Protein Intermediate Trapped by the Simultaneous Crystallization Process

CRYSTAL STRUCTURE OF AN IRON-SATURATED INTERMEDIATE IN THE Fe$^{3+}$ BINDING PATHWAY OF CAMEL LACTOFERRIN AT 2.7 Å RESOLUTION

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This is the first protein intermediate obtained in the crystalline state by the simultaneous process of Fe$^{3+}$ binding and crystal nucleation and is also the first structure of an intermediate of lactoferrin in the Fe$^{3+}$ binding pathway. Lactoferrin is an iron-binding 80-kDa glycoprotein. It binds Fe$^{3+}$ very tightly in a closed interdomain cleft in both lobes. The iron-free structure of lactoferrin, on the other hand, adopts an open conformation with domains moving widely apart. These studies imply that initial Fe$^{3+}$ binding must be in the open form. The protein intermediate was crystallized by the microdialysis method. The protein solution, with a concentration of 100 mg/ml in 10 mM Tris-HCl, pH 8.0, was loaded in a capillary and dialyzed against the same buffer containing 26% (v/v) ethanol placed in a reservoir. FeCl$_3$ and CO$_3^{2-}$ in excess molar ratios to that of protein in its solution were added to the reservoir buffer. The crystals appeared after some hours and grew to the optimum size within 36 h. The structure was determined by molecular replacement method and refined to final R- and R-free factors of 0.187 and 0.255, respectively. The present structure showed that the protein molecule adopts an open conformation similar to that of camel apolactoferrin. The electron density map clearly indicated the presence of two iron atoms, one in each lobe with 4-fold coordinations: two by the protein ligands of Tyr-92(433) OH and Tyr-192(526) OH and two other coordination sites occupied by oxygen atoms of bidentate CO$_3^{2-}$ ions leading to a tetrahedral intermediate. The CO$_3^{2-}$ anion is stabilized through hydrogen bonds with the synergetic anion-binding site Arg-121(463) and with Ser-122 O$_y$ in the N-lobe and Thr-464 O$_y$ in C-lobe. The third oxygen atom of CO$_3^{2-}$ interacts with a water molecule in both lobes.

Lactoferrin is an iron-binding 80-kDa glycoprotein. It consists of two homologous N- and C-terminal lobes, which are made up of two domains, N-1 (1–90 and 252–320), N-2 (91–251) and C-1 (345–433 and 596–663), C-2 (434–595), respectively. So far, amino acid sequences from eight species, i.e. human (1), bovine (2), goat (3), equine (4), buffalo (5), porcine (6), murine (7), and camel (8, 9), have been determined. The sequence identity among these lactoferrins varies from 60 to 90%. The sequence comparison also reveals that the C-lobes of lactoferrins are highly conserved, whereas the N-lobes show larger variations (10). Despite sequence similarities, lactoferrins differ in important functional details including their relative affinities for iron, propensity for iron release, and receptor binding properties (9, 11). The crystal structures of fully iron-saturated dfferic forms of lactoferrins (4, 5, 11–13) have revealed that the two domains of each lobe are closed over an Fe$^{3+}$ ion. Four of the six Fe$^{3+}$ coordination sites are occupied by protein ligands (2 Tyr, 1 Asp, and 1 His residue) and the other two by a bidentate carbonate ion. The structure of the iron-saturated form of camel lactoferrin is not yet known. However, the structure of the iron-free form of camel lactoferrin has been determined recently (9). The structure of camel apolactoferrin (UALF) shows that both of its lobes adopt open conformations. The N-2 and C-2 domains move away from the N-1 and C-1 domains, respectively, almost as rigid bodies. Thus, the transition from the open to closed conformations is expected to pass through an intermediate in which the Fe$^{3+}$ ion might bind initially to the protein in the open form before being transformed into the closed holoform. The four protein ligands have different locations, and the iron-loaded open form is a possible transient intermediate toward the fully saturated holoform with closed domains. The postulated reaction scheme of iron binding is presented in Fig. 1. The study of intermediate III is necessary to reveal the pathway of Fe$^{3+}$ uptake/release in lactoferrins and for the understanding of protein folding. The main difficulty encountered in the structure determination of such an intermediate is in obtaining the stable protein. There is no way to trap it in solution and stabilize it over a period for the purpose of crystallization. On the other hand, the binding of Fe$^{3+}$ with the protein in the crystalline state is bound to hurt the crystals if it introduces even moderate structural changes. Indeed, the soaking experiments with apolactoferrin crystals using natural cation Fe$^{3+}$ and anion CO$_3^{2-}$ did not succeed, as the crystals dissolved within a few minutes. The structure of the proteolytically generated N-2 domain of an iron-saturated duck ovotransferrin has indicated the association of Fe$^{3+}$ ion with N-2 domain (14). The structure of an iron-saturated open form of the N-lobe of hen ovotransferrin with an unnatural anion nitritetriacetate has been reported recently (15).

