A new system for the bacterial expression of a variant of bovine α-lactalbumin has been developed. Eighteen mutant proteins containing single site substitutions in a cluster of predominantly aromatic residues adjacent to the cleft (aromatic cluster I) and in the hydrophobic box were expressed. The proteins were extracted from inclusion bodies and treated to generate native folding and disulfide bonds to provide appropriately folded protein samples for nine of the mutants. These were characterized with respect to kinetic parameters reflecting aspects of α-lactalbumin activity in modulating the specificity of bovine galactosyltransferase. In aromatic cluster I, changes at tryptophan 118 or glutamine 117 were found to specifically reduce affinity for galactosyltransferase, whereas substitutions for phenylalanine 31 or histidine 32 have major effects on the ability to promote glucose binding (13-200-fold) and lesser effects on galactosyltransferase affinity (1.6-70-fold). Substitutions in the hydrophobic box were found to affect folding rather than activity. Thus, the binding of α-lactalbumin to galactosyltransferase and its ability to promote glucose binding can be separately perturbed and are associated with distinct but adjacent structures. Aromatic cluster I is directly involved in activity whereas the hydrophobic box appears to have a structural rather than functional role.

The milk whey protein, α-lactalbumin (LA) functions as the regulatory component of the lactose synthase enzyme system (EC 2.4.1.32) which catalyzes and regulates the biosynthesis of lactose in the lactating mammary gland (for reviews, see Refs. 1-3). The catalytic component of lactose synthase is the enzyme UDP-galactose β-N-acetylgalactosaminide β-4-galactosyltransferase (GT; EC 2.4.1.38), an intrinsic component of the trans-golgi membranes which, in most cells, has the function of catalyzing the transfer of galactose from UDP-galactose to non-reducing terminal β-linked N-acetylgalcosaminyl residues in the carbohydrate chains of glycoproteins and glycolipids. Although GT can catalyze lactose synthesis (transfer of galactose to free glucose) in vitro, it is ineffective as a catalyst for this reaction under physiological conditions because of its low affinity for glucose, reflected in a $K_m$ of about 2 mM LA, a specific product of the lactating mammary gland, binds reversibly with GT to form 1:1 complexes with a concomitant 1000-fold decrease in the $K_m$ for glucose, thereby allowing efficient production of lactose under physiological conditions.

The relationship of structure and function in LA is of particular interest for a number of reasons: (a) it is similar in sequence and three-dimensional structure to the type c lysozymes (4), providing a case of extreme functional divergence in a pair of homologous proteins, (b) it represents an example of enzyme regulation at the level of specificity through a heterologous protein-protein interaction, (c) it contains a tightly bound Ca$^{2+}$ ion (5) that has a major influence on its molecular stability and folding properties (6-8), and (d) under mildly denaturing conditions it can assume a stable conformational state with the properties of a "molten globule," an early intermediate in protein folding (2, 3, 7).

Here, we describe a facile bacterial expression system for a variant of bovine LA with which fully active recombinant LA can be generated in high yields. This system succeeds an earlier system in which bovine LA was expressed as a fusion protein (C-LA) from which a native, active protein was generated by limited proteolysis after extraction and treatment to generate native folding and disulfide bond formation (9). With the new system, site-directed mutagenesis has been used to investigate the roles of structural elements of the bovine LA molecule in its activity. One region, defined by x-ray crystallographic and NMR studies, is designated "aromatic cluster I" (4, 10) and contains the side chains of the invariant amino acids Phe-31, His-32, Gln-117, and Trp-118, together with Tyr-36. This structure is adjacent to the cleft and has been implicated by chemical studies to be involved in the interaction with GT (11-13). An alternative candidate for the GT binding site is a structure, designated the "hydrophobic box" or "aromatic cluster II" which includes Trp-26, Phe-53, Trp-60, Tyr-103, and Trp-104 (14). This structure is found both in LA and lysozyme, but is distinct in LA because of the presence of Tyr-103 (alanine in most lysozymes), which blocks the LA cleft, making it a less open structure than in lysozyme. To probe the roles of these substructures, we have constructed and expressed LA mutants with substitutions at selected sites in both clusters I and II. Mutant proteins that allowed the generation of significant...
quantities of native protein after folding, in vitro, were characterized with respect to the values of parameters that reflect different aspects of LA action. The relevance of the results to structure-function relationships in LA and the mechanism of action of LA are discussed as well as the facility of the expression system in comparison to others that have been previously described (9, 15, 16).

