross-linking.

Minimal Size of Reagent for Effective Cross-linking—Following the procedure described under "Experimental Procedures," a homologous series of bisimidoesters were surveyed for their ability to cross-link the components of lactose synthase. The formation of covalent complexes is indicated by a peak of lactose synthase activity (assayed at 5 mM glucose)

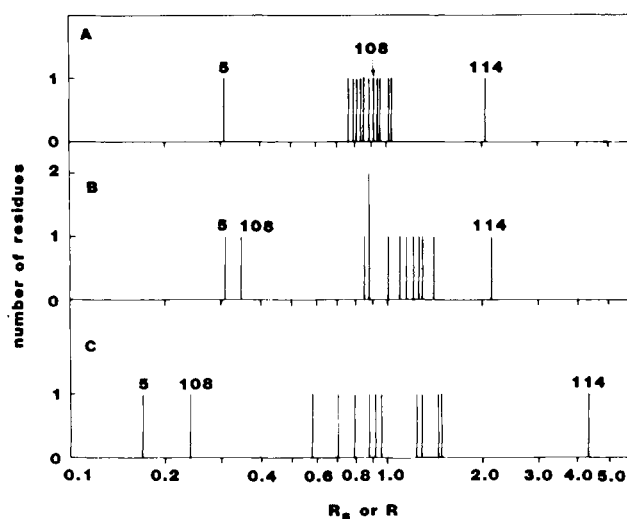


FIG. 15. Values of the selection factor (R_s) obtained from first (B) and second (C) cross-linking experiments. A, the inverse of the "protection factor" (R) obtained in the differential labeling experiment (Table II of Ref. 8) for comparison of the results. The numbers identify the positions in the α -lactalbumin sequence of lysine residues discussed in the text.

TABLE III

$^3\text{H}/^{14}\text{C}$ ratios for individual amino acids from complex (r_{XL}) and control (r_{C}) samples from first experiment and selection factor (R_s) values from first and second experiments

Amino groups	α -NH ₂	Lys 5	13	16	58	62	79	93	94	98	108	114	122
r_{XL}	0.74	2.47	3.78	14.7	1.69	2.63	1.10	ND ^a	ND	24.40	0.50	5.05	5.09
								2.94 ^b					
r_{C}	0.87	7.86	2.92	10.5	1.67	2.09	1.00	ND	ND	19.90	1.44	2.38	4.39
								3.35 ^b					
$R_s = \frac{r_{\text{XL}}}{r_{\text{C}}}$ (first experiment)	0.85	0.31	1.30	1.41	1.01	1.27	1.10	ND	ND	1.22	0.35	2.13	1.16
								0.88 ^b					
$R_s = \frac{r_{\text{XL}}}{r_{\text{C}}}$ (second experiment)	0.58	0.17	1.23	1.44	0.92	1.49	0.79	0.88 ^c	0.70 ^c	1.28	0.24	4.30	0.96
								0.54 ^b					

^a ND, not determined.

^b Values from peptides containing two lysines. As indicated in the text, the values from the cross-linked material may not be a good measure of the labeling in these residues because of the effect of nonspecific chemical modification.

^c Determined by Edman degradation of a sample of CN2.

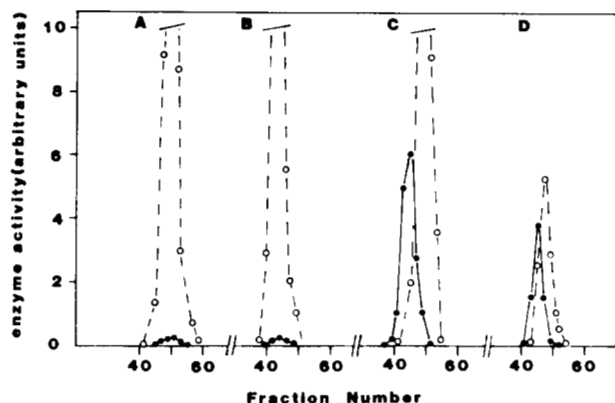


FIG. 16. Isolation of cross-linked complex of α -lactalbumin and galactosyltransferase with various bisimidoesters. Cross-linking was performed in each case as described under "Experimental Procedures." Separation of reaction mixtures was carried out by gel filtration with columns (1.6 \times 94 cm) of Sephadex G-75 (superfine) equilibrated and eluted with 0.02 M Tris-HCl, pH 7.4, at 4 $^{\circ}$ C. Fractions of 1.9 ml were collected at a flow rate of 5.7 ml/h. \circ - \circ , N-acetyllactosamine synthase activities; \bullet - \bullet , lactose synthase activities, indicating the formation of cross-linked complex. The bisimidoesters used, along with their maximal effective reagent lengths, were as follows: A, dimethyl succinimide, 4.9 \AA ; B, dimethyl glutarimide, 6.1 \AA ; C, dimethyl adipimide, 7.3 \AA ; D, dimethyl pimelimide, 8.5 \AA . Reaction with dimethyl malonimide, 3.7 \AA , was also carried out, but the results are not shown here as the result was essentially identical with that of A and B.

eluting at the leading edge of the galactosyltransferase peak on gel filtration. It can be seen from Fig. 16 that the lower molecular weight reagents: dimethyl malonimide, dimethyl succinimide, and dimethyl glutarimide did not produce significant cross-linking. However, the higher homologues, dimethyl adipimide and dimethyl pimelimide were effective cross-linkers. It can be seen that the highest yield of cross-linked complex, as shown by lactose synthase activity, is obtained with dimethyl adipimide. This was observed in three separate experiments. Cross-linking with dimethyl pimelimide results in major losses in total enzyme activity (N-acetyllactosamine synthase activity) possibly through formation of cross-linked oligomeric forms of galactosyltransferase. SDS-gel electrophoresis of samples from these separations confirmed the presence of large proportions of covalent cross-linked 1:1 complexes after treatment with dimethyl adipimide and dimethyl pimelimide, and the absence of such species after cross-linking with the shorter reagents (data not shown).

