Structural Evidence for the Presence of a Secondary Calcium Binding Site in Human α-Lactalbumin[†,‡]

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Received December 5, 1997; Revised Manuscript Received January 26, 1998

ABSTRACT: The high-resolution X-ray crystal structure of human α-lactalbumin (at 1.8 Å) in the presence of an elevated level of calcium reveals a new secondary calcium binding site, 7.9 Å away from the primary calcium binding site known in all α-lactalbumin structures so far. The new calcium binding site is different from the zinc and sulfate binding sites [Ren, J., et al. (1993) *J. Biol. Chem. 268*, 19292−19298] but shares common features with the manganese binding site as described by Gerkin [Gerkin, T. A. (1984) *Biochemistry 23*, 4688−4697]. The proximity of the manganese and calcium binding region and the location of the functional site on one side of the charged surface of the α-lactalbumin molecule suggest that these binding sites might play a role in the formation of the lactose synthase complex.

α-Lactalbumin (LA)[1] is a well-studied protein that is expressed only in the lactating mammary gland and is secreted into the whey fraction of milk (reviewed in refs *1*−*4*). As the specifier protein of the lactose synthase enzyme system (EC 2.4.1.22), LA interacts with a galactosyltransferase (GT), UDPgalactose:*N*-acetylglucosamine β-1,4-galactosyltransferase (EC 2.4.1.38), to modulate its acceptor substrate specificity so that it can catalyze the transfer to glucose, a very poor substrate in the absence of LA, to synthesize lactose during lactation. LA is also similar in sequence and closely similar in structure to the type-c lysozymes, providing an extreme example of functional divergence in homologous proteins.

LA derived from milk contains a single tightly bound Ca^{2+} ion (*5*); the K_{app} for calcium binding to apo-LA under physiological conditions is on the order of 10^{-7} M (*2*, *6*−*8*). The crystal structures of different species variants of LA (*9*−*13*) and human LA grown under different conditions of pH and temperature (*14*) showed the presence of a calcium binding site (we refer to this site as the primary or high-affinity site). The observation that the residues in LA that ligand calcium in the high-affinity site are conserved in all currently known LA sequences suggests the possibility that Ca^{2+} binding might be linked to the biochemical function of LA. However, at least two subgroups of lysozymes possess similar Ca^{2+} binding sites, suggesting that Ca^{2+} binding is a common (although not universal) feature of members of the LA/lysozyme superfamily (*15*). Calcium binding has a large influence on the molecular stability of LA and is required for refolding and native disulfide bond formation in the reduced, denatured protein (*16*, *17*). This could reflect a biological regulatory mechanism or merely result from the low intrinsic stability of LA and its strong Ca^{2+} dependence (*4*).

LA can bind different metal ions in addition to calcium (*2*, *7*, *18*−*27*); these have varying effects on the conformational state and physicochemical properties of LA (reviewed in ref *2*). The presence of a secondary weaker binding site for Ca^{2+} in bovine LA was originally reported by Kronman et al. (*6*) and confirmed by later studies (*23*). However, a similar site was not found in human LA (*28*). Multiple binding sites for other ions and combinations of ions have also been described. The high-resolution (1.7 Å) X-ray crystal structure of the human LA−Zn^{2+} complex, which also contains the primary Ca^{2+} ion, shows that the Zn^{2+} ion does not significantly perturb the conformation (*29*). The Zn^{2+} site was found to be located in the cleft of the LA molecule, about 17 Å from the primary Ca^{2+} site. NMR studies with ^{13}C-reductively methylated LA also suggest that a unique Mn^{2+} site is located close to the N terminus of the protein (*19*). The involvement of metal ions in catalysis by the lactose synthase system and their effects on interactions between LA and GT raise the possibility that a shared metal ion might play a role in the formation of the lactose synthase complex. Secondary metal binding sites in LA provide an indication of regions on the LA structure that could participate in this type of interaction.

We have performed extensive crystallization trials using human LA in the presence of elevated levels of calcium. We report here the structure of human LA with two calcium

[†] This work was supported by a Leverhulme Trust (U.K.) research grant to K.R.A., a NATO collaborative grant to K.R.A. and K.B., and partial support from NIH Research Grant GM21363 to K.B.

[‡] The atomic coordinates have been deposited with the Brookhaven Protein Data Bank (accession number 1A4V).