* This work was supported by a grant from the Department of Biotechnology, Government of India, New Delhi. The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement is made up of two domains, N-1 (1–90 and 252–320), N-2 (91–251) and C-1 (345–433 and 596–663), C-2 (434–595), respectively. So far, amino acid sequences from eight species, i.e. human (1), bovine (2), goat (3), equine (4), buffalo (5), porcine (6), murine (7), and camel (8, 9), have been determined. The sequence identity among these lactoferrins varies from 60 to 90%. The sequence comparison also reveals that the C-lobes of lactoferrins are highly conserved, whereas the N-lobes show larger variations (10). Despite sequence similarities, lactoferrins differ in important functional details including their relative affinities for iron, propensity for iron release, and receptor binding properties (9, 11). The crystal structures of fully iron-saturated dfferic forms of lactoferrins (4, 5, 11–13) have revealed that the two domains of each lobe are closed over an Fe$^{3+}$ ion. Four of the six Fe$^{3+}$ coordination sites are occupied by protein ligands (2 Tyr, 1 Asp, and 1 His residue) and the other two by a bidentate carbonate ion. The structure of the iron-saturated form of camel lactoferrin is not yet known. However, the structure of the iron-free form of camel lactoferrin has been determined recently (9). The structure of camel apolactoferrin (UALF) shows that both of its lobes adopt open conformations. The N-2 and C-2 domains move away from the N-1 and C-1 domains, respectively, almost as rigid bodies. Thus, the transition from the open to closed conformations is expected to pass through an intermediate in which the Fe$^{3+}$ ion might bind initially to the protein in the open form before being transformed into the closed holoform. The four protein ligands have different locations, and the iron-loaded open form is a possible transient intermediate toward the fully saturated holoform with closed domains. The postulated reaction scheme of iron binding is presented in Fig. 1. The study of intermediate III is necessary to reveal the pathway of Fe$^{3+}$ uptake/release in lactoferrins and for the understanding of protein folding. The main difficulty encountered in the structure determination of such an intermediate is in obtaining the stable protein. There is no way to trap it in solution and stabilize it over a period for the purpose of crystallization. On the other hand, the binding of Fe$^{3+}$ with the protein in the crystalline state is bound to hurt the crystals if it introduces even moderate structural changes. Indeed, the soaking experiments with apolactoferrin crystals using natural cation Fe$^{3+}$ and anion CO$_3^{2-}$ did not succeed, as the crystals dissolved within a few minutes. The structure of the proteolytically generated N-2 domain of an iron-saturated duck ovotransferrin has indicated the association of Fe$^{3+}$ ion with N-2 domain (14). The structure of an iron-saturated open form of the N-lobe of hen ovotransferrin with an unnatural anion nitritetriacetate has been reported recently (15).

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The abbreviations used are: UALF, camel apolactoferrin; ULFI, camel lactoferrin intermediate; HLF, human lactoferrin; BLF, buffalo lactoferrin.
Structure of an Intermediate of Camel Lactoferrin

In the present study, a complete molecule of native apolactoferrin and natural cation Fe\(^{3+}\) and anion CO\(_3^{2-}\) have been used. Furthermore, here we have employed an entirely new strategy to trap an intermediate in the crystalline state. The introduction of Fe\(^{3+}\) and CO\(_3^{2-}\) into the protein and the process of crystal nucleation were initiated simultaneously so that the iron bound intermediate in the open conformation was trapped by crystalline forces. We report here the crystal structure of such an intermediate in the binding pathway of Fe\(^{3+}\). The observations clearly suggest that the two tyrosine residues are the initial binding ligands in the open conformation and that some of the hydrogen-bonding interactions of anion with protein are different than those observed in diferric lactoferrins.