**EXPERIMENTAL PROCEDURES**

**Materials**

The sources of reagents for lactose synthase assays, protein, and DNA sequencing, and protein purification are the same as in previous publications (9, 17, 18). T7 promoter and T7 terminator primers and pET3a vector were purchased from Novagen. HP-PEI anion exchange HPLC columns (7.8 x 100 mm) were obtained from International Chromatography Inc., San Jose, CA. Taq polymerase, DNA ligase, and Magic™ PCR Preps DNA purification systems were purchased from Promega Corp., Madison WI. DNA ligase, BamHI, and NdeI were from New England Biolabs. N,N'-Diacetylcysteine was supplied by Sigma. Other reagents were of analytical grade.

**Bacterial Expression and Site-directed Mutagenesis**

Bovine LA was expressed in *Escherichia coli* strain BL21(DE3) by using a vector (pMLA) generated by cloning the coding sequence for LA into the pET3a vector at a site adjacent to the codon for the initiator methionine. The coding region for mature bovine LA in which the codon for methionine 90 was modified by M13 site-directed mutagenesis (19) to valine was amplified by PCR using the primers designated Nf-N and Nf-L. Table I lists the primers that were used for the mutagenesis. Table I lists the mutagenic primers that were used for the generation of the expression vector (pET3a) that had been previously generated and cloned into the pET3a vector at a site adjacent to the codon for the initiator methionine. The expression vectors for mLAs and variants were characterized by restriction mapping and DNA sequencing (21).

**Generation and Purification of Native Recombinant LAs**

**Extraction and Initial Purification—Cultures of *E. coli* strain BL21(DE3) transformed with pMLA were grown and induced for the expression of lactose synthase by conducting lactose synthase assays with increasing concentrations of LA at a fixed concentration of GT and 10 mM glucose and as inhibitors of galactose transfer to N,N'-diacetylcysteine (ChB) where the acceptor concentration was 0.5 mM. The enzyme (GT) concentration was the same for each set of assays, and comparisons between assays conducted at different times with GT preparations of different activity were affected by varying the concentration of bovine LA. For inhibition assays with ChB, the activity obtained in the absence of LA at 0.5 mM ChB was arbitrarily designated as 1.0. Kinetic data were analyzed by fitting to appropriate rate equations using the ENZFITTER computer program (Bioisoft).

**GT Assays**

GT assays were carried out by a radiochemical procedure using UDP-[[H]galactose, as in previous studies (17, 18). All assays were performed in 10 mM tris-HCl buffer, pH 7.5, 10 mM MnCl₂, 0.1% BSA, and 0.3 mM UDP-galactose at 37 °C. Recombinant LAs were compared with bovine LA as activators of lactose synthesis by conducting lactose synthase assays with increasing concentrations of LA at a fixed concentration of GT and 10 mM glucose and as inhibitors of galactose transfer to N,N'-diacetylcysteine (ChB) where the acceptor concentration was 0.5 mM. The enzyme (GT) concentration was the same for each set of assays, and comparisons between assays conducted at different times with GT preparations of different activity were affected by varying the concentration of bovine LA. For inhibition assays with ChB, the activity obtained in the absence of LA at 0.5 mM ChB was arbitrarily designated as 1.0. Kinetic data were analyzed by fitting to appropriate rate equations using the ENZFITTER computer program (Bioisoft).

**Interpretation of Steady State Kinetic Measurements of LA Activity**

LA modulates the affinity of GT for acceptor substrates. It acts as a competitive inhibitor with respect to extended substrates such as N,N'-diacetylcysteine and enhances the binding of glucose and other monosaccharides (17, 18, 24). With weaker binding substrates, such as glucose, inhibition effects resulting from effects on *Vₐ* are observed at high concentrations of LA and/or acceptor when dead-end inhibitory complexes are formed (25). We have assumed here that the mutant LAs act in a similar manner to bovine and other wild-type LAs.