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[1] Abbreviations: LA, α-lactalbumin; mLA, recombinant bovine LA; GPLA, guinea pig LA; GOLA, goat LA; BLA, baboon LA; BULA, buffalo LA; HLA, human milk LA; GT, β-1,4-galactosyltransferase; NMR, nuclear magnetic resonance; PIPES, 1,4-piperazineethanesulfonic acid; rms, root-mean-square.

S0006-2960(97)03000-6 CCC: $15.00
Published on Web 03/13/1998

binding sites (primary and secondary) occupied and discuss the potential significance of the second site for the structure and activity of LA. Throughout the paper, the previously reported structures of human LA with Ca^{2+} bound only at the primary sites (*11*, *14*) will be designated as the native structure.

EXPERIMENTAL PROCEDURES

Human LA was obtained from Sigma and used without further purification. The crystals were grown in the presence of 100 mM calcium chloride using the hanging drop vapor diffusion method. This protocol was slightly different from that previously described for native human LA (grown in the presence of 10 mM calcium chloride) using the batch method which contains only the primary calcium binding site (*11*). Briefly, drops containing protein (20 mg/mL) at pH 7.5 in 0.1 M PIPES, 1.8 M ammonium sulfate, and 100 mM calcium chloride were equilibrated against a reservoir solution containing 2.0 M ammonium sulfate, 100 mM calcium chloride, and 0.2 M PIPES buffer at pH 7.5. The crystallization trays were left at 35 °C, and after 1 week, they were brought to room temperature (about 20 °C). One brick-like crystal grew to 0.5 mm × 0.3 mm × 0.3 mm after 3 weeks, and the systematic absences and symmetry were consistent with space group $P2_12_12$ with the following unit cell dimensions: $a = 35.47$ Å, $b = 69.56$ Å, and $c = 45.96$ Å (isomorphous with the native human LA crystals; see ref *11*).

Diffraction data were recorded from a single crystal using a Siemens area detector. Graphite monochromated CuKα X-rays were produced by a Siemens rotating anode generator operating at 45 kV and 70 mA. The data frames (0.25° per frame, 200 s per frame exposure) were measured at room temperature. A total of 800 frames were collected in order to obtain a complete data set to 1.8 Å resolution. These frames were processed and reduced using the XENGEN software package (*30*) (Table 1). The structure of native human LA at 1.7 Å (*11*) was used as the starting model. The refinement of the structure by simulated annealing was carried out with XPLOR (version 3.1) (*31*) to a conventional crystallographic *R*-factor (R_{cryst}) of 21% and to an R_{free} (*32*) of 26.5% for all the data from 8 to 1.8 Å resolution. The model exhibits reasonable stereochemistry (rms deviation from ideal covalent bond lengths = 0.011 Å, Table 1) and consists of all the protein atoms, two Ca^{2+} ions, and 106 water molecules.

RESULTS AND DISCUSSION

The structure reported here for human LA at an elevated concentration of Ca^{2+} is closely similar to that previously described for human LA at lower calcium concentrations (Figure 1). The rms difference between the two structures (including all atoms) is only 0.11 Å. Most of the new structure is well-defined in the electron density map with the exception of the loop region between residues 44 and 46 and the flexible C terminus. In all currently known LA structures, both of these regions have high temperature factors and display alternative conformations, suggesting that they represent regions that are flexible in the LA molecule.

The unique feature of the present structure is the presence of two calcium binding sites in close proximity (Figure 1).

Table 1: Crystallographic Data Processing and Refinement Statistics

space group	orthorhombic, $P2_12_12$
cell dimensions (Å)	$a = 35.47$, $b = 69.56$, $c = 45.96$, one human LA molecule/AU
resolution range (Å)	10.0−1.8
N_m[a]	40 180
N_u[b]	9928
overall completeness (%)	82.0
redundancy	4.1
completeness in the outer shell (%)	64 (1.9−1.8 Å)
R_{sym}[c] (%)	6.99 (18%, 2.0−1.8 Å)
no. of reflections used in refinement (8−1.8 Å)	8907
R_{cryst}[d] (%) ($F > 2\sigma$)	21.0
R_{free}[e] (%)	26.3
no. of protein atoms	983
no. of water molecules	106
no. of calcium ions	2
rms deviation in bond lengths (Å)	0.011
rms deviation in bond angles (deg)	1.40

[a] Number of measurements. [b] Number of unique reflections. [c] $R_{sym} = \sum_{hkl}\sum_i|I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $\langle I\rangle$ is the averaged intensity of the i observations of reflection hkl. [d] $R_{cryst} = \sum||F_o| - |F_c||/\sum|F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively. [e] R_{free} is equal to R_{cryst} for a randomly selected 5% subset of reflections not used in the refinement (*32*).