EXPERIMENTAL PROCEDURES

Purification—A locally modified procedure (16) was used for the purification of camel lactoferrin. Fresh colostrum was obtained from the National Research Center on Camels, Bikaner, India. Twice diluted colostrum in water was defatted by skimming. Skimmed milk was further diluted twice with 50 mM Tris-HCl, pH 8.0. Cation exchanger CM-Sephadex C-50 was added to it (7 g/l) and stirred slowly for 1 h using a mechanical stirrer. The gel was allowed to settle, and the milk was decanted. The gel was washed with an excess of 50 mM Tris-HCl, pH 8.0. It was packed in a column (25 \(\times\) 2.5 cm) and washed with the same buffer containing 0.1 M NaCl, which facilitated the removal of some of the protein impurities. Finally, the lactoferrin was eluted with same buffer containing 0.3 M NaCl. The protein solution was dialyzed against triple distilled water. The protein was again passed through a CM-Sephadex C-50 column (10 \(\times\) 2.5 cm) pre-equilibrated with 50 mM Tris-HCl, pH 8.0, and eluted with a linear gradient of 0.05–0.3 M NaCl in the same buffer. The protein was concentrated using an Amicon ultrafiltration cell. The concentrated protein was passed through a Sephadex G-150 column (100 \(\times\) 2 cm) using 50 mM Tris-HCl buffer, pH 8.0. The purified protein was saturated with iron to obtain a homogenous iron saturated form. The protein could be saturated by iron to the order of 98%.

Preparation of Apolactoferrin—The iron was removed from the lactoferrin using the procedure of Masson et al. (17). The purified iron-saturated lactoferrin solution (1%) in 50 mM Tris-HCl buffer at pH 8.0 was dialyzed against an excess of 0.1 M citric acid with regular changes every 6 h. The dialysis was carried out at different pH values from pH 8.0 to 2.0 for 24 h. Finally, citric acid was removed by dialysis against an excess of distilled water with regular changes for 24 h at 4°C. The colorless apoprotein was obtained. These samples were lyophilized.

Cryostabilization of Protein Intermediate—The apo form of protein was cryostabilized using microdialysis (9). For soaking Fe\(^{3+}\) into the crystals of apolactoferrin, the crystals were first transferred to the precipitant solution at pH 8.0. The original precipitant solution was replaced gradually by fresh precipitant solutions containing excess amounts FeCl\(_3\) and CO\(_3^{2-}\) at 4°C. As soon as the crystals were introduced to the precipitant solution containing FeCl\(_3\) and CO\(_3^{2-}\), they cracked badly. Instead of the original precipitant solutions, other stabilizing solutions containing 2-methylpentane-2,4-diol and polyethylene glycol were used also, but the crystals cracked and dissolved rapidly. Therefore the crystals of the intermediate were grown by a modified technique of microdialysis at 4°C. 100 mg of purified and lyophilized camel apolactoferrin was dissolved in 1 ml of 10 mM Tris-HCl at pH 8.0. 20 \(\mu\)l of protein solution was placed in the glass capillaries separated by dialysis membrane from the plastic tubing. These were placed in the vials containing the same buffer with 26% (v/v) ethanol. To this reservoir solution the excess amounts of FeCl\(_3\) and CO\(_3^{2-}\) were added. These vials were put aside for microdialysis at 4°C. These setups allowed the simultaneous diffusion of Fe\(^{3+}\) and CO\(_3^{2-}\) ions as well as the precipitating agent, ethanol, to the protein solution. The crystal nucleations were observed almost instantaneously, and the crystals with pale yellow color grew to the final dimensions of 0.4 \(\times\) 0.3 \(\times\) 0.3 mm\(^3\) within 36 h. It may be noted that the crystals of apolactoferrin were colorless whereas those of iron-saturated lactoferrin, as observed in other cases, had a dark brown color. Therefore, the pale yellow color of the crystals indicated a different state of iron association with the protein. The iron content of the crystals was estimated from the ratio of \(A_{465}/A_{280}\) (18). The iron saturation was found to be of the order of 80%. Unlike other lactoferrins studied so far, it is an important feature of camel lactoferrin that its iron-free form was crystallized successfully, whereas its iron-saturated form could not be crystallized despite repeated efforts for the past several years (9). This fact indicates that the structure of apolactoferrin with both lobes in open conformations is more homogenous and less mobile. Therefore, a postulated iron-saturated intermediate in the apo form (Fig. 1, III) is also expected to be a stable structure, about which its successful crystallization provides appropriate evidence.

\[\text{Apo-Lf} + 2\text{CO}_3^{2-} \rightarrow \text{Lf-2CO}_3^{2-} \]
\[(\text{Open}) \quad (\text{Open})\]

I

II

\[\text{Lf-2CO}_3^{2-} + 2\text{FeCl}_3 \rightarrow \text{Lf-2Fe}^{3+}\text{-2CO}_3^{2-} \]
\[(\text{Open}) \quad (\text{Open tetrahedral})\]