DISCUSSION

Although cross-linking with bisimidoesters has been widely used in studies of protein interactions, few attempts have been made to identify specific sites involved in cross-linking. The aim of the present procedure was to identify the site of cross-linking on α -lactalbumin for galactosyltransferase in the lactose synthase complex using the mutual exclusivity of acetylation and amidination; as such it represents an extension of our previous studies of the location of the interaction site on α -lactalbumin (8).

Before discussing the results, there are some aspects to the methodology that should be mentioned. First, the experimental procedure draws on previous studies in which the conditions for cross-linking the components of lactose synthase to form a specific 1:1, functional complex were developed together with the procedure for purifying this covalent complex (5).

In the present case, it is clear that a proportion of the

galactosyltransferase present aggregates to form high molecular weight complexes, some of which cross-link with α -lactalbumin (Fig. 1). This probably reflects the tendency of bovine colostrum galactosyltransferase to aggregate into largely inactive species at protein concentrations greater than about 0.1 mg/ml.⁴ However, the presence of specifically cross-linked lactose synthase complex is shown by a peak of lactose synthase activity, measured in the absence of exogenous α -lactalbumin that is included on gel filtration with columns of Sephadex G-75, but elutes on the leading edge of the main peak of N-acetyllactosamine synthase activity (uncross-linked galactosyltransferase). After purification by absorbing uncross-linked enzyme with α -lactalbumin-Sepharose in the presence of GlcNAc, the covalent complex is obtained in a pure state by re-separation by gel filtration (Fig. 2). It is characterized as the appropriate complex by: 1) lactose synthase activity, assayed in the absence of α -lactalbumin at 5 mM glucose, which exceeds N-acetyllactosamine synthase activity by a factor of 2; 2) tritium radioactivity which coincides with these activities; 3) migration of tritium radioactivity on SDS-gel electrophoresis with an apparent molecular weight of 65,000 (galactosyltransferase, M_r = 50,000; α -lactalbumin, M_r = 14,000; complex, M_r = 65,000) with a shift to 15,000 after reductive cleavage of the cross-links.

In the analysis of the [^3H]acetylation patterns of the amino groups, we have used a strategy similar to that in protective labeling studies as described under "Results." The isolation of highly purified peptides containing each labeled amino group was essential because of range of reactivities of lysines (up to 50-fold). In our hands, only column chromatographic techniques had sufficient resolving power to separate these peptides adequately. Peptides were characterized by amino acid analysis and knowledge of the CNBr fragment of origin. If cleavage yields peptides containing multiple residues of lysine, there is a potential that the $^3\text{H}/^{14}\text{C}$ ratio can be distorted by nonspecific reaction with the cross-linking reagent as such a reaction at one amino group, where the other bears a [^3H]acetyl label, will produce a labeled peptide of different structure and chromatographic behaviour from the ^{14}C -peptide used as an internal standard. This in fact may be the case for the peptide containing lysines 93 and 94 from the second sample, where the ratio is lower than expected, and indicates an apparent perturbation in labeling, which is not borne out by the ratios of the individual residues determined by Edman degradation of the CNBr fragment. The first sample which was cross-linked at lower reagent concentration and lower temperature, conditions that should minimize nonspecific reactions, does not show a decreased ratio.

As discussed previously (see the introduction to the text), there are two possible causes of the changes in specific activity of lysines 5, 108, and 114 in this experiment: first, an exclusion of [^3H]acetyl label from a major site of cross-linking and, second, alterations in the affinity for galactosyltransferase as a result of acetylation of residues close to the interaction site. To clarify the basis of these changes, it is revealing to compare the present results with those of previous protective labeling studies. These are displayed in Fig. 15 on a logarithmic scale to show evenly negative ($R < 1$) and positive ($R > 1$) changes. In differential labeling studies, lysine 5 was decreased in reactivity by 3 and lysine 114 increased in reactivity by 2 (Fig. 15A). In the present study, the increased specific activity of lysine 114 can only be attributed to an effect on the affinity for galactosyltransferase, as the alternative mechanism of perturbation (a site of cross-linking) can only lower the $^3\text{H}/^{14}\text{C}$ ratio. Previously, the increased reactivity of this residue

⁴ S. K. Sinha and K. Brew, unpublished observations.

to acetylation had been attributed to either its proximity to a positively charged group in the lactose synthase complex or to a conformation change resulting from binding to galactosyltransferase that produced greater exposure or a lowered pK_a of this group. As acetylation of the ϵ -amino group of lysine 114 can now be said to increase binding to galactosyltransferase, the most probable explanation is that lysine 114 is sufficiently close to a positively charged group on galactosyltransferase in the complex to generate charge repulsion and lower the pK . Acetylation of lysine 114 increases the strength of interaction by negating the charge repulsion. However, it is not entirely possible to exclude the alternative hypothesis of a localized conformational change that is favored by acetylation (*e.g.* disruption of an electrostatic interaction) and promotes the interaction of the two proteins.