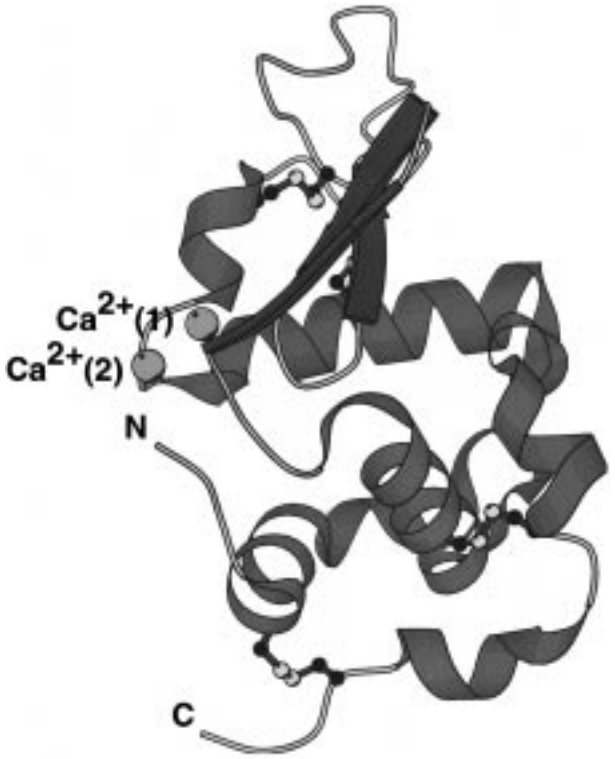

FIGURE 1: Overall topology of the human LA structure. The four disulfide bridges are shown. The primary and secondary calcium binding sites are marked as (1) and (2) in spheres. This figure was drawn with the program MOLSCRIPT (*42*).

The high-affinity Ca^{2+} site (designated as the primary site) could clearly be identified as a strong feature in the electron density map and forms the calcium binding elbow (*9*), one of the most rigid parts of the molecule. The location of the primary Ca^{2+} ion and its coordination (distorted pentagonal bipyramid) are essentially identical to those observed in the previously published structure of human LA (*11*). The secondary Ca^{2+} binding site was located as a persistent