Although there have been alternative proposals that the mechanism of lactose synthase is best represented by (a) a partially ordered mechanism in which UDP-galactose binds to a GT-Mn²⁺ complex prior to a random equilibrium binding of LA and glucose (17, 18) or (b) a completely random equilibrium mechanism (24), there is agreement that the effect of LA involves a random highly synergistic binding with glucose to GT complexes or, in the case of larger acceptor substrates, exemplified by ChB, a partially ordered mechanism of LA and acceptor with GT. Under the conditions used here for enzyme assays, GT is essentially saturated with Mn²⁺ at both metal binding sites (Mn²⁺ concentration of 10 mM versus *K₂* values of 2 μM and 2 mM) and with UDP-galactose (concentration of 350 μM versus *K₂* values of 25 and 60 μM, respectively (17, 25)) so that this formative of what may become the best model for enzyme system, essentially all of the GT will be distributed in complexes containing both Mn²⁺ and UDP-galactose. For lactose synthase assays, low glucose concentrations were also used (10 mM or less as compared with a *Kₐ* of 2 μM) so that enzyme complexes containing glucose but not LA are insignificant. The section of the mechanism investigated can be consequently represented by the following steps:
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**Equation 1**

\[ \text{GT}^* + \text{LA} \rightarrow \text{GT}^* \cdot \text{LA} \]

**Equation 2**

\[ \text{GT}^* \cdot \text{LA} + \text{glc} \rightarrow \text{GT}^* \cdot \text{LA} \cdot \text{glc} \]

**Equation 3**

\[ \text{GT}^* \cdot \text{LA} \cdot \text{glc} \rightarrow \text{GT}^* + \text{LA} + \text{products} \]

where GT* represents a GT-(Mn²⁺)-UDP-galactose complex.

If rapid equilibrium binding of LA and glucose is assumed, as indicated by previous kinetic studies (17, 18, 24) the rate equation (in the absence of products) becomes:

\[ \frac{V_m[\text{LA}][\text{glc}]}{K^2 + 1} \]

\[ \frac{K^*_{\text{app}}}{K^*_{\text{true}}} = \frac{1 + [\text{S}]}{K_m^*} \]

where \( V_m \) is the maximum velocity, \( K^*_{\text{true}} \) is the dissociation constant for lactose synthase activity, and \( K_m^* \) is the dissociation constant for glucose. From the above equation, it can be seen that a double reciprocal plot of lactose synthase activity versus glucose will have an intercept/slope ratio equal to:

\[ \frac{[\text{glc}]}{K^2 + 1} \]

from which, \( K^2 \) can be determined, using the value for \( K^* \) obtained from the inhibition of galactose transfer to ChB. Alternatively, the apparent \( V_m \) for lactose synthesis can be determined separately as an inhibition constant \( K^* \) for that reaction. The action of LA (and its mutants) in activating the catalysis of lactose synthesis by GT at a fixed concentration of glucose reflects an increase in the proportion of enzyme complexes containing both glucose and LA which can give rise to products (i.e. also containing Mn²⁺ and UDP-galactose), and is consequently dependent on both the affinity of LA for GT and its effects on glucose binding. From the above equation, it can be seen that a double reciprocal plot of lactose synthase activity versus [LA] will have an intercept/slope ratio equal to:

\[ \frac{[\text{glc}]}{K^2 + 1} \]

where \( V_m \) is the maximum velocity, \( K^*_{\text{true}} \) is the dissociation constant for lactose synthase activity, and \( K_m^* \) is the dissociation constant for glucose.

Near and far UV CD spectra of bovine LA and mutant LAs were determined with a JASCO J-710/720 spectropolarimeter. Twenty spectra were scanned for each sample at a speed of 100 nm/min which were subsequently averaged and smoothed. Near UV CD spectra (250–320 nm) were determined using a cell with a path length of 1 cm, and far UV spectra (200–250 nm) using a cell with a path length of 0.1 cm. Proteins were dissolved in 0.02 M tris-HCl, pH 7.4, containing 0.1 M CaCl₂, 0.01 M HCl (pH 2) or 6 M guanidine hydrochloride at concentrations between 190 and 500 μg/ml.

**Other Methods**

The NH₂-terminal sequence of mLA was determined using an Applied Biosystems model 470A Protein/Peptide Sequencer fitted with a model 120A analyzer and a model 900A data analysis system for phenylthiohydantoin-derivative analysis and sequence assignment. Synthetic oligonucleotide synthesis was carried out by Dr. Rudolf Werner, Department of Biochemistry and Molecular Biology, University of Miami. Molecular models of mA and some variants (lacking the NH₂-terminal methionine) were constructed using the Hyperchem software package (Autodesk Inc., Sausalito, CA) by substituting its sequence into the baboon LA structure (4) and optimizing the geometry by AMBER force field energy minimization to an RMS gradient of <0.05 kcal/Å mol.

