Functional Site in α-Lactalbumin Encompasses a Region Corresponding to a Subsite in Lysozyme and Parts of Two Adjacent Flexible Substructures†

Vladimir A. Malinovskii, Jie Tian, Jay A. Grobler,‡ and Keith Brew*

Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101

Received February 22, 1996; Revised Manuscript Received May 8, 1996⊗

ABSTRACT: Aromatic cluster 1 of α-lactalbumin (LA), a substructure adjacent to the cleft, is important for its interaction with galactosyltransferase (GT) and effects on glucose binding in the lactose synthase complex [Grobler, J. A., Wang, M., Pike, A. K., & Brew, K. (1994) *J. Biol. Chem. 269*, 5106−5114]. The full extent of the functional region in LA has been probed by mutagenesis of residues that are near aromatic cluster 1 or within the cleft that corresponds to the active site in the homologous type c lysozymes. The conserved residues Val42, Gln54, and Ile59, which correspond to residues of lysozyme that act in substrate binding in subsites C to E, together with residues adjacent to aromatic cluster 1, were found to be not required for activity. In contrast, replacing Leu110, a component of the region corresponding to lysozyme subsite F, with His or Glu greatly reduces the affinity of LA for GT while the introduction of Arg lowers the synergism of LA and glucose binding to GT and also reduces the affinity of LA for GT. Substitutions for Ala106, which is adjacent to Leu110 in the structure, also perturb activity. The region of the cleft corresponding to subsite F is important for function in LA as well as in lysozyme since other components of this subsite, His32 and Phe31, are also crucial for LA activity. The qualitatively different effects of various substitutions for Leu110 may be mediated by their influence on His32 or by changes in the structure of the lactose synthase complex.

Lactose synthase provides an unusual example of enzyme regulation in which substrate specificity is modulated by the highly reversible interaction of a regulatory protein, α-lactalbumin (LA),[1] with a glycosyltransferase, UDP-galactose *N*-acetylglucosamine β-1,4-galactosyltransferase, GT [reviewed by Hill and Brew (1975) and Grobler and Brew (1992)]. In isolation, GT binds glucose weakly as reflected in a K_m of about 2 M. LA binds to GT synergistically with glucose to produce a 1000-fold reduction in the K_m for glucose and consequently supports high rates of lactose synthesis at physiological concentrations of glucose. At the same time LA competitively inhibits the binding of monosaccharides extended on the C1 (β) or C2 positions, as well as glycoprotein or oligosaccharide substrates, to GT.

LA is homologous with the type c lysozymes, being 35−40% identical in sequence and closely similar in 3D structure to the mammalian and avian lysozymes (Grobler et al., 1994a; Acharya et al., 1989). However, LA differs from the lysozymes significantly in molecular stability (Ikeguchi et al., 1986) and local flexibility (Acharya et al., 1989, 1991; Harata & Muraki, 1992) as well as in function. The crystals structures of different species variants of LA (Acharya et al., 1989, 1991; Pike et al., 1996) or of human LA grown under different conditions of pH and temperature (Harata & Muraki, 1992) or in the presence of Zn^{2+} ions (Ren et al., 1993) indicate that LA can exist in different structural states in which regions of the molecule exhibit different conformations. The greater flexibility of regions of LA, as compared with the corresponding regions of lysozyme, has been suggested to be an adaptation associated with a requirement for local structural adjustments during the interaction with GT (Acharya et al., 1989).

The LA molecule is divided by a cleft which separates an α-helical lobe, composed from the amino- and carboxy-terminal parts of the polypeptide chain, and a lobe containing an irregular antiparallel β-sheet and a disulfide loop that is composed from the central part of the polypeptide chain (Acharya et al., 1989, 1991). In lysozyme, the cleft contains the catalytic site that includes six monosaccharide-binding subsites, A−F (Blake et al., 1967); see Figure 1. While the structural basis of activity is less well understood in LA than in lysozyme, previous studies with LA have shown that components of a surface cluster of predominantly aromatic residues, aromatic cluster 1 (AC1), are crucial for LA function in lactose synthase (Grobler et al., 1994b). This region is in the α-helical lobe of LA and extends over a surface that corresponds to subsite F of lysozyme (Figure 1). Gln117 and Trp118, which are components of the C-terminal region, and Phe31 and His32, which are part of α-helix B, are components of the active site. The upper part

† This work was partly supported by Grant GM21363 from the National Institutes of Health and by a National Research Service Award to J.A.G.

* To whom correspondence should be addressed at the Department of Biochemistry and Molecular Biology, R-629, University of Miami School of Medicine, P.O. Box 016129, Miami, FL 33101. Telephone: (305)-243-6297. FAX: (305)-243-3065.

‡Present address: Laboratory of Molecular Biology, R-433, NIDDK, NIH, Bethesda, MD 20892-6600.

⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations: LA, α-lactalbumin; bLA, bovine α-lactalbumin; mLA, recombinant bovine α-lactalbumin with NH_2-terminal methionine and valine substituted for methionine 90; GT, UDP-galactose *N*-acetylglucosamine β-1,4-galactosyltransferase; AC1, aromatic cluster 1; CD, circular dichroism; PCR, polymerase chain reaction. Variants of mLA are designated X*n*Y-mLA, where *n* is the sequence position in bLA, X is the original amino acid at that site, and Y the amino acid substituted at the site. C-Terminal deletion mutants in which the I-VIII disulfide bond is removed by mutation of Cys6 as well as the deletion of Cys120 and subsequent residues are designated C6S, Δ*n*-mLA, where Δ*n* indicates that a stop codon replaces the codon for amino acid X at position *n*.

S0006-2960(96)00437-0 CCC: $12.00

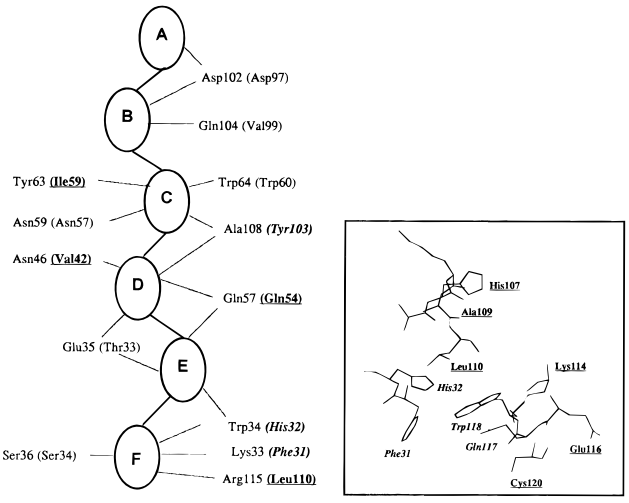

FIGURE 1: Schematic diagram of the cleft region showing residues in lysozyme that are in contact with the oligosaccharide substrate in subsites A−F and the corresponding residues in LA. The insert shows the arrangement of the side chains of residues that are part of or are adjacent to AC1. LA residues in italics were mutated in a previous study (Grobler et al., 1994b) while bold-faced, underlined residues were investigated in the present study.

of the LA cleft, corresponding to lysozyme subsites A and B, is blocked by the substitution of a Tyr at position 103 for Ala or Pro in the lysozymes (see Figure 1), but several residues in other parts of the cleft are conserved between LA and the lysozymes, raising the possibility that, by analogy with its function in the lysozymes, part of the cleft in LA may act to facilitate glucose binding in the lactose synthase complex (Shewale et al., 1984).

The goal of this investigation was to delineate the functional roles of selected residues in the cleft and adjacent to AC1 in the activity of LA. The residues that were chosen and their locations are shown schematically in Figure 1 together with the functional roles of the corresponding residues in lysozyme. The choice of substitutions was, where possible, based on the nature of residues present in lysozymes from different sources. This was to explore the structural basis of functional divergence of the two homologues and also to introduce mutations into the relatively unstable LA molecule that are compatible with the LA/lysozyme fold.

EXPERIMENTAL PROCEDURES

Materials. Reagents and kits for molecular biology and reagents for enzyme assays were from the same sources as in previous studies (Wang et al., 1989; Grobler et al., 1994b).

Mutagenesis. Single-site mutations were introduced by the PCR megaprimer method (Sarkar & Sommer, 1990) as described previously (Grobler et al., 1994b). In constructing the mutants with C-terminal deletions and with Ser substituted for Cys6, two megaprimers were generated, one using a T7 terminator primer and a mutagenic primer and the other with a T7 promoter primer and the second mutagenic primer. These were subsequently used in a third reaction with original pMLA template. Single-site mutants were constructed using mutagenic primers that were designed to avoid adventitious mutations arising from 3′-terminal transferase activity of Taq polymerase by ending the primer at a base immediately prior to the third (wobble) position of a codon where base changes do not introduce protein sequence changes. All mutants were characterized by DNA sequencing of the expression vector.