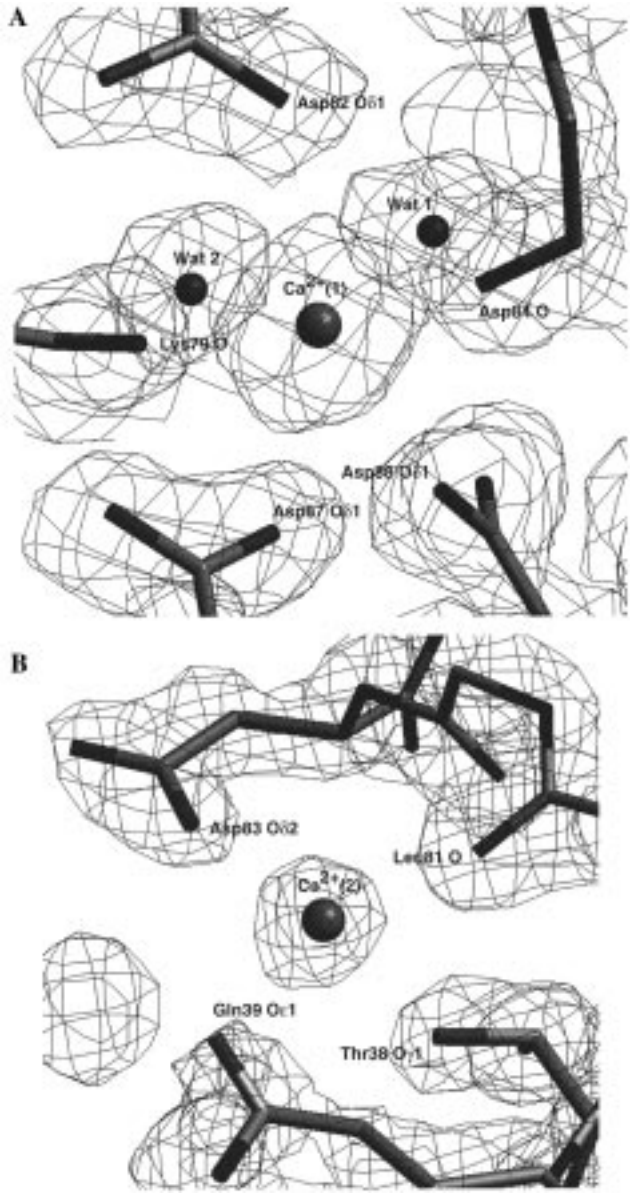

FIGURE 2: Diagram of the electron density in the vicinity of the two calcium ions in the human LA−Ca^{2+} complex structure: primary calcium ion (A) and secondary calcium ion (B). The electron density map is calculated after refinement with amplitude coefficients 2|Fo|-|Fc|. The contour level is 1.6σ. Drawn with the program SETOR (*43*).

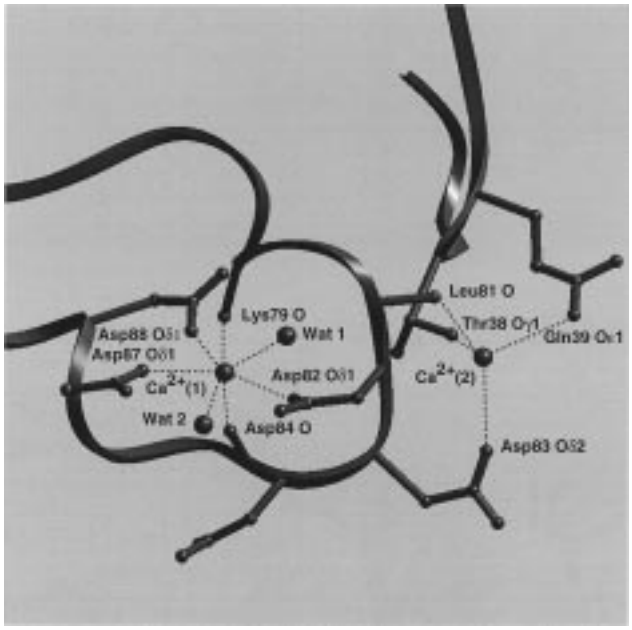

FIGURE 3: Diagram showing the details for the two calcium binding sites in human LA. The figure was drawn with the program SETOR (*43*).

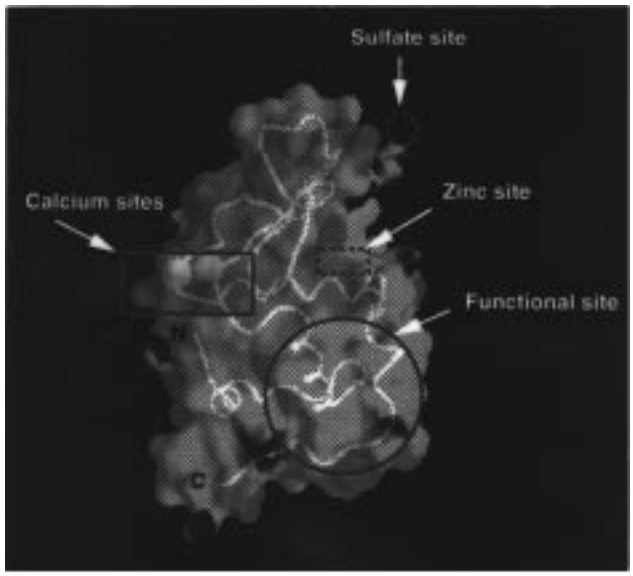

FIGURE 4: Electrostatic surface potential of human LA. Regions of negative potential are colored in red and positive potential in blue. The surface is transparent to reveal the underlying C^{α} backbone of the protein (shown in white). The various metal binding and functional sites established from crystallographic studies are shown. The figure was drawn with the program GRASP (*44*).

feature of a difference Fourier map at the 6σ level (the only "difference" in the difference map) which could not be adequately accounted for by a water molecule. Further refinement of the structure fully confirmed this feature, resulting in a significant drop in the R_{free} value by about 0.7% with the inclusion of a Ca^{2+} ion rather than a water molecule in the secondary site. Refinement of the site occupancy factor for the two Ca^{2+} ions gave values of 0.99 and 0.92 for the primary and secondary sites, respectively. The electron density maps (at 1.8 Å resolution) for the two sites are shown in Figure 2; the detailed atomic structures around the two ions are shown in Figure 3, and the coordination parameters are listed in Table 2.