III

\[\text{Lf-2Fe}^{3+}\text{-2CO}_3^{2-} \rightarrow \text{Lf-2Fe}^{3+}\text{-2CO}_3^{2-} \]
\[(\text{Open tetrahedral}) \quad (\text{Closed octahedral})\]

IV

FIG. 1. The postulated reaction scheme of iron binding in camel lactoferrin.

| Crystallization method | Microdialysis |
|-----------------------|--------------|
| Crystallization conditions | Camel apolactoferrin 100 mg/ml in 10 mM Tris-HCl, pH 8.0 |
| | Reservoir: 10 mM Tris-HCl, pH 8.0 |
| | 26% (v/v) ethanol, FeCl\(_3\), and CO\(_3^{2-}\) (in excess) at 4°C |
| | Crystals: irregular in shape and pale yellow in color |
| | C\(_2\) |
| | 175.9 |
| | 80.6 |
| | 56.3 |
| | 92.4 |
| | 2.66 |
| | 25.0–2.7 |
| a (Å) | 44,276 |
| b (Å) | 19,901 |
| c (Å) | 9.6 |
| Matthews coefficient (V_n) (Å\(^3\)/Da) | 5.6 |
| Resolution range (Å) | 92 |
| Total number of measured reflections, 0 | 47.0 |
| Number of unique observed reflections | 10.5 |
| \(I_0\alpha(I)\) for all data | 25.0 |
| \(I_0\alpha(I)\) for the outer shell (2.8–2.7) (Å) | }
| Overall completeness (%) | }
| Completeness (%) in the highest resolution shell (2.8–2.7) (Å) | }
| R_{merge} for all data (%) | }
| R_{merge} in the highest resolution shell (2.8–2.7) (Å) | }

TABLE I

Crystallgraphic data.
The intensity data were collected at 4 °C using a MAR Research imaging plate scanner mounted on an RU-200 rotating anode x-ray generator equipped with a graphite monochromator. The data were integrated using DENZO (19, 41) and scaled using MARSCALE (20). The crystals belong to monoclinic space group C2 with cell parameters \(a = 175.9\) Å, \(b = 80.6\) Å, \(c = 56.3\) Å, and \(\beta = 92.4^\circ\) with 4 molecules in the unit cell. The solvent content was estimated to be 54%. The data have an \(R\)sym of 10.5% and an overall completeness of 92% to 2.7 Å resolution (Table I).

The structure determination was carried out by molecular replacement method using the program AMoRe (21). Because iron was present in the crystals, the initial attempts were made to determine the structure with models of diferric lactoferrin, but it did not yield a solution. Finally, the structure was determined with a model of camel apolactoferrin. The solution appeared to be very distinct with a final correlation coefficient of 68.5%.

**Model Building and Refinement**—The model was built using the program O (22) and refined with X-PLOR (23) using all the data between 20.0 and 2.7 Å resolution. A test set of about 5% of the data was kept aside to monitor the \(R\)-free factor during the course of refinement (24). The positions of two Fe\(^{3+}\) ions and two carbonate anions, one in each lobe, were clearly identified in the difference density \((F_0 - F_c)\) map of the first refinement round. These were included in the model followed by more than 10 rounds of refinement and manual model building. The quality of phases was further improved by solvent flattening using the program DM (25, 26), allowing more residues to be built based on the improved electron density map. The omit maps \((2F_o - F_c)\), contoured at 1 \(\sigma\) and \(F_0 - F_c\) contoured at 4 \(\sigma\) were calculated using all of the reflection data after refinement of the model in which the Fe\(^{3+}\) was excluded (Fig. 4, A and B). The next set of omit maps was obtained in which CO\(^3-\) was excluded (Fig. 4, C and D). Water molecules were added where the difference density \((F_o - F_c)\) had values of more than 3 \(\sigma\) above the mean level, and the \(2F_o - F_c\) map showed density at more than 1 \(\sigma\) level. This resulted in the determination of 232 water molecules. The final \(R\)-factor and \(R\)-free factor were 0.187 and 0.255, respectively. The refinement statistics are summarized in Table II.