Protein Extraction, Folding, and Purification. mLA and its variants were expressed in *Escherichia coli* strain BL21-(DE3) as inclusion bodies. Proteins were extracted from inclusion bodies, partially purified, and folded by a progressive dialysis procedure essentially as described previously (Grobler et al., 1994b). Folded protein was concentrated by adsorption on DE52 DEAE-cellulose equilibrated with 20 mM Tris-HCl, pH 8, and eluted stepwise with 0.5 M NaCl in the same buffer. Subsequently, purification was carried out by gel filtration with columns (5 × 25 cm) of Superdex G75, equilibrated and eluted with 20 mM Tris-HCl, pH 7.4, containing 0.1 mM $CaCl_2$, at a flow rate of 1.5 mL/min. The peak corresponding to monomeric mLA eluted at about 300 mL and was pooled and finally purified by anion-exchange chromatography with columns (2.5 × 5 cm) of DEAE-cellulose equilibrated with 20 mM Tris-HCl, pH 7.4, containing 0.1 mM $CaCl_2$. After application of the sample, the column was washed with equilibration buffer for 5 min and eluted with a linear gradient from 0 to 0.4 M NaCl in the same buffer over 120 min. A flow rate of 2.5 mL/min was used throughout. Native mLA (and variants) eluted after about 20 min.

Functional Characterization of Proteins. The activities of mLA variants as competitive inhibitors of the transfer of galactose to *N*,*N*′-diacetylchitobiose and as activators of lactose synthesis by bovine GT were determined as described previously (Grobler et al., 1994b; Khatra et al., 1974). The assays of inhibitory activity were performed with chitobiose at a concentration of 0.5 mM, while assays of the activation of lactose synthesis were carried out using a glucose concentration of 10 mM. However, with L110R-mLA, where initial studies indicated that the ability to promote glucose binding was impaired, a higher concentration of glucose (40 mM) was used in another set of assays to obtain reliable values for the kinetic parameter associated with glucose binding, K_m^{glc}. A mutant constructed in a previous study, F31Y-mLA, was also reassayed at the higher glucose concentration for the same reason. Lactose synthase activities are expressed relative to the V_m value obtained with bLA and activities with chitobiose relative to the activity observed in the absence of LA. Apparent K_i and K_m values determined directly from these assays were used to calculate values for K_i^{LA} (a measure of LA affinity for GT) and K_m^{glc}, the K_m for glucose at saturating concentrations of LA, as described by Grobler et al. (1994b). Data were fitted to appropriate equations using the Curve Fit function of SigmaPlot for Windows (Jandel Scientific, San Rafael, CA).

Other Methods. Oligonucleotide synthesis and automated DNA sequencing were performed by Dr. Rudolf Werner, Department of Biochemistry and Molecular Biology, University of Miami School of Medicine. CD spectra of mLA variants were determined with a JASCO J720 spectropolarimeter as described previously (Grobler et al., 1994b).

RESULTS

Design and Production of Mutant Proteins. Two types of mLA mutant were constructed and characterized. The first group had substitutions for residues of the cleft region that are functionally important in lysozyme and are either conserved in both LA and lysozyme (Gln54, of subsites D and E) or conserved in LA but different from lysozyme (Val42 that corresponds to Asn44 of subsite D, Ile59 that corresponds to Trp or Tyr63 of lysozyme subsite C, and

Leu110 that corresponds to Arg115 of subsite F). A second group includes residues that are proximal to AC1, the substructure previously identified as being crucial to LA action (Grobler et al., 1994b). Residues in this group are Ala106, His107, and Ala109 of the flexible loop,[2] Lys114 and Asp116 which are adjacent to Trp118, and residues 119−123 of the C-terminus. Leu110 falls into both groups since it is adjacent to His32 of AC1 as well as being part of the subsite F region. While the primary basis for the choice of residues to substitute at the various sites was the corresponding residue in different lysozymes and in some of the more divergent LAs (e.g., V42N, I59W, H107W, L110R, L110H, K114Q, K114D, K114N, D116E), some substitutions were directed by structural considerations. Thus, Ala and Gly were also substituted for Val42 to progressively truncate the side chain; Glu and Ala were substituted for Gln54 to alter the charge and size, respectively. Ala106 was replaced by Lys to preserve the helix propensity while altering size and charge and with Ser to alter the conformational properties. Additional substitutions of Glu for Lys114 and Asn for Asp116 were designed to retain the hydrophilic character but modify the charge.

The I−VIII disulfide bond between Cys6 and Cys120 is the most solvent-exposed of disulfide bonds in LA and is readily reduced in the absence of denaturants (Schechter et al., 1973). The residues following Cys120 are not necessary for structure or activity in LA since they are variable or absent in certain LAs and lysozymes (Grobler et al., 1994a) and, when present, are disordered in electron density maps (Acharya et al., 1989, 1991; Ren et al., 1993). To investigate the minimal requirements in the C-terminal region for LA activity, Cys6 was changed to Ser to remove the I−VIII disulfide bond and the polypeptide chain was truncated at positions 119, 118, and 116.