Of the two groups that show lower specific activities in the cross-linked complex, the ϵ -amino groups of lysines 5 and 108, the former group corresponds to one that is decreased in reactivity on binding to galactosyltransferase and is therefore proximal to the interaction site, while the latter group was unaffected in reactivity (Fig. 15A). As it is reasonable to expect that groups whose modification affects the extent of binding to be close to the interaction site, whereas sites of cross-linking should be at some distance from the interaction site, we can deduce that acetylation of lysine 5 decreases the strength of interaction either through steric effects or through the disruption of favorable interactions. Lysine 108, on the other hand, was not perturbed on complex formation, and so is some distance from the binding site, whereas the acetylated species is largely excluded from cross-linking. We may therefore conclude that the side chain amino group of this residue is a major site of cross-linking. It is clearly not the only cross-linking site, as some labeling of this residue is found in the cross-linked complex, but as the decrease in labeling is close to 75%, it must be the kinetically dominant position of cross-linking.

It is interesting to note the greater perturbations in the labeling pattern in the second cross-linking experiment, which was conducted at a higher temperature, and lower protein concentration. Both of these alterations tend to decrease the extent of interaction between the two proteins (6) and so will magnify any variations in affinity between the different acetylated α -lactalbumin species. It will be interesting to conduct cross-linking studies under conditions where binding is less favorable but cross-linking can still occur (*e.g.* fewer ligands) to see if these differences become even more exaggerated.

The results (Fig. 16) of the cross-linking with imidoesters of varying maximal effective reagent lengths, 3.7–8.5 Å (12) indicates clearly that, whereas under similar experimental conditions reaction with dimethyl malonimide, dimethyl succinimide (Fig. 16A), or dimethyl glutarimide (Fig. 16B) does not produce significant amounts of cross-linked α -lactalbumin-galactosyltransferase complex, reaction with dimethyl adipimide (Fig. 16C) or dimethyl pimelimide (Fig. 16D) does produce such cross-linked complexes. Although it is difficult to correlate quantitatively the relative effectiveness of the imidoesters because of possible inherent difference in their reactivities, one can safely conclude that the adipimide with a maximal effective reagent length of 7.3 Å is the minimum size cross-linker to produce a cross-linked complex. Given the specific steric requirements for cross-linking, namely an amino group on each protein outside the interaction site, but sufficiently close to permit cross-linking, it is reasonable to conclude that lysine 108 is the site of cross-linking for dimethyl adipimide, as well as for the longer reagent (dimethyl 3,3'-dithiobispropionimide) used in our label selection study. Therefore, it can be proposed that the ϵ -NH₂ group

of lysine 108 is approximately 7 Å from an amino group on galactosyltransferase in the lactose synthase complex.

These results, which confirm and extend conclusions drawn from previous differential labeling studies (8), give an indication of the location and extent of the interaction site on α -lactalbumin. As yet, the precise three-dimensional structure of α -lactalbumin has not been determined by x-ray crystallography, although structures for the protein modified from that of lysozyme by model building (13) and energy minimization (14) have been reported. Unfortunately, and perhaps significantly, the three groups under discussion are located in a region whose structure is uncertain owing to major sequence differences from lysozyme, including deletions and an insertion. No attempt was made to define a unique structure for this region by model building (13), but Warne and co-workers (14) reported three alternative structures based on energy minimization that they designated T₂, T₃, and T₄. As discussed previously (8), differential labeling studies are more consistent with structure T₂ which will be used as a basis for this discussion, although essentially similar conclusions would result from the other two structures.

The side chains of lysines 5, 108, and 114 are all located on the right-hand side of the cleft region in the lysozyme-based structure for α -lactalbumin. In contrast, the other 10 amino groups, which were "unselected" in the cross-linking experiment, are widely distributed on the remaining surface of the protein (see Fig. 9 of Ref. 8). A topographical representation of the region of the molecule in the vicinity of these amino groups in conformation T₂ is given in Fig. 17. Also shown in the figure are the most prominent features (*i.e.* highest x coordinate) of the side chains of a number of other residues in this region which are conserved in the amino acid sequences of all α -lactalbumins of known structure: the bovine (11), goat (15), human (16), guinea pig (17), and rabbit (18) variants.