The distance between the Ca^{2+} ions bound at the two sites is 7.9 Å. The secondary site is located on the surface of the LA molecule and has a coordination number smaller than the ideal value for a Ca^{2+} ion. Four protein groups are involved in coordinating the Ca^{2+} ion at this site in a tetrahedral arrangement: Oγ1 of Thr38, Oϵ1 of Gln39, Oδ2 of Asp83, and the carbonyl O of Leu81. No water molecules are present in the coordination sphere, although one internal water molecule is 3.8 Å from the secondary metal ion. However, we do not rule out the possibility that there may be disordered water molecules that are not observed in the electron density map that would add to the coordination sphere.

This finding demonstrates definitively that human LA has a second specific Ca^{2+} binding site that is occupied in the presence of higher concentrations of calcium. The binding of the second Ca^{2+} ion does not produce any significant

Table 2: Comparison of Calcium−Protein Ligand Distances (Å) in Diffferent LA Species[a]

residue	atom	mLA	GPLA	GOLA	BLA	BULA	HLA	this study
Lys79	O	2.15 (39.6)	2.24 (10.8)	2.30 (14.0)	2.2 (13.9)	2.27 (18.0)	2.34 (8.40)	2.28 (7.06)
Asp82	Oδ1	2.40 (35.4)	2.42 (12.1)	2.50 (34.4)	2.4 (11.7)	2.29 (23.7)	2.35 (11.0)	2.46 (4.12)
Asp84	O	2.24 (32.7)	2.16 (10.3)	2.28 (20.3)	2.3 (12.6)	2.37 (17.2)	2.24 (5.8)	2.20 (5.31)
Asp87	Oδ1	2.49 (30.8)	2.44 (10.1)	2.41 (27.9)	2.3 (7.9)	2.26 (14.3)	2.40 (10.4)	2.49 (7.57)
Asp88	Oδ1	2.59 (33.1)	2.33 (10.8)	2.47 (12.5)	2.3 (18.8)	2.55 (9.0)	2.42 (7.3)	2.52 (5.81)
water 1		—[b]	2.44 (8.6)	—[b]	2.4 (16.1)	—[b]	2.30 (7.9)	2.55 (4.30)
water 2		—[b]	2.33 (21.8)	—[b]	2.6 (21.0)	—[b]	2.50 (19.1)	2.50 (3.63)
Ca^{2+} (1)		(35.2)	(8.3)	(17.5)	(11.5)	(14.6)	(6.2)	(7.46)
Ca^{2+} (2)								(35.1)
Coordination around the Low-Affinity Ca^{2+} (2) Ion (This Study)								
Thr38					Oγ1		2.67 (12.0)	
Gln39					Oε1		3.06 (10.14)	
Leu81					O		2.75 (10.63)	
Asp83					Oδ1		2.84 (19.86)	
Ca^{2+} (1)−Ca^{2+} (2) distance = 7.86 Å (this study)								

[a] The temperature factors (Å^2) are given in parentheses. Abbreviations: mLA, recombinant bovine LA (*12*); GPLA, guinea pig LA (*12*); GOLA, goat LA (*12*); BLA, baboon LA (*10*); BULA, buffalo LA (*13*); HLA, human milk LA (*11*). [b] The water molecules were not well defined due to limited resolution.

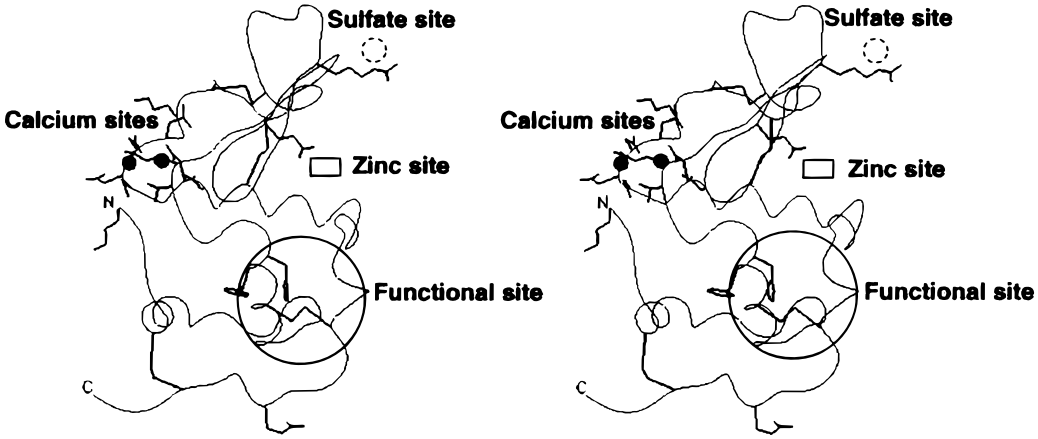

FIGURE 5: Stereodiagram of human LA showing the calcium, sulfate, and zinc binding and functional sites. The figure was prepared with the program SETOR (*43*).