Preparation and Properties of mLA Mutants. Folded forms of the mLA variants were generated from inclusion bodies as described. The expression yields of different mutants, calculated from the absorbance at 280 nm after separation by ion exchange in urea, varied from 21 to 116 mg/L, and final yields of folded protein ranged from 18% to 162% (mean 67%) of that obtained with mLA. Thus, there are significant differences between them in folding efficiency and/or stability. The mutants described here had far- and near-CD spectra that indicate that their secondary and tertiary structures are similar to those of bLA and mLA. We have also expressed a number of other mutants, including several with different substitutions at the same sites, whose CD spectra indicate disturbed tertiary and/or secondary structures. Since functional perturbations that are accompanied by global structural changes are not a reliable guide to structure−function relationships, these proteins were not used in this study and will be described elsewhere. Although the parent protein with a C-terminal deletion in which the I−VIII disulfide bond is also missing, C6S, ΔC120-mLA, has a native-like CD spectrum, the structural properties of the two mutants with additional residues deleted from the C-terminus were not characterized by CD spectroscopy because of the low yields of folded protein. The assumption that they have native structures is based on their similar behavior to mLA on gel filtration and ion-exchange chromatography [see Grobler et al. (1994b)].

[2] Residues 105−110 of LA which correspond to α-helix 4 of lysozyme are designated the "flexible loop" since this region displays partially helical and loop conformations in different crystal forms.

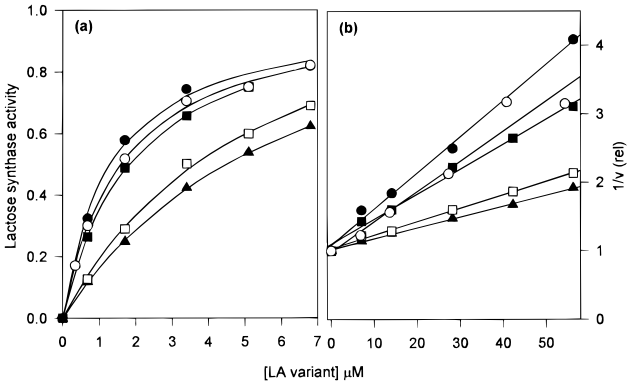

FIGURE 2: Effects of substitutions for Val42 and Gln54 on the activity of LA as (a) an activator of lactose synthase activity and (b) an inhibitor of galactose transfer to chitobiose. Symbols: (●) bLA; (■) V42N-mLA; (□) V42G-mLA; (▲) V42A-mLA; (○) Q54A-mLA. In (a), lactose synthase activities are given relative to the activity obtained with the same quantity of GT and a saturating concentration of bLA while in (b) activities for galactose transfer to *N,N′*-diacetylchitobiose are relative to the activity in the absence of LA.

Functional Properties of mLA Mutants. Mutagenesis of residues of the LA cleft whose counterparts in lysozyme are components of subsites C through E produce minor changes in activity. Figure 2 compares the activities of mutants with substitutions for Val42 and Gln54 with bLA as inhibitors of galactose transfer to chitobiose and activators of lactose synthesis with bovine GT. Changes in inhibitory activity are a measure of changes in the affinity of LA for GT while changes in lactose synthase activity can reflect effects on both the affinity for GT and ability of the GT−LA complex to bind glucose. The fact that the same order of activities is observed in both assays indicates that changes in activity in these mutants principally reflect a reduced affinity for GT. Table 1 gives values for two parameters calculated from these data, K_i^{LA} and K_m^{glc}. These measure respectively the affinity of LA for GT and the ability of the LA variant to promote glucose binding when present at saturating concentrations. Activity plots are not shown for I59W-mLA, which is very similar to mLA in functional properties, but the kinetic parameters for this mutant are given in Table 1.

In contrast, all substitutions for Leu110 generate major changes in activity (Figure 3 and Table 1). Two substitutions for Ala106, which is adjacent to Leu110 in the structure, both reduce the affinity for GT, although the change is much less with Ser (data not shown) than with Lys (Figure 3). Mutations were introduced at two other sites in the flexible loop, His107 and Ala109. Substitutions for His107 produced 2−10-fold reductions in affinity for GT, while the A109P-mLA was essentially unchanged in activity (data not shown; see Table 1 for values of kinetic parameters). The reduced activities of the Ala106 and His107 mutants result primarily from decreases in the affinity for GT (parameter K_i^{LA}). In contrast, the substitution of Arg and His and Glu for Leu110 had differential effects on GT affinity and ability to promote glucose binding. This is shown in Figure 3, where L110H-mLA is more active in promoting lactose synthesis than L110R-mLA, but L110R-mLA is more effective than L110H-mLA as an inhibitor of galactose transfer to chitobiose (a measure of affinity for GT). To obtain a more reliable value for K_m^{glc} for L110R, it was characterized as