**RESULTS**

*Preparation and Properties of Recombinant Bovine LA (mLA)—The system described here was designed to directly express the mature bovine LA sequence in E. coli. The substitution of valine for the single methionine at position 90 in the mature LA sequence produced a molecule which is resistant to CNBr cleavage. mL A was expressed, treated and purified as described in "Experimental Procedures." Fig. 1 shows typical elution profiles for the separation of two mA variants (W118Y-mLA and W104Y-mLA) by gel filtration with Sephadex G-75 after folding. The profile obtained with W118Y-mLA (Fig. 1a) is typical of that obtained with recombinant LAs that folded efficiently, including mL A. The product from this step showed a single band with the same mobility as bovine LA on SDS-gel electrophoresis even at high loadings. The product also ran as a single component on anion exchange HPLC analysis. Final yields of mL A reproducibly exceeded 25 mg/liter of bacterial culture. When mL A was subjected to automated amino-terminal sequencing, a single sequence was obtained with methionine as the amino-terminal residue, followed by the expected amino-terminal sequence of bovine LA.

Because mL A was constructed to be devoid of internal methionine residues, the NH₂-terminal methionine can be specifically removed by CNBr cleavage without cleaving any internal peptide bonds. However, as described below, mL A is very similar in activity and physical properties to bovine milk LA. Therefore, it was concluded that it is appropriate to use variants of mL A to investigate structure-function relationships in LA without removing the amino-terminal methionyl residues.

*Generation and Purification of Mutants of mL A—The mutations introduced into mL A are listed in Table I together with...
the oligonucleotides used in generating them. Sites of mutation included all invariant positions in cluster I (Phe-31, His-32, Gln-117, and Trp-118) together with Tyr-103 and Trp-104 of cluster II. Other residues in cluster II were not altered because of variability in different LA sequences (3, 13) or buried nature in both LA and lysozyme (4, 10). The choice of substitutions was based on either the nature of residues found at the corresponding site in various lysozymes (e.g. Glu for Phe-31, Asn and Tyr at position 32, Pro and Ala at position 103, Ser and Ala at position 117, and Tyr at position 118), while other substitutions were designed to be structurally conservative or to examine the effects of different side chain types at a particular site. Table I summarizes the yields obtained at different stages during their production. For mLAs and variants that folded efficiently in vitro, high yields of monomeric protein were obtained on purification by gel filtration (Fig. 1a) and further purification by HPLC anion exchange chromatography gave a single peak that eluted at a similar NaCl concentration to bovine LA and mLA (see Fig. 2a). Variants of mLAs that did not fold efficiently in vitro in some cases showed a large proportion of aggregated protein that eluted at the void volume on separation by gel filtration. With other mutants, aggregated protein was present together with a peak of monomeric protein which, on separation by HPLC, did not show a peak eluting at the same time as mLAs but did show a major peak eluting at high NaCl concentration. Some variants were analyzed directly by HPLC after folding and were found to be devoid of native protein (H32E-mLA, H32N-mLA, W104A-mLA, and W104L-mLA). Fig. 1b shows the separation of W104Y-mLA by gel filtration after folding in the presence of 10% glycerol; monomeric protein was present which, on separation by HPLC, showed a major component that eluted at a later time and as a broader peak than mLA (Fig 2b); additional material eluted at high NaCl concentrations. The main component was used in further characterization. In the case of H32A-mLA, the final yield of folded protein increased 5-fold when glycerol (10%) was included during the folding process; however, the inclusion of glycerol did not allow the production of native protein from Y103A-mLA.