Although the amino groups of lysines 5 and 114 are suffi-

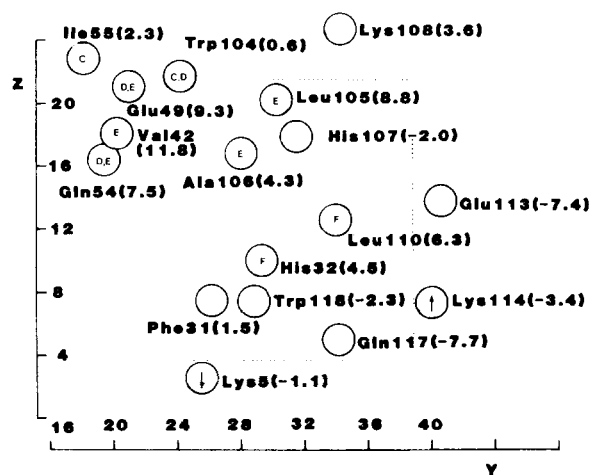


FIG. 17. A topographical representation of some of the amino acid residues of bovine α -lactalbumin in or near its interaction site with galactosyltransferase. Each circle represents the relative position of the relevant side chain of the amino acid in the y,z plane; numbers in parentheses give the values of their x coordinates. The circles are centered on the most prominent group (most positive x coordinate) of each side chain, apart from phenylalanine 31, where the center of the aromatic ring is used. The nature of the residues and their positions in the sequence are indicated. \uparrow and \downarrow , residues that increased and decreased, respectively, in reactivity in the lactose synthase complex. The letters inside circles are residues that correspond to components of subsites for substrate binding in lysozyme. The representation is constructed from the coordinates for the T₂ conformation of α -lactalbumin (14). Distances are in angstroms.

ciently close to the interaction site for their reactivities to be perturbed in the complex and for their modification by acetylation to affect the strength of interaction, the evidence indicates that they are not essential components of the binding site, as their acylation does not inactivate α -lactalbumin and they are not invariant in all species variants of α -lactalbumin, being replaced in rabbit α -lactalbumin by arginine (for lysine 5) and asparagine (for lysine 114). Therefore, it is reasonable to use these residues as indicators of the boundary of the interaction site. Lysine 108 is sufficiently far from the binding site that its reactivity is not affected on binding to galactosyltransferase but is close enough to an amino group on galactosyltransferase to be cross-linked by a reagent as short as dimethyl adipimidate (*i.e.* 7 Å). Presumably this cross-link projects away from lysine 108 towards the interaction site, so the upper boundary of the interaction site can be provisionally defined by including only groups more than 7 Å distant from lysine 108. The area so-defined is indicated by the *dotted line* in Fig. 17; it should be emphasized that this represents the maximum extent of the binding site.

The hydrophobic nature of the surface of this region of the α -lactalbumin molecule has been previously discussed (8, 14). Prominent surface groups include phenylalanine 31, histidine 32, leucine 105, alanine 106, and leucine 110. The degree of accessibility of tryptophan 118 to the surface depends on the precise conformation of this region and so must be regarded as uncertain. Physical evidence appears to favor the view that the indole ring of this residue is not in contact with solvent (see Ref. 19 for discussion). Evidence from chemical modification studies (20) supports the view that the side chain of histidine 32 is a component of the binding site. Carboxymethylation of the 3 position of the imidazole ring of His 32 produces some loss of activity along with physical evidence of conformational alterations. In contrast, ethoxyformylation of the 1-N renders α -lactalbumin inactive for binding to galactosyltransferase. As in the structure for α -lactalbumin under consideration the 3-N of the imidazole ring is somewhat buried, it is consistent that carboxymethylation of this position would result in a local conformation disturbance; the slow rate of reaction of this histidine relative to histidine 68 supports the view that this nitrogen is not readily accessible. As the 1-N of this ring is the most prominent feature, the attachment of an ethoxyformyl group can be expected to sterically hinder the binding of galactosyltransferase to this region. The other surface residues in this region are of a type not readily amenable to chemical modification. This may be a contributing factor to the equivocal results of many chemical modification studies of structure-function relationships in α -lactalbumin. Part of this extended hydrophobic binding site in α -lactalbumin corresponds to the monosaccharide binding subsite F in lysozyme, a region that has undergone major structural changes through amino acid substitutions (13). The upper region overlaps the adjacent subsites D and E. Although this region is somewhat modified from lysozyme, one of these sites could still function in providing favorable interactions for monosaccharides in a complex with galactosyltransferase, as discussed previously (8). At present, studies are in progress aimed at identifying the region of galactosyltransferase to which lysine 108 becomes cross-linked by bisimidoesters.

In terms of applicability to other systems, the label selection procedure has some advantages over other approaches for investigating interaction sites and some disadvantages. It has an advantage over the protective labeling approach in that it is applicable to systems where interaction is relatively weak and reversible, although susceptibility to cross-linking by amino group-specific reagents is essential. It is important that specifically cross-linked complexes should be readily purified and characterized and that the protein under study should be readily cleaved, preferably into fragments containing single amino groups. Finally, it is clear from our study with lactose synthase that the information obtained is "degenerate" in that it indicates positions where modification affects binding, as well as any major cross-linking site. However, this in itself can represent valuable information, as in the present case, particularly when complementary studies of other types (*e.g.* chemical modification, differential labeling) can be performed. Application of the procedure to proteins trace-labeled in positions other than amino groups may be useful for examining the effects of other types of chemical modification on protein interactions.