Table 1: Summary of the Functional and Structural Properties of mLA and Its Variants[a]

original residue(s) in mLA	substitution	K_i^{LA} (μM)	K_m^{glc} (mM)
	none	15.3 ± 0.9	1.6 ± 0.1
Val42	Ala	39 ± 1	2.0 ± 0.1
	Gly	32 ± 2	2.0 ± 0.4
	Asn	18 ± 2	1.0 ± 0.2
Gln54	Ala	18 ± 2	1.0 ± 0.1
Ile59	Trp	18 ± 2	2.1 ± 0.4
Ala106	Lys	3600 ± 500	3.3 ± 0.9
	Ser	84 ± 13	1.4 ± 0.3
His107	Tyr	146 ± 26	0.7 ± 0.2
	Trp	34 ± 4	4.1 ± 0.9
	Ala	175 ± 40	1.1 ± 0.5
Ala109	Pro	19 ± 6	1.3 ± 0.5
Leu110	His	635 ± 156	2.9 ± 1.1
	Arg	180 ± 19	29 ± 7
	Glu	2050 ± 280	3.9 ± 0.8
Lys114	Glu	24 ± 1	3.0 ± 0.3
	Gln	21 ± 1	2.6 ± 0.1
	Asn	610 ± 240	4.7 ± 2.7
Asp116	Glu	17 ± 1	0.9 ± 0.1
	Asn	32 ± 2	1.6 ± 0.3
Cys6, Gln117−Leu123	Ser6, Δ120−123	59 ± 4	5.9 ± 0.7
	Ser6, Δ119−123	nd[b]	active[c]
	Ser6, Δ117−123	nd	inactive[c]
Phe31	Tyr	26 ± 1	20 ± 7
	Ser	328 ± 30	10.0 ± 2
His32	Tyr	190 ± 17	inactive
Gln117	Ala	139 ± 5	3.1 ± 0.3
Trp118	Tyr	1488 ± 509	1.7 ± 0.7

[a] K_i^{LA} and K_m^{glc} and standard errors were determined as described in the text. Values for K_m^{glc} given for L110R-mLA and F31Y-mLA are based on lactose synthase assays carried out at 40 mM glucose; all other lactose synthase assays were performed with 10 mM glucose. The properties of F31Y, F31S, H32Y, Q117A, and W118Y are as reported previously (Grobler et al., 1994b) except for F31Y, which was recharacterized at the higher glucose concentration. [b] nd = not determined. [c] The C-terminally truncated mutants S6, ΔL119-mLA and S6, ΔQ117-mLA were obtained in low yields and assayed for lactose synthase activity only so that quantitative values for their kinetic parameters were not determined.

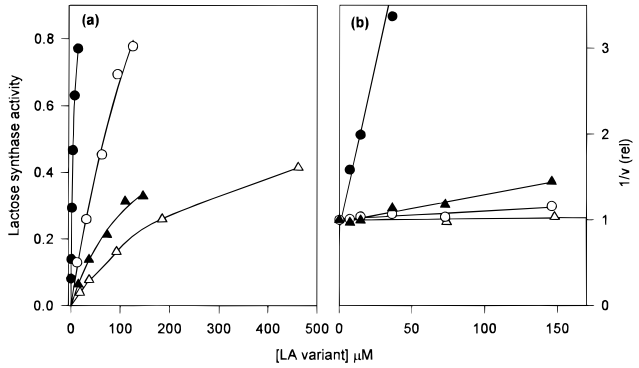

FIGURE 3: Effects of some substitutions for Ala106 and Leu110 on the activity of LA as (a) an activator of lactose synthase activity and (b) an inhibitor of galactose transfer to chitobiose. Symbols: (●) bLA; (○) L110H-mLA; (▲) L110R-mLA; (△) A106K-mLA. The K_i value given for A106K-mLA in Table 1 was determined using additional data obtained with protein concentrations up to 461 μM. Relative activities are as described in Figure 2.

an activator of the lactose synthase reaction at a higher concentration of glucose (40 mM) to give the values shown in Table 1.

The substitution of Glu or Gln for Lys114 had little effect on activity in either reaction whereas the substitution of Asn produced a more than 30-fold reduction in activity that is largely attributable to a decrease in affinity for GT. In the case of Asp116, the introduction of Asn or Glu had little effect (Table 1). The removal of residues 120−123 together with the I−VIII disulfide produced a variant with reduced activity (Figure 4). On the removal of Leu119, the activity decreased while the removal of Trp118 and Gln117 eliminated lactose synthase activity.

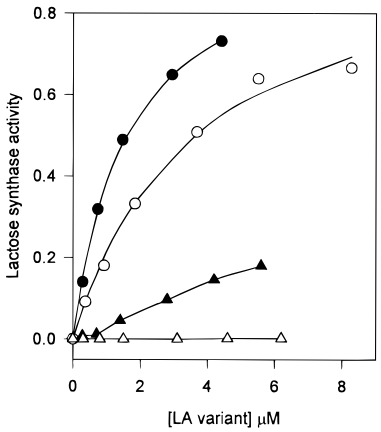

FIGURE 4: Effects of C-terminal truncations on the activity of LA as an activator of lactose synthase. Symbols: (●) bLA; (○) C6S, ΔC120-mLA; (▲) C6S, ΔL119-mLA; (△) C6S, ΔQ117-mLA. Relative lactose synthase activities are as described for Figure 2.