SDS-gel electrophoresis showed that only low levels of protein contaminants were present after the initial separation by anion exchange chromatography in 4 M urea so that the initial levels of expression of the different proteins varied only slightly although there were major differences in the final yields of the native protein. Based on minimal yields of folded protein, the following mutations were incompatible with native folding under the conditions used here: F31E, F31L, H32E, H32N, Y103A, W104L, and W104A. Proteins obtained in sufficient yield for detailed characterization had the following substitutions: F31S, F31Y, H32A, H32Y, Y103P, W104Y, Q117D, Q117A, W118Y, and W118H. W104Y-mLA is not included in the list because the major product isolated by HPLC after folding has

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### Table I

| Primer | Sequence | Orientation |
|--------|----------|-------------|
| NF-N   | CCAAGCCACCATATGGACAGTTAACG | Coding |
| NF-C   | GCAGAGTACGCAATGGTCATCCAC | Complementary |
| T7 promoter | ?ATACGACTCTACTATTAGGG | Coding |
| T7 terminator | GCACGTTCACGCAATCCAC | Complementary |

### Table II

| Protein | Yield from O60 mg/liter of culture | Yield from G-75 | Yield% |
|---------|-----------------------------------|----------------|--------|
| mLAs    | 107.0 40                         | 100            |
| F31E    | 77.5 8.8 (0)                      | 0              |
| F31L    | 62.5 5.5 (0)                      | 0              |
| F31S    | 60.0 16.5 (1.0)                   | 4              |
| F31Y    | 32.0 5.5                         | 46             |
| H32A    | 55.5 10.0 (1.0)                   | 5              |
| H32A*   | 73.0 29.5 (5.7)                   | 21*            |
| H32E    | 65.5 ND (0)                       | 0              |
| H32N    | 55.8 ND (0)                       | 0              |
| H32Y    | 47.0 17.5                        | 100            |
| Y103A   | 26.7 1.7                         | 100            |
| Y103P   | 53.3 17.7                        | 29             |
| W104A   | 53.3 ND (0)                       | 0              |
| W104L   | 42.5 ND (0)                       | 0              |
| W104Y   | 58.8 11.3 (2)                     | (9)            |
| Q117A*  | 53.9 42.4                        | 210            |
| Q117D*  | 62.2 29.3                        | 126            |
| W118H*  | 54.0 15.6                        | 77             |
| W118Y*  | 53.0 29.8                        | 150            |

* Yield for mLAs after final purification was set at 100%. Yields for all other proteins are relative to that of met-LA.

* Values in parentheses are yields after ion exchange HPLC.

* Yields calculated for protein after HPLC separation.

* Proteins were folded in the presence of 10% glycerol.

* ND, protein was separated by HPLC directly after treatment to generate native fold.

* The major component isolated for W104Y met-LA was not native based on chromatographic and physical properties.
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**Fig. 2.** Separation of representative recombinant LAs by HPLC anion exchange chromatography. a, mLA; b, W104Y-mLA. The conditions are those described in the text.

properties that suggest it is in a different conformational state from native mLA.

**Structural and Physical Characterization of Mutant LAs—**
The purified proteins were all homogeneous on SDS-gel electrophoresis and anion exchange HPLC even at high loadings. The entire sequences of mLA and the mutants were checked by DNA sequencing, confirming the presence of the desired mutation in each case. In two cases, an additional single base change was present resulting in an additional amino acid substitution, presumably being introduced by the PCR mutagenesis procedure. These were in F31E-mLA where methionine was substituted for valine 8, and Q117D-mLA where lysine was substituted for glutamate 113.

The near and far UV CD spectra of mLA, and selected mutant proteins are shown in Fig. 3 in comparison with those of bovine LA at pH 2 (acid or molten globule state), pH 7.4 (native state), and in 6 M guanidine HCl (denatured state). These indicate that most of the mutant proteins which were characterized with respect to functional properties are closely similar in conformation to the native state of bovine LA. W118Y-mLA had a slightly modified spectrum in the near UV range, a feature that can be reasonably attributed to the nature of the sequence change. There are pronounced differences between the CD spectra of the mutants with changes in the hydrophobic box residues, tyrosine 103 and tryptophan 104. Y103P-mLA, after purification by HPLC had near and far UV spectra that are closely similar to those of bovine LA and mLA, whereas the monomeric form of Y103A-mLA, isolated by gel filtration in low yield, had a molten globule spectrum, similar to that of bovine LA at pH 2 (see Fig. 3, a and c). The major component isolated from W104Y-mLA by HPLC was quite distinct. It has a pronounced near UV CD spectrum of similar magnitude to mLA, but with major differences (Fig. 3c). The small trough at about 295 nm is missing and the main trough is red-shifted and altered in shape. The far UV CD spectrum also differs in shape from that of mLA.