structural change in the protein. Previous binding studies have indicated that bovine apo-LA has two Ca^{2+} binding sites (*6*, *33*). However, a second site was not detected in human LA by ^{43}Ca NMR, although two sites were found in bovine LA (*28*). The second site in bovine LA must be different from that described here since it probably includes His68. Binding to this site is also blocked at higher salt concentrations (*2*) and would be unlikely to be occupied at the concentrations of ammonium sulfate used in crystallization. The site described here appears to be a lower-affinity site that was not observed at the concentrations of calcium used in previous binding studies.

The second Ca^{2+} binding site is distinct from the Zn^{2+} binding site described by Ren et al. (*29*) and, on initial examination, appears to be at a location different from that of a Mn^{2+} binding site described by Gerken (*19*) on the basis of NMR studies with ^{13}C-reductively methylated bovine LA. The Zn^{2+} binding site is at a dimer interface in the crystal and involves residues 49 and 116 from different LA molecules, together with water molecules. A sulfate ion is also present in the Zn^{2+}−LA structure at an intermolecular surface, but this ion is also present in Zn^{2+}-free crystals. Ligands of the sulfate ion are Arg70 from one molecule, Gln39 and Lys94 from a symmetry-related molecule, and two water molecules (*29*). A water molecule which bridges the sulfate and the second protein molecule interacts with LA in a fashion similar to that of the second Ca^{2+} ion described here, forming H bonds with Gln39, Asp83, and Lys1. The NMR results of Gerken (*19*) indicated that the Mn^{2+} ion is 7.4 Å from the α-amino group (Glu1 of bovine LA). Since a crystal structure for LA was not available at that time, a model for the Mn^{2+} binding site was developed using a predicted structure for LA based on the structure of the homologous chicken lysozyme (*34*, *35*). In this model, the ligands for the Mn^{2+} ion are the side chain carboxyl groups of Glu1, Glu7, and Glu11 and the peptide carbonyl group of Asp84. The crystal structure of LA has a fold similar to lysozyme as envisaged by model building (*9*−*11*, *36*) but the energy-minimized model (*35*) used by Gerken is incorrect in detail (reviewed in ref *37*). It appears to be significant that the second Ca^{2+} ion in the present structure is 9.5 Å from the α-amino group, a distance remarkably similar to the Mn^{2+}−α-amino distance determined by Gerken of 7.4 Å. Considering differences in the liganding requirements of Mn^{2+} and Ca^{2+} as well as the fact that the two studies were conducted with different species variants under somewhat different conditions, these distances agree sufficiently to represent compelling evidence that the Mn^{2+} and second Ca^{2+} sites are closely similar. Although the exact nature of the Mn^{2+} site requires clarification with ongoing

Table 3: Variability of Amino Acid Residues at the Second Calcium Binding Site among Known LA Species

human LA residue	variability
Thr38	Ser (rat and platypus)
Gln39	Glu (camel) and Lys (rabbit)
Leu81	invariant
Asp83	invariant

structural studies, available data indicate that the second Ca^{2+} site represents a surface region of LA that can bind both inorganic cations and water molecules. The affinity for cations can be partly attributed to the electrostatic potential of this part of the LA surface (Figure 4). A comparison of known LA sequences indicates that there is a partial conservation of the metal liganding residues, and the types of substitutions observed appear to be compatible with the retention of a partial metal binding site in all LAs (Table 3).

Could this site have any functional relevance? The interaction of LA with GT is synergistic with monosaccharides, an arrangement that is manifested functionally in a 1000-fold reduction of the K_m for glucose in the presence of LA (*38*, *39*). GT is also a metal-binding protein, and two metal binding sites are implicated in its activity. Catalysis by GT is absolutely dependent on the binding of Mn^{2+} to a high-affinity (micromolar K_d) binding site, while the binding of a second metal ion affects the interaction of GT with UDPgalactose (*40*). Zn^{2+} and some other ions can replace Mn^{2+} at the primary site, although with loss of catalytic efficiency, but Ca^{2+} does not bind to this site. Calcium, organic cations such as spermidine, and even cationic proteins can replace Mn^{2+} at the second site, and it is possible that an endogenous cationic protein may act at this site in vivo (*41*). The interaction of LA with GT is also affected by metal ions and substrates, and the congruence in metal binding properties of the two proteins has led to speculation that a metal ion could bridge them in the lactose synthase complex (*2*).