Several substitutions result in particularly low affinities for GT, for example, Lys for Ala106 and His or Glu for Leu110. Protein solubility and availability limited the concentration range over which it was feasible to perform inhibition studies (e.g., concentrations of up to 5 mg/mL were required in some cases) resulting, in the case of these mutants, in an increased error in K_i^{LA} values and, in turn, K_m^{glc} values, which are calculated using the former. Table 1 shows standard errors for K_i^{LA} together with the compounded standard errors for K_m^{glc}. In the case K114N-mLA, the proportional errors in both parameters are too large to support reliable conclusions regarding the basis of the low activity, although it is clear that the affinity for GT is greatly reduced.

DISCUSSION

Previous work has shown that substitutions for Gln117 or Trp118 specifically affect GT binding while mutagenesis of Phe31 or His32 reduces the affinity of lactose synthase for glucose or, more correctly, reduces the synergism between LA and glucose for binding to GT and also exerts variable effects on GT affinity (Grobler et al., 1994b). As shown in Figure 5, although these pairs of residues are part of aromatic cluster 1, they are separated structurally as well as having distinct functional influences and can be regarded as parts of separate but adjacent subsites. The present results show that the surface around residues 117 and 118 that selectively influences the affinity of LA for GT is small since changes in the size and charge of two residues adjacent to Trp118, Lys114 (changed to Glu and Gln), and Asp116 (to Glu and Asn) have only minor effects on the activity of LA. Another substitution for Lys114, Asn, does perturb activity, but because the effects of the two other substitutions are minor, it is reasonable to conclude that Lys114 is not directly involved in activity in LA. Progressive C-terminal truncations in a variant that lacks the I−VIII disulfide bond between Cys6 and Cys120 show that residues C-terminal to Trp118 are not essential for LA activity but Gln117 and Trp118 are essential for lactose synthase activity. The

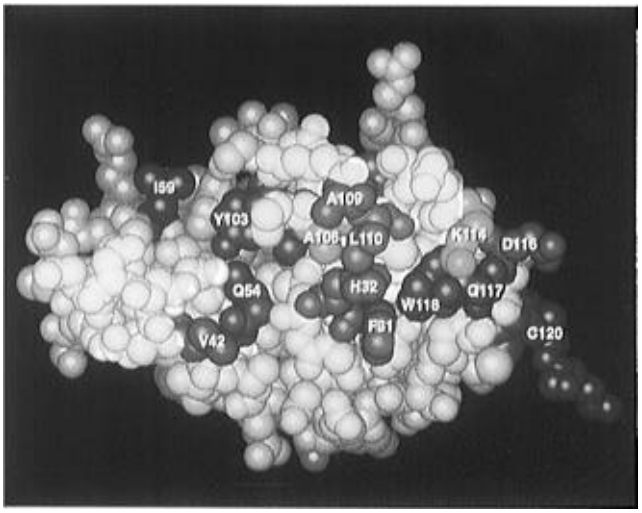

FIGURE 5: Surface structure of baboon LA showing the functional effects of mutations at different sites. Residues that have a minimal influence on activity are shown in green, Ala106 and Lys114 which appear to affect or influence LA activity indirectly are in azure, residues that specifically influence GT binding are in dark purple, and residues that can affect glucose binding are in light purple.

disulfide bond, itself, is not necessary for the structure or activity of LA as shown by earlier chemical studies (Schechter et al., 1973). Therefore, in the region carboxy-terminal to Cys111, only Trp118 and Gln117 are crucial for the interaction with GT. A small interaction surface is consistent with the relatively low affinity of LA for GT in the absence of monosaccharide, as indicated by a K_d of about 10^{-5} M and the fact that 40% of the free energy of the LA−GT interaction is lost on replacing this residue with either His or Tyr [a reduction from 28 to 17 kJ/mol, calculated from K_i^{LA} values given by Grobler et al. (1994b)].