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SUPPLEMENTARY MATERIAL TO:

A Label Selection Procedure for Determining the
Location of Protein-Protein Interaction Sites by Crosslinking
with Bis-imidoesters. Application to Lactose Synthase

by

Sudhir K. Sinha and Keith Brew

EXPERIMENTAL PROCEDURES

Preparation of trace labelled N- 14 C-acetyl α -lactalbumin

To a solution of α -lactalbumin (4 ml, 9.85 mg/ml) in 0.02 M tris-HCl buffer pH 8.0 containing 0.01 M KCl, a solution of 14 C-acetic anhydride (16 mCi, specific activity 6.0 Ci/mole) in 200 μ l of freshly-distilled dry acetonitrile was added. The reaction was allowed to proceed at room temperature for 2h, and the labelled protein separated from other reaction products by gel filtration with a column (2.2 x 32 cm) of Sephadex G25, equilibrated and eluted with 0.1 M ammonium bicarbonate. The protein peak, detected by absorbance at 280 nm, and by tritium content, was pooled, freeze dried, and stored at -20° .

Preparation of Uniformly labelled N- 14 C-acetyl α -lactalbumin

α -Lactalbumin (50 mg) was dissolved in 0.2 M tris-HCl buffer pH 8.6 containing 8 M urea (10 ml) and 100 μ l of acetic anhydride containing 500 μ Ci of 14 C-acetic anhydride (118.6 mCi/mole) was added in 20 μ l aliquots at 10 min intervals, with constant stirring at room temperature. The pH was maintained between 8.5 to 9.0 by the addition of 2M NaOH. After a total of 2h, the acetylated protein was separated by gel filtration with a column of Sephadex G25 equilibrated with 0.1 M ammonium bicarbonate, and freeze dried. The dried protein was redissolved in 4 ml of 0.5 M hydroxylamine in 0.1 M tris pH 7.8 and left for 3h at room temperature to remove O-linked acetyl groups. The solution was subsequently dialyzed against 4 changes of distilled water (1 l each), and freeze dried.

Preparation and Separation of CNBr fragments

Mixed samples containing 14 C-labelled, 14 C-labelled and cold carrier α -lactalbumin were completely acetylated with unlabelled acetic anhydride, reduced and carboxymethylated and cleaved at the single methionyl residue with CNBr as described previously (8).

Cyanogen bromide fragments (CN1 and CN2) were separated by gel filtration with columns (2.6 x 98 cm) of Sephadex G50-superfine equilibrated and eluted with 0.1 M ammonium bicarbonate. Fractions of 3.5 ml were collected and 50 μ l aliquots taken for scintillation counting. The fragments were pooled as indicated and freeze dried.

Digestion with thermolysin

Cyanogen bromide fragments (CN1 or CN2) was dissolved in 2.0 ml of water and the solution adjusted to pH 8.2 with 1 M NaOH. Thermolysin (42 u/w of fragment) was added and the digestion allowed to proceed for 4h at 37° . The pH was checked at intervals and adjusted as necessary to pH 8.2 with 1 M NaOH. Digests were applied directly to columns of DEAE cellulose for the separation of peptides.

Ion-exchange chromatography

The initial separations of peptides from thermolysin digests of CN1 and CN2 were performed by ion exchange chromatography with columns (0.9 x 27 cm) of DEAE cellulose (DE-52) equilibrated with 5 mM ammonium bicarbonate. The digests of the two fragments were eluted with different gradients. In each case, after applying the sample, the column was washed with 15 ml of 5 mM ammonium bicarbonate before applying the gradient. Peptides from CN1 were eluted with a concave gradient in ammonium bicarbonate concentration produced with three similar interconnected vessels containing equal volumes (150 ml) of 5 mM, 50 mM and 500 mM ammonium bicarbonate respectively. Peptides from CN2 were eluted with a linear gradient composed of equal volumes (200 ml) of 5 mM and 500 mM ammonium bicarbonate. Peptides were detected by subjecting aliquots to scintillation counting.

Reverse Phase Liquid Chromatography

Each peptide pool from ion exchange chromatography was subjected to re-separation by reverse phase liquid chromatography with columns (0.4 x 50 cm) of Spherisorb 5 μ ODS. Columns were equilibrated with 10 mM ammonium acetate adjusted to pH 6.5 with acetic acid and containing 1% methanol. Peptide pools were dried by rotary evaporation at temperatures below 35° and dissolved in approximately 2 ml of the equilibration buffer. The solution was dried again, dissolved in approximately 400 μ l of the same solvent and applied to the column. The column was washed with 15 ml of equilibration buffer and eluted with a linear gradient (300 ml) of 1% to 50% methanol in 10 mM ammonium acetate pH 6.5 at a flow rate of 30-35 ml/h.

Some peptide fractions from this separation, in particular those that emerged from the column unretarded, were subjected to rechromatography under similar conditions, except that the ammonium acetate buffer was adjusted to pH 5.0 or 4.0. Following each separation, the column was flushed with 70% methanol (30 ml) and re-equilibrated with the starting buffer. Peptides were detected by subjecting appropriate aliquots (see Figure legends) to scintillation counting.

Other Methods

Amino acid analyses of peptides were performed as described previously (8). 3 H/ 14 C ratios in residues 93 and 96 were determined in one case by manual Edman degradation of CN2 (residues 91-123). Edman degradations (4 cycles) were performed as previously described (16); the organic phase from each cycle was re-washed with water, and a portion of it dried in a scintillation vial and redissolved in a mixture of water and scintillation fluid (0.75:7.5 ml) for measurement of radioactivity.