**Functional Properties of Mutant LAs—**
The activities of different mutant LAs and mLA as activators of glucose binding are compared in Fig. 4. mLA and Y103P-mLA are closely similar in activity whereas other mLA variants are less active than the parent protein. Bovine LA is closely similar in activity to mLA (Table III). Visual inspection of the activity profiles suggests that some variants have reduced apparent $V_m$ values (e.g. F31Y-mLA) while others have increased apparent $K_m$ values.
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Figure 3. Near (250-320 nm) and far (200-250 nm) ultraviolet CD spectra of bovine LA and selected mLA variants. a, bovine LA at pH 7.0. ——; pH 2. ——; and in 6 M guanidine hydrochloride. b, mLA. ——; H32A-mLA. ——; and W118Y-mLA. ——; all at pH 7.0. c, mLA. ——; Y103A-mLA. ——; and W104-mLA. ——; all at pH 7.0. All spectra are adjusted to represent protein concentrations of 0.5 mg/ml.

(e.g. W118Y-mLA). Mutants with substitutions for histidine 32 (tyrosine or alanine) showed very little activity over a wide concentration range. W104Y-mLA also had a low activity, but was not isolated in sufficient quantity to perform assays at high concentrations or to perform inhibition assays. The double mutant, Q117D/E113K-mLA displayed an activity profile that is essentially superimposable on that of Q117A-mLA (data not shown).

The different levels of activity observed in the lactose synthase reaction can reflect changes in either or both of two parameters associated with LA function, the affinity for GT (Kₘₐ) and the ability at saturation to promote glucose binding (Kₑ). As discussed in "Experimental Procedures," changes in the apparent Vₐ in the lactose synthase reaction at a fixed concentration of glucose are expected to reflect changes in the latter parameter. Differences in the affinity for GT were determined using the LAs as inhibitors of the catalysis of galactose transfer to ChB (Fig. 5). Arranged in terms of decreasing effectiveness as inhibitors, the mutant LAs are: Y103P > bovine LA = mLA > F31Y > Q117D > H32A > F31S > W118Y = W118H, the latter two mLA mutants having very low activities in this assay. A comparison of the activities of the various proteins in the two reactions (Figs. 4 and 5) indicates that the relative effectiveness of pairs of mutants, Q117A/F31Y, Q117A/F31S, and W118Y/H32A are reversed. The explanation of this is shown in Table III, which gives the values for Kₘₐ and Kₑ for the various proteins. The deficiencies in Q117A-mLA and W118Y-mLA specifically reflect increased values for Kₘₐ, whereas F31Y-mLA has a slightly decreased affinity for GT (<2-fold) combined with a much lower ability to promote glucose binding (15-fold increase in Kₑ). The latter deficiency is displayed as a reduced lactose synthase activity at high LA concentrations. A similar change is found in other mutants with substitutions for phenylalanine 31 and histidine 32, but combined with major reductions in affinity for GT. Although the low activity levels obtained with F31S-mLA, H32A-mLA, and H32Y-mLA introduce uncertainty regarding the precise values of their kinetic parameters, it appears that the position 32 mutants are even more deficient than the position 31 mutants in their effects on lactose synthase activity at high LA concentrations. A similar change is found in other mutants with substitutions for phenylalanine 31 and histidine 32, but combined with major reductions in affinity for GT. Although the low activity levels obtained with F31S-mLA, H32A-mLA, and H32Y-mLA introduce uncertainty regarding the precise values of their kinetic parameters, it appears that the position 32 mutants are even more deficient than the position 31 mutants in their effects on glucose binding. Y103P-mLA has a slightly higher affinity for GT than mLA and bovine LA and W118Y (Fig. 4 and Table III) in conjunction with a slightly decreased ability to promote glucose binding. However its overall activity in the lactose synthase reaction is closely similar to that of mLA (Fig. 4).