The data in Table 1 also show that Val42, Gln54, and Ile59, of the region corresponding to lysozyme subsites C through E (Figures 1 and 5), are not required for LA activity. Our previous observation that the substitution of Pro for the conserved Tyr103 that corresponds to Ala108 of subsite C in human lysozyme does not impair activity (Grobler et al., 1994b) also supports this conclusion. In contrast, substitutions for Leu110 and Ala106 of the lower cleft have large effects on lactose synthase activity. Two other residues, His107 and Ala109, in this region of the LA molecule (the flexible loop, residues 105−110) can be replaced by some amino acids with little impact on activity. Replacing Ala109 with Pro has essentially no effect, and substitution of Trp for His107 has only a 2-fold effect on the affinity for GT. His107 is thought to be a key to the helix−coil transconformation of the flexible loop, being buried in the helical conformer and exposed to solvent in the coil structure (Harata & Muraki, 1992; Pike et al., 1996), and the introduction of Tyr or Ala at this site is likely to affect the structure or flexibility of this substructure. As summarized in Figure 5, our results show that the functionally crucial region of LA is a contiguous surface formed from the side chains of Leu110, Phe31, His32, Gln117, and Trp118, and possibly Ala106, while other residues surrounding AC1 and other parts of the cleft are not required for activity.

The three residues in LA that can influence glucose binding by the LA−GT complex, Phe31, His32, and Leu110, are the counterparts of residues in lysozyme that are parts of monosaccharide binding subsite F. The exact nature of carbohydrate−protein interactions in this lysozyme subsite is controversial. Initially, model building studies indicated that Phe33 (LA, His32), Asn37 (LA, Gly35), and Arg115 (LA, Leu110) were involved (Blake et al., 1967), but the recently published crystal structure of a complex of human lysozyme with both a tetrasaccharide and disaccharide bound in the cleft (products of hydrolysis of a hexasaccharide substrate) implicates Phe31, Asn37, and Lys33 (Phe31 in LA), but not Arg115 (Song et al., 1994). However, this discrepancy may reflect differences in interactions in the Michaelis and enzyme−product complexes, and it is possible that all of these residues could function at different stages of the catalytic cycle in lysozyme.

Previously, we have proposed a "monosaccharide bridge" model (Grobler et al., 1994b) for the action of LA in lactose synthase. In this, distinct but adjacent sites on LA and GT interact with the cognate protein and glucose in the GT−LA complex. The linked synergistic equilibria for the binding of LA and glucose to GT that result are consistent with the known effects of LA on acceptor substrate specificity. Substrates that have extensions on the C2 position or have β-linked groups on C1 obstruct the LA binding site on GT so that such substrates and LA bind antagonistically with GT (Lambright et al., 1985; Berliner et al., 1984). If it is assumed that the LA−GT interaction is not accompanied by conformational changes, this model predicts that mutations in the GT interaction site of LA will specifically perturb the affinity for GT (K_i^{LA}) while not affecting the ability of LA to promote glucose binding in the lactose synthase complex (K_m^{glc}). Conversely, mutations in the glucose interaction site will specifically affect glucose binding without affecting the interaction with GT. While substitutions for Gln117 and Trp118, identified as representing the GT interaction site, produce perturbations in GT binding unaccompanied by effects on glucose binding, only one mutation (F31Y) shows a large change in K_m^{glc} that is not accompanied by a large change in K_i^{LA}. Replacement of the key residue, His32, with Tyr essentially eliminates lactose synthase specifier activity but also reduces the affinity for GT (Grobler et al., 1994b). The new results reported here are even less consistent with these predictions since two substitutions (His and Glu) for Leu110 selectively reduce the affinity for GT while the introduction of Arg reduces K_m^{glc} by a factor of 19 and K_i^{LA} by a factor of 12.

The monosaccharide bridge model does not invoke conformational changes to explain the ligand-modulated LA−GT interaction that underlies specificity regulation in lactose synthase. However, it is very likely that structural adjustments occur in either or both proteins on heterodimer formation and there is indirect evidence for such changes. The ability of functionally important regions of LA to undergo conformational adjustments is demonstrated by their multiple conformational states in different crystal forms (Pike et al., 1996). Also, localized conformational changes provide a reasonable, albeit not unique, explanation of the increased reactivities of lysine 114 in LA and one or more of lysines 230, 237, and 241 of GT produced by their mutual interaction (Richardson & Brew, 1980; Yadav & Brew, 1991). LA could possibly act as an allosteric effector by inducing a conformational change in GT to alter its acceptor substrate binding properties, but this seems unlikely because of the rapid equilibrium binding of LA with GT during the catalytic cycle (Khatra et al., 1974; Bell et al., 1976; Powell & Brew, 1976). While the issue of conformational changes can only be definitively resolved by structural studies of the lactose