RESULTS

Figures 3-14 show separations of peptides from successive stages of the cleavage of labelled α -lactalbumin. As the crosslinked and control samples gave essentially identical patterns, only one set of separations, namely those from the crosslinked sample, are shown. In the separation of the fragments from cyanogen bromide cleavage, (Fig. 3), three peaks of absorbance (280 nm) can be seen. From previous work, these can be identified as uncleaved protein, CN1 and CN2 in order of elution. A considerable amount of radioactivity (3 H and 14 C) is found associated with the first peak; the fractions that were pooled as CN1 were chosen on the basis of constant 3 H/ 14 C ratio as well as correspondence to the second, partially resolved absorbance peak. Some contamination of this fragment with uncleaved material was evident through the generation from it, in low yield, of peptides corresponding to CN2. The elution profile of the cyanogen bromide fragments of the control α -lactalbumin sample was closely similar. In further cleavages and separations care was taken to treat the crosslinked and control samples at the same time and under similar conditions.

The fractionation of most peptide pools from the initial DEAE-cellulose separations of the thermolysin digests of CN1 and CN2 by reverse phase chromatography gave most peptides in a pure form. A pair of peptides containing lysines 16 and 58, respectively, which were present in one pool gave some difficulty in separation. Fig. 6 illustrates the separation of these peptides by reverse phase liquid chromatography; unmarked peaks in this separation contained either different forms of peptides isolated in higher yields in other separations, or impurities derived from contamination with CN2. The main peak contains a partially resolved mixture of the peptides containing lysines 16 and 58. As shown by the 3 H/ 14 C ratios marked on the figure, fractions 13 and 14 (ratios 14.6 and 14.7) contained mainly lysine 16, whereas 16 and 17 (ratios 3.8 and 3.0) contained predominantly lysine 58. Fraction 15 (ratio 6.1) represents a mixture of these peptides. Accordingly, fractions 13-14 and 16-17 were separately combined and re-separated by reverse phase chromatography at pH 4.0 using a 300 ml gradient from 1 to 25% methanol. In the case of the peptide containing lysine 16 (CN1 Th2 R1) showed a constant 3 H/ 14 C ratio across the peak, whereas the peptides containing lysine 58 (CN1 Th2 R2) the initial part of the peak showed higher ratios than the latter half. The last two fractions of the peak (1.5 ml each), which had identical 3 H/ 14 C ratios were combined. As shown by the amino acid compositions in Tables I and II, this procedure resulted in successful purification of the appropriate peptides. All peptides from CN1 (Figs. 7-11) were repurified by chromatography at pH 4.0. Of the peptides from CN2, CN2 Th1 R1 (Fig. 12, peak 1), CN2 Th2 R1 and CN2 Th2 R2 (Fig. 13, peaks 1 and 2) were re-separated at pH 4.0 while CN2 Th3 R1 and CN2 Th3 R2 (Fig. 14, peaks 1 and 2) were re-separated at pH 5.0. Tables I and II show, respectively, the amino acid compositions of peptides from the crosslinked and control samples of the first experiment. Similar levels of purity were obtained in the second experiment, although details are not given here because of its repetitive nature. 3 H/ 14 C ratios of peptides are given in the main text.

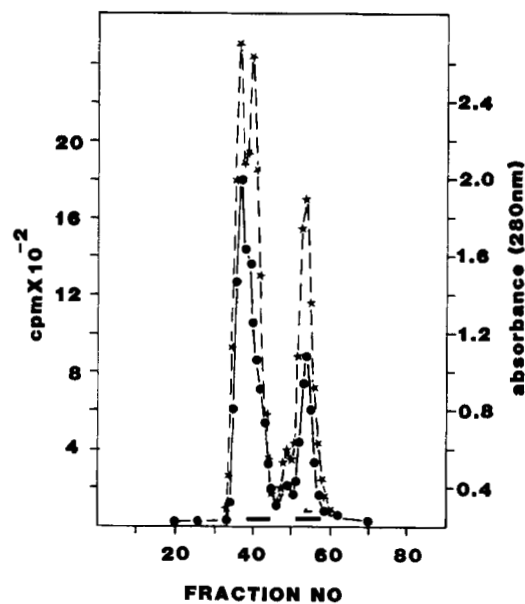


Figure 3 Separation of the cyanogen bromide fragments of the crosslinked α -lactalbumin preparation by gel filtration with a column of Sephadex G50. The column was equilibrated and eluted with 0.1 M ammonium bicarbonate at a flow rate of approximately 10 ml/h. Fractions of 3.5 ml were collected. \bullet — \bullet 14 C content of 50 μ l aliquots; \star — \star A_{280} . Fractions were pooled as indicated by the bars on the abscissa.

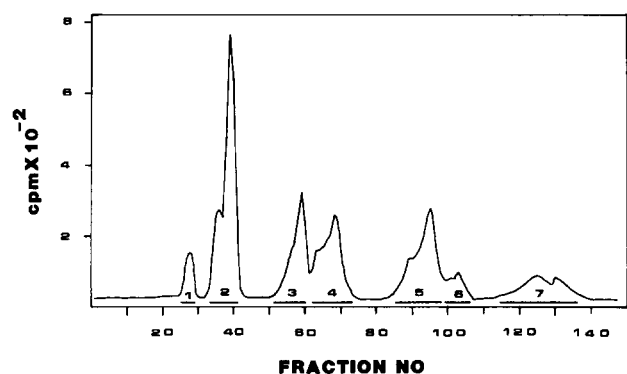


Figure 4 Separation of the products of thermolysin digestion of XL- α -lactalbumin CN1 by ion exchange chromatography with a column of DEAE cellulose. Elution was performed with a concave gradient in ammonium bicarbonate as described in Experimental Procedures. Fractions of 3.0 ml were collected and 100 μ l aliquots taken for 14 C determination.