The functional site in LA identified by mutational and chemical studies is shown in Figure 5. This encompasses part of the lower cleft that corresponds to monosaccharide-binding subsite F of lysozyme and an adjacent surface that has no functional equivalent in lysozyme (*38*, *39*). Structural studies show that these regions have alternative conformational states in different crystal forms of human LA and in the structures of other species variants (*10*−*14*). Their high regional flexibility is also indicated by relatively high crystallographic temperature factors. Mutagenesis of functional site residues indicates that Gln117 and Trp118 are specifically involved in the protein−protein interaction with GT while Phe31, His32, and Leu110 (components of the region corresponding to lysozyme subsite F) influence the affinity of the LA−GT heterodimer for glucose. The action of LA as a competitive inhibitor of the binding of monosaccharide derivatives with extensions at C1 (*β*) or C2 indicates that the functional site in LA is close to the acceptor substrate binding site of GT in this complex and appears to be likely to form part of a hybrid glucose binding site at the LA−GT molecular interface (*38*).

As shown in Figure 5, the second Ca^{2+} binding site described here is located in the same surface of LA as the mutationally defined functional site. It is close to (17 Å from His32) but not contiguous with this region and also appears to be unlikely to form a functionally important independent metal-binding site. However, components of the site could participate in a metal-binding site at the LA−GT interface. In this regard, a likely role for the catalytically important Mn^{2+} ion in GT is the activation of the 4-OH group of the acceptor substrate for nucleophilic attack on C1 of the galactose moiety of UDPgalactose. This mode of action would require that the Mn^{2+} ion be adjacent to the acceptor binding site where it could interact with residues of LA. The urgent need for structural studies of lactose synthase is emphasized by the indirect nature of evidence on which this and other hypotheses about LA action are based.

ACKNOWLEDGMENT

We thank Richard Ellis and Evangelia Chrysina for their contribution to the LA project.

REFERENCES

1. Hill, R. L., and Brew, K. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 411−489.
2. Kronman, M. J. (1989) *CRC Crit. Rev. Biochem. Mol. Biol. 24*, 565−667.
3. McKenzie, H. A., and White, F. H., Jr. (1991) *Adv. Protein Chem. 41*, 173−315.
4. Brew, K., and Grobler, J. A. (1992) in *Advances in Dairy Chemistry* (Fox, P., Ed.) Vol. 1, pp 191−229, Elsevier Applied Science, London and New York.
5. Hiroaka, Y., Segawa, Y., Kuwajima, K., Sugai, S., and Murai, N. (1980) *Biochem. Biophys. Res. Commun. 93*, 1098−1104.
6. Kronman, M. J., Sinha, S. K., and Brew, K. (1981) *J. Biol. Chem. 256*, 8582−8587.
7. Permyakov, E. A., Yarmolenko, V. V., Kalinichenko, L. P., Morozova, L. A., and Burstein, E. A. (1981) *Biochem. Biophys. Res. Commun. 100*, 191−197.
8. Schaer, J.-J., Milos, M., and Cox, J. A. (1985) *FEBS Lett. 190*, 77−80.
9. Stuart, D. I., Acharya, K. R., Walker, N. P. C., Smith, S. G., Lewis, M., and Phillips, D. C. (1986) *Nature 324*, 84−87.
10. Acharya, K. R., Stuart, D. I., Walker, N. P. C., Lewis, M., and Phillips, D. C. (1989) *J. Mol. Biol. 208*, 99−127.
11. Acharya, K. R., Ren, J., Stuart, D. I., Phillips, D. C., and Fenna, R. E. (1991) *J. Mol. Biol. 221*, 571−581.
12. Pike, A. C. W., Brew, K., and Acharya, K. R. (1996) *Structure 4*, 691−703.
13. Calderone, V., Giuffrida, M. G., Viterbo, D., Napolitano, L., Fortunato, D., Conti, A., and Acharya, K. R. (1996) *FEBS Lett. 394*, 91−95.
14. Harata, K., and Muraki, M. (1992) *J. Biol. Chem. 267*, 1419−1421.
15. Grobler, J. A., Rao, K. R., Pervaiz, S., and Brew, K. (1995) *Arch. Biochem. Biophys. 313*, 360−366.
16. Rao, K. R., and Brew, K. (1989) *Biochem. Biophys. Res. Commun. 163*, 1390−1396.
17. Ewbank, J. J., and Creighton, T. E. (1993) *Biochemistry 32*, 3677−3693.
18. Permyakov, E. A., Kalinichenko, L. P., Morozova, L. A., Yarmolenko, V. V., and Burstein, E. A. (1981) *Biochem. Biophys. Res. Commun. 102*, 1−7.
19. Gerkin, T. A. (1984) *Biochemistry 23*, 4688−4697.
20. Musci, G., and Berliner, L. J. (1985) *Biochemistry 24*, 3852−3856.
21. Musci, G., and Berliner, L. J. (1985) *Biochemistry 24*, 6945−6948.
22. Musci, G., and Berliner, L. J. (1986) *Biochemistry 25*, 4887−4889.
23. Berliner, L. J., Koga, K., Nishikawa, H., and Scheffler, J. E. (1987) *Biochemistry 26*, 5769−5774.
24. Permyakov, E. A., Morozova, L. A., Kalinichenko, L. P., and Derezhkov, V. Y. (1988) *Biophys. Chem. 32*, 37−42.
25. Permyakov, E. A., Shnyrov, V. L., Kalinichenko, L. P., Kuchar, A., Reyzer, I. L., and Berliner, L. J. (1991) *J. Protein Chem. 10*, 577−584.