**DISCUSSION**

In this study we describe an expression system for bovine LA that is much more expedient than fusion protein systems previously

| Table III | Kinetic parameters determined for mLA and variants with bovine GT |
|-----------|---------------------------------------------------------------|
| Protein   | Kₘₐ | Change in Kₘₐ | Kₑ   | Change in Kₑ   |
| Bovine LA | 1.7 | 1             | 0.9  | 0.9             |
| mLA       | 2.6 | 1             | 0.8  | 0.7             |
| F31S      | 2.4 | 1             | 1.6  | 1.5             |
| F31Y      | 5.1 | 1             | 1.7  | 1.5             |
| H32A      | 7.1 | 1             | ND   | ND              |
| H32Y      | 12  | 2             | 2.1  | 2               |
| Y103P     | 15  | 1             | 2.0  | 2               |
| Q117A     | 15  | 1             | 3.2  | 3               |
| Q117D     | 15  | 3             | 2.1  | 2               |
| W118H     | 176 | 84            | ND   | ND              |
| W118Y     | 1488| 71            | 1.7  | 1.5             |

* Change in Kₘₐ is value for Kₘₐ divided by corresponding value for mLA.
* Change in Kₑ is value for Kₑ divided by corresponding value for mLA.
* ND, could not be reliably determined from data.
developed in this laboratory (9, 27). Two sequence changes introduced into the parent recombinant protein (mLA), the presence of an additional methionine at the NH₂ terminus and the substitution of valine for methionine 90, have insignificant effects on its functional properties. Also, as shown in Fig. 3, mLA and variants used in studies of structure-function relationships have near- and far-UV CD spectra that are closely similar to those of bovine LA, and other studies indicate that the molecular stability of mLA is closely similar to that of bovine milk LA. These properties and the relative ease with which high yields of pure native protein can be generated indicate that the mLA expression system is suitable for investigating the structural basis of activity and stability in LA. Construction of mutants using the PCR "megaprimer" method resulted in the introduction of unintended mutations in two cases, a relatively low error rate when balanced against the possibility that lysines 5 and 114, which flank cluster I, proximate histidine 32 would be expected to be associated with such a region where LA activity of LA (31), the possibility that cluster II plays a functional role in LA could not be excluded by previous data. To facilitate the characterization of the functional properties of mutant LAs a procedure was developed that allows the distinction of effects on affinity for GT from effects on the enhancement of glucose binding. The validity of the values elucidated for $K_a$ and $K_c$ with the various mutants are supported by the close agreement between the values obtained for bovine LA and mLA with those previously obtained from more detailed kinetic studies with bovine LA (see Table III). To ensure that any observed functional differences reflect local structural changes, only protein preparations that appear native based on chromatographic behavior, homogeneity on HPLC anion exchange chromatography and near and far UV CD spectra, were considered suitable for probing structure-function relationships. Chromatography was performed with columns containing an organic matrix with that is derivatized with polyethyleneimine. Chromatography on this support therefore involves adsorption as well as ion exchange and, from our observations, separates protein forms with similar ionic properties but different conformations. As shown in Table II, despite similar expression yields, the final yields of folded protein varied greatly. Although a limited number of variants were generated, it appears that, in general, the replacement of aromatic residues in the clusters with non-aromatics resulted in low or insignificant folding yields presumably reflecting a destabilization of the native fold. In one case (H32A-mLA) glycerol was found to improve the yield (Table II) as previously reported for marginally stable mutants of other proteins (e.g. see Ref. 32). The properties of the mutant proteins confirm and extend previous chemical studies of LA, the distinct effects of substitutions in clusters I and II serving to clarify the respective roles of these structures. In the case of cluster II one of five mutants, namely Y103P-mLA, was isolated in native form in good yield. Monomeric protein obtained from Y103A-mLA did not chromatograph like native LA on HPLC and showed a CD spectrum characteristic of the molten globule state of LA. Two of the three mutants with substitutions for tryptophan 104 (alanine and leucine) also did not fold. However, some monomeric protein was isolated from W104Y-mLA by gel filtration (Fig. 1b) which on further separation by HPLC gave a major peak that eluted later than the native protein. Because of the low yield, this protein was not characterized as fully as other mutants. However, it was found to have a significant level of activity in the lactose synthase reaction and to have a CD spectrum which is distinct from that of mLA but indicates the presence of secondary and tertiary structure (Fig. 3c). Tryptophan 104 is conserved in all known LA and lysozyme sequences. In LA it is buried (4, 10) and is therefore unlikely to play a direct role in interacting with GT or glucose. Its structural importance is supported by a previous study with human LA in which the nitration of tryptophan 104 abolished activity and altered the far UV CD spectrum (31). The changed CD spectrum of W104Y-mLA can be, in part, attributed to the nature of the sequence change. It is more pronounced than the change seen in W118Y-mLA (Fig. 3a and c) which could reflect the different environments of these two tryptophans in the native structure or a conformational change in W104Y-mLA. Energy minimization studies suggest that the substitution of tyrosine in position 104 may result in a rearrangement of the hydrophobic box and a more open cleft, which may serve to explain the increased retention time on HPLC. Studies are in progress aimed at characterizing W104Y-mLA in detail but, based on present evidence, it appears that the reduced activity of this mutant results from a localized conformational change. The ability of the Y103P mutant to produce fully active native protein, in contrast with Y103A-mLA, is interesting since alanine and proline are found at this site in various lysozymes. This may reflect the stabilization of protein native structures