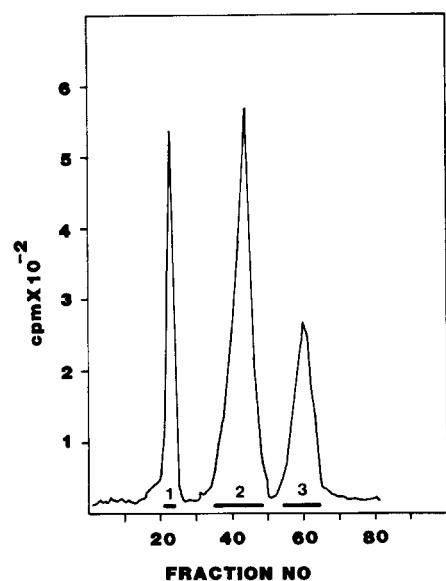


Figure 5 Separation of the products of thermolysin digestion of XL- α -lactalbumin CN2 by ion exchange chromatography with DEAE cellulose. Elution was performed with a linear gradient of ammonium bicarbonate as described in Experimental Procedures. Fractions of 2.8 ml were collected and aliquots of 50 μ l taken for 14 C determination.

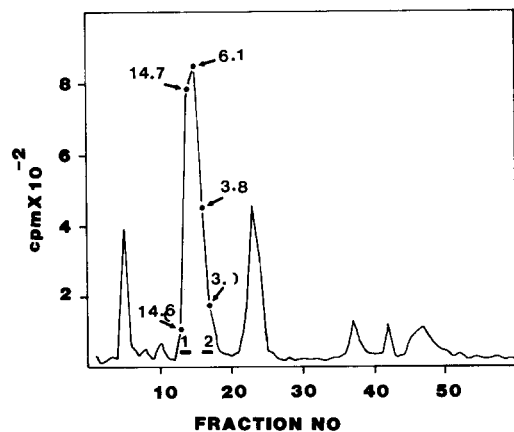


Figure 6 Reseparation of fraction CN1 Th2 (Fig. 4, peak 2) by reverse phase liquid chromatography. Elution was performed with a linear gradient of methanol (1% - 50%) in 10 mM ammonium acetate pH 6.5 as described under Experimental Procedures. Fractions of 2.0 ml were collected and 200 μ l aliquots taken for radioactivity measurements. These conditions also apply to Figs. 7-14. The numbers adjoining fractions 12-17 indicate their $^3\text{H}/^{14}\text{C}$ ratios.

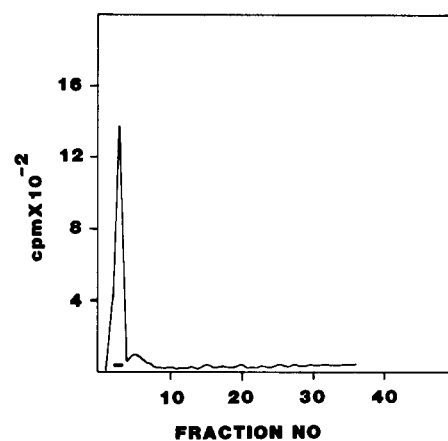


Figure 7 Reseparation of fraction CN1 Th3 (Fig. 4, peak 3) by reverse phase liquid chromatography at pH 6.5.

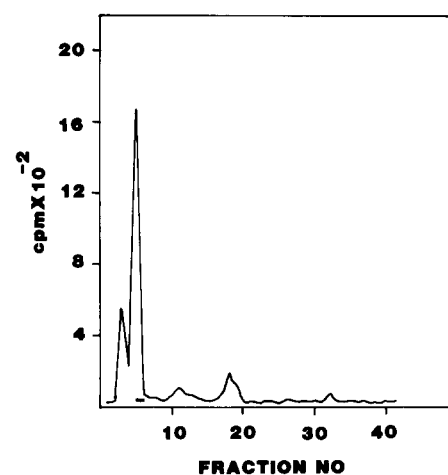


Figure 8 Reseparation of fraction CN1 Th4 (Fig. 4, peak 4) by reverse phase liquid chromatography at pH 6.5.

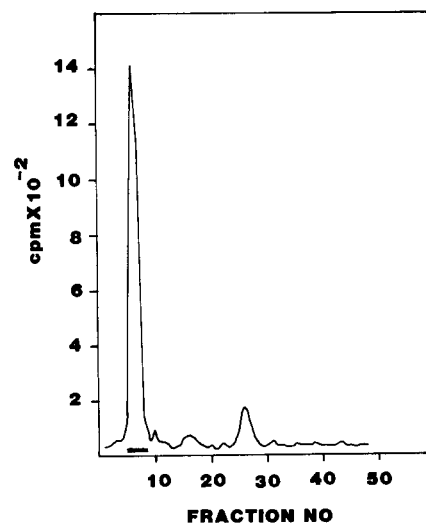


Figure 9 Reseparation of fraction CN1 Th5 (Fig. 4, peak 5) by reverse phase liquid chromatography at pH 6.5.