26. Permyakov, E. A., and Berliner, L. J. (1994) *J. Protein Chem. 13*, 277−281.
27. Veprintsev, D. B., Permyakov, E. A., Kalinichenko, L. P., and Berliner, L. J. (1996) *Biochem. Mol. Biol. Int. 39*, 1255−1265.
28. Aramini, J. M., Drakenberg, T., Hiraoki, T., Ke, Y., Nitta, K., and Vogel, H. J. (1992) *Biochemistry 31*, 6761−6768.
29. Ren, J., Stuart, D. I., and Acharya, K. R. (1993) *J. Biol. Chem. 268*, 19292−19298.
30. Howard, A., Gilliland, G., Finzel, B., Poulos, T., Ohlendorf, D. H., and Salemme, F. R. (1987) *J. Appl. Crystallogr. 20*, 383−387.
31. Brünger, A. T. (1992) *X-PLOR manual. Version 3.1. A System for Crystallography and NMR*, Yale University Press, New Haven, CT.
32. Brünger, A. T. (1992) *Nature 355*, 472−474.
33. Murakami, K., Andree, P. J., and Berliner, L. J. (1982) *Biochemistry 21*, 5488−5494.
34. Browne, W. J., North, A. C. T., Phillips, D. C., Brew, K., Vanaman, T. C., and Hill, R. L. (1969) *J. Mol. Biol. 42*, 65−86.
35. Warme, P. K., Momany, F. A., Rumball, S. V., Tuttle, R. W., and Scheraga, H. A. (1974) *Biochemistry 13*, 768−782.
36. Smith, S. G., Lewis, M., Aschaffenburg, R., Fenna, R. E., Wilson, I. A., Sundaralingam, M., Stuart, D. I., and Phillips, D. C. (1987) *Biochem. J. 242*, 353−360.
37. Acharya, K. R., Stuart, D. I., Phillips, D. C., and Scheraga, H. A. (1990) *J. Protein Chem. 9*, 549−563.
38. Grobler, J. A., Wang, M., Pike, A. C. W., and Brew, K. (1994) *J. Biol. Chem. 269*, 5106−5114.
39. Malinovskii, V. A., Tian, J., Grobler, J. A., and Brew, K. (1996) *Biochemistry 35*, 9710−9715.
40. Powell, J. T., and Brew, K. (1976) *J. Biol. Chem. 251*, 3653−3663.
41. Navaratnam, N., Virk, S. S., Ward, S., and Kuhn, N. J. (1986) *Biochem. J. 239*, 423−433.
42. Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.
43. Evans, S. V. (1993) *J. Mol. Graphics 11*, 134−138.
44. Nicholls, A., Bharadwaj, R., and Honig, B. (1991) *Biophys. J. 239*, 423−433.

BI973000T