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2 J. A. Grobler, W. K. Linnerooth, and K. Brew, unpublished observations.
that results from the introduction of proline at a compatible site which has been attributed to a resulting decrease in entropy, and concomitant increase in free energy, of the unfolded state (34). Overall, these observations indicate that the hydrophobic box (aromatic cluster II) has a structural rather than functional role in LA (Fig. 6).

Mutant LAs with substitutions for components of cluster I are distinct in properties as a class from those involving components of cluster II. A larger proportion of them (8 out of 12) allow the generation of native protein, and all of these show significant changes in functional properties. Therefore, this region, which is more exposed to solvent than cluster II, is of functional importance in LA. In agreement with previous results, it is shown that histidine 32 and tryptophan 118 play particularly important roles in LA action, all substitutions made at these sites producing major perturbations of activity. Two conserved residues adjacent to these, phenylalanine 31 and glutamine 117, which had not been previously probed by chemical modification, are also implicated in the activity of LA. The kinetic properties of Q117A, W118Y and W118H-mLAs are consistent with direct or indirect roles for residues 117 and 118 in the interaction with GT. Although the activity of W118H-mLA was so low that its effects on glucose binding were not determined, W118Y-mLA appears to be specifically reduced in affinity for GT (Table III). In contrast, the position 31 and 32 mutants show changes in both kinetic parameters. The most informative protein in this group is F31Y-mLA whose low lactose synthase activity is almost entirely attributable to a reduced (15-fold) ability to promote glucose binding. With the position 32 mutants the effect on glucose binding is decreased by up to two orders of magnitude. As far as we are aware, significant effects on this aspect of LA action resulting from a structural modification have not been noted previously. Therefore, the effects of changes in the residues composing cluster I divide them into two groups: residues 117 and 118 which specifically influence the strength of binding with GT, and residues 31 and 32 which influence both GT binding and the enhancement of glucose binding or in the case of F31Y-mLA, affect glucose binding with minimal effects on GT binding.

These results are relevant to the mechanism of action of LA. In one model previously proposed for its mechanism of action (35), LA binds to GT at a site adjacent to the acceptor binding site and provides additional stabilizing interactions for a monosaccharide located at the interface of the two protein components (a "monosaccharide bridge" arrangement as shown schematically in Fig. 7). The same mode of binding sterically prevents the binding of more extended acceptor substrates. The model implies that LA has a binding site for GT (Sgt) and an adjacent site which can interact favorably with a bound monosaccharide in the lactose synthase complex (Sgec). Because the cleft in LA contains several conserved residues whose counterparts in lysozyme act in carbohydrate binding, a region within the shortened cleft in LA may support glucose binding in the lactose synthase complex (12). In this model, the interaction of LA with GT and the enhancement of glucose binding to this complex are associated with distinct but neighboring regions of the LA molecule and would be separately affected by mutagenesis. An alternative mechanism would be for LA to modulate the substrate specificity of GT through an allosteric effect. Although reasonable, this hypothesis implies an unusually extreme level of functional divergence at the molecular level between LA and lysozyme and is also difficult to reconcile with the rapid equilibrium association of LA and GT (17, 24). The properties of the mutant LAs are more consistent with the former model for LA action since single-site substitutions are found to separately change the two aspects of LA activity. If the action of LA on GT was allosteric the two facets of LA action would be intrinsically linked. Therefore, these results tentatively support the "monosaccharide bridge" model although more information regarding the structure of GT and the mo-
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The proximity of phenylalanine 31 and histidine 32 to the cleft suggest that part of this region may act in supporting glucose binding. However, a contact site with GT that includes the two proteins. Studies are currently in progress to attempt amino acid sequence analysis, Adriana Vasquez count for the strength and specificity of the interaction between Dr. Richard, H., and Brew, K. (1974) Eur. J. Biochem. 44, 537-550.

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