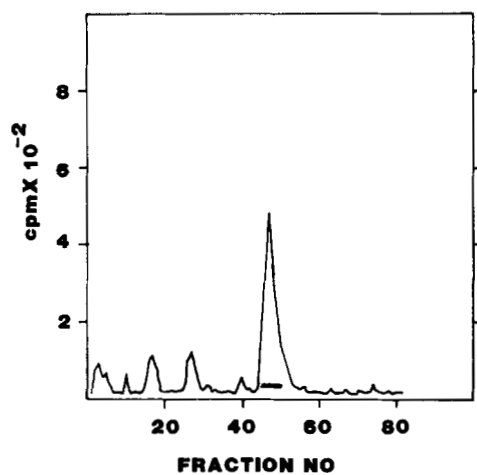


Figure 10 Reseparation of fraction CN1 Th6 (Fig. 4, peak 6) by reverse phase liquid chromatography at pH 6.5.

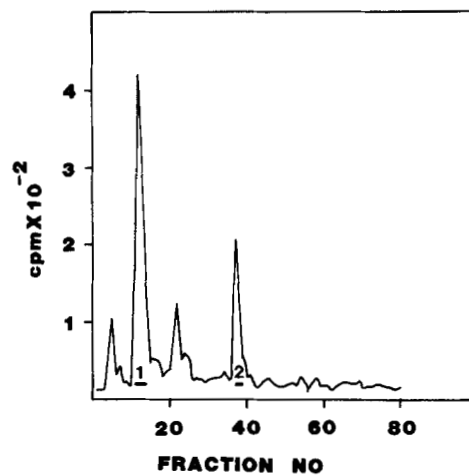


Figure 13 Reseparation of fraction CN2 Th2 (Fig. 5, peak 2) by reverse phase liquid chromatography at pH 6.5.

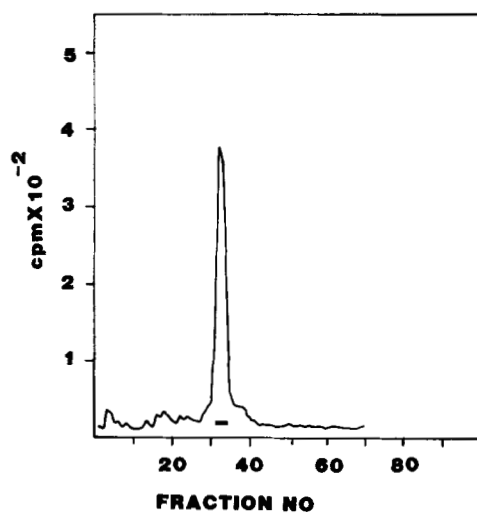


Figure 11 Reseparation of fraction CN1 Th7 (Fig. 4, peak 7) by reverse phase liquid chromatography at pH 6.5.

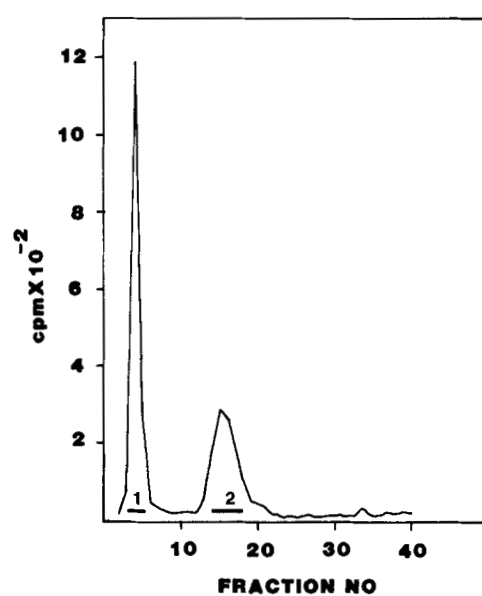


Figure 14 Reseparation of fraction CN2 Th3 (Fig. 5, peak 3) by reverse phase liquid chromatography at pH 6.5.

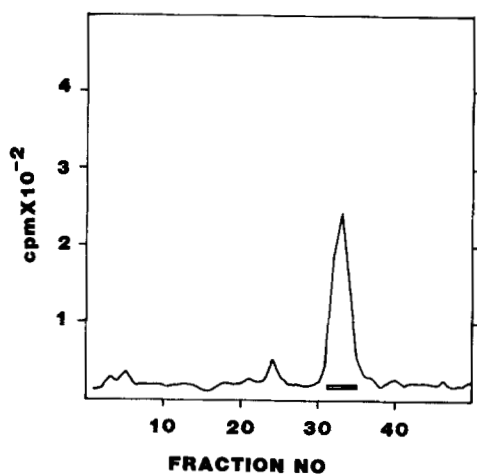


Figure 12 Reseparation of fraction CN2 Th1 (Fig. 5, peak 1) by reverse phase liquid chromatography at pH 6.5.

Table II

Amino Acid Compositions of Labelled Peptides from Control α -Lactalbumin Sample

[illegible]