synthase complex, the hypothesis that the LA component plays a direct role in glucose binding remains attractive because of the close structural similarity between LA and lysozyme. On the basis of the structures of carbohydrate-binding sites (Quiocho, 1986) and previous mutational studies (Grobler et al., 1994b), His32 is the most likely of the residues in the LA active site to interact directly with a monosaccharide in the lactose synthase complex; Leu110 is an unlikely candidate for such a role, yet the substitution of Arg at this site has a strong effect on glucose binding. Several considerations suggest an indirect, more structural role for Leu110 in lactose synthase. Its side chain is in contact distance of the imidazole side chain of His32, which is particularly critical for glucose binding (Grobler et al., 1994b), and substitutions may exert variable effects on activity by influencing the environment, p*K*, or orientation of this residue, by modulating the conformation of the flexible loop (Pike et al., 1996), or by altering the ability of LA to induce a change in structure in GT. A structural role for Leu110 is indicated by the large structural influence of the equivalent residue in lysozyme, Arg115; substitution of histidine in chicken lysozyme increases stability (Shih & Kirsch, 1995) while glutamate reduces activity and alters the structure of human lysozyme (Harata et al., 1993). Interestingly, the substitution of Asn for Lys114, which uniquely among the mutations at this site perturbs activity in a major way, also produces a large increase in thermal stability (L. Greene and K. Brew, unpublished experiments) and probably affects the structure and/or dynamics of the flexible C-terminus. The effects of substitutions for both Leu110 and Lys114 should be clarified by ongoing structural studies of these mutants (K. R. Acharya, personal communication) and the functional effects of other substitutions at these sites.

ACKNOWLEDGMENT

We thank Vera Ondricek for help with CD spectroscopy, Adriana Vasquez and Baoping Pang for technical assistance, and Dr. Ravi Acharya for helpful discussions.

REFERENCES

Acharya, K. R., Stuart, D. I., Walker, N. P. C., Lewis, M., & Phillips, D. C. (1989) *J. Mol. Biol. 208*, 99−127.

Acharya, K. R., Stuart, D. I., Phillips, D. C., & Fenna, R. E. (1991) *J. Mol. Biol. 221*, 571−581.

Bell, J. E., Beyer, T. A., & Hill, R. L. (1976) *J. Biol. Chem. 251*, 3003−3013.

Berliner, L. J., Davis, M. E., Ebner, K. E., Beyer, T. A., & Bell, J. E. (1984) *Mol. Cell. Biochem. 62*, 37−42.

Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1967) *Proc. R. Soc. London, B 167*, 378−388.

Brew, K., & Grobler, J. A. (1992) in *Advanced Dairy Chemistry-1* (Fox, P. F., Ed.) pp 191−229, Elsevier Applied Science, London and New York.

Grobler, J. A., Rao, K. R., Pervaiz, S., & Brew, K. (1994a) *Arch. Biochem. Biophys. 313*, 360−366.

Grobler, J. A., Wang, M., Pike, A. C. W., & Brew, K. (1994b) *J. Biol. Chem. 269*, 5106−5114.

Harata, K., & Muraki, M. (1992) *J. Biol. Chem., 267*, 1419−1421.

Harata, K., Muraki, M., & Jigami, Y. (1993) *J. Mol. Biol. 233*, 524−535.

Hill, R. L., & Brew, K. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 411−490.

Ikeguchi, M., Kuwajima, K., & Sugai, S. (1986) *J. Biochem. (Tokyo) 99*, 1191−1201.

Khatra, B. S., Herries, D. G., & Brew, K. (1974) *Eur. J. Biochem. 44*, 537−560.

Kuwajima, K. (1989) *Proteins 6*, 87−103.

Lambright, D. G., Lee, T. K., & Wong, S. W. (1985) *Biochemistry 24*, 910−914.

Pike, A. C. W., Acharya, K. R., & Brew, K. (1996) *Structure* (in press).

Pincus, M. R., & Scheraga, H. A. (1981) *Biochemistry 20*, 3960−3965.

Powell, J. T., & Brew, K. (1976) *J. Biol. Chem. 251*, 3653−3663.

Prieels, J. P., Bell, J. E., Schindler, M., Castellino, F. J., & Hill, R. L. (1979) *Biochemistry 18*, 1771−1776.

Quiocho, F. A. (1986) *Annu. Rev. Biochem. 55*, 287−315.

Ren, J., Stuart, D. I., & Acharya, K. R. (1993) *J. Biol. Chem. 268*, 19292−19298.

Richardson, R. H., & Brew, K. (1980) *J. Biol. Chem. 255*, 3377−3385.

Sarkar, G., & Sommer, S. S. (1990) *BioTechniques 8*, 404−407.

Shechter, Y., Patchornik, A., & Burstein, Y. (1973) *Biochemistry 12*, 3407−3413.

Shih, P., & Kirsch, J. F. (1995) *Protein Sci. 4*, 2063−2072.

Song, H., Inaka, K., Maenaka, K., & Matsushima, M. (1994) *J. Mol. Biol. 244*, 522−540.

Wang, M., Scott, W. A., Rao, K. R., Udey, J., Conner, G. E., & Brew, K. (1989) *J. Biol. Chem. 264*, 21116−21121.

Yadav, S. P., & Brew, K. (1991) *J. Biol. Chem. 266*, 698−703.

BI960437C