**RESULTS AND DISCUSSION**

**Quality of the Model**—The final model consists of 5284 protein atoms from 689 amino acid residues, 2 Fe\(^{3+}\) ions, 2 CO\(^3-\) ions, and 232 water molecules. The protein structure has geometry close to the ideal values with root mean square deviations of 0.013 Å and 1.8° from the standard values of bond lengths and angles, respectively. The overall mean B-factor for
the structure is 57.0 Å². It may be mentioned here that the values of overall mean B-factors for the structures of various iron-saturated forms of lactoferrins have been observed between 39 and 72 Å² (10). These values have been found even larger (41–93 Å²) in the case of iron-free forms of lactoferrins (27). Therefore, the mean B-value for the present structure lies within the limits of those observed for the structures of other lactoferrins and represents a typical value for lactoferrins. The structure is well defined, and in the final 2Fo − Fc electron density map, there are no breaks in the main chain and side chain densities when contoured at the 1σ level. A Ramachandran plot of the main chain torsion angles (ϕ, ψ) (28) shows that 84.4% of the residues are in the most allowed regions as defined in the program PROCHECK (29). Only two residues, Leu-299 and Leu-640, are in the normally disallowed regions, which are the central residues in two γ-turns (30), and have (ϕ, ψ) values that are typical of such conformations (70°, -60°). The two γ-turns are conserved in the N- and C-lobes of lactoferrins and transferrins (31).

Overall Molecular Structure—For the iron-free apo form, the solution-scattering (32–35) analyses have revealed that all of the transferrins and lactoferrins studied so far assume a conformation with a widely open interdomain cleft. The polypeptide chain of the present iron-saturated camel lactoferrin intermediate (ULFI) folds into two lobes (N- and C-lobes, representing the N-terminal and C-terminal halves of the molecule), each of which is folded into two domains (N-1 and N-2; C-1 and C-2) (Fig. 2). This structure, when compared with the holo (Fe³⁺ and CO₃²⁻ loaded form) structures of human (HLF) (12), equine (ELF) (4), and buffalo (BLF) (5, 11) lactoferrins, comprises an open domain conformation. The extent and mode of opening were almost the same as in UALF. The root mean
square shifts for all the Cα atoms of ULFI with those of UALF was found to be 0.54 Å (Fig. 3). In this structure, the iron atoms exist in the opened up interdomain clefts. The two Fe³⁺ ligating tyrosine residues in the holoform Tyr-92(433) and Tyr-192(526) appear to participate in the iron coordination, whereas the other two protein ligands of Asp-60(395) and His-253(595) are located far away from the iron atoms. The bidentate carbonate ions provide two oxygen atoms in each lobe to complete the tetrahedral coordinations of Fe³⁺ ions. Overall, the present structure of lactoferrin intermediate is almost indistinguishable from that of camel apolactoferrin. The molecular packing in the present crystal structure is also identical to that of apolactoferrin. The crystal nucleation of the open conformation seems to be an efficient process, and as soon as the iron binding occurs, the molecules of the iron-saturated intermediate tend to pack instantaneously. Once they are packed into a crystalline arrangement, the conversion to the next step of closing of domains is prevented by molecular packing forces.

The Structure of the Fe³⁺ Binding Sites—The iron coordination and hydrogen bonding structure of CO₃²⁻ in the ULFI are
illustrated in (Fig. 4, A–D). As summarized in Table III, the distances from the iron of Tyr-92 OH and Tyr-192 OH in the N-lobe and of Tyr-433 OH and Tyr-526 OH in the C-lobe of 2.7 and 2.7 Å and 2.8 and 2.4 Å, respectively, clearly show the involvement of tyrosine residues in the coordinations with FeIII. The other two protein ligands of Asp-60(395) and His-253(595) are not involved in the coordination with the iron atom; the distances of Asp Oδ1 and His Nε2 from iron in the N- and C-lobe are 10.5 and 8.6 Å and 10.1 and 8.2 Å, respectively. The other two coordinations of the tetrahedrally coordinated iron atom are provided by CO3– anion. The distances of CO3– O–1 and CO3– O–2 from iron in the N- and C-lobe are 2.8 and 2.8 Å and 2.6 and 2.6 Å, respectively. The coordination distances in the present structure are slightly larger than those generally observed in the structures of lactoferrins (4–5, 11–13) suggesting the formation of slightly loose coordinations.

The carbonate ion is stabilized by hydrogen-bonding interactions with Arg-121(463) involving Ne and NH2 at distances of 3.2 and 3.0 Å in the N-lobe and 3.0 and 2.7 Å in the C-lobe. The other anion interactions that are different in ULFI involve a hydrogen bond with Ser-122 Oγ in the N-lobe and Thr-464 Oγ in the C-lobe. Additionally, a water molecule is present in each lobe to form a hydrogen bond with CO3–. In the present structure, the anion lacks two hydrogen bonds with backbone nitrogen atoms of residues 123(465) and 124(466), which are present in the iron-saturated forms of other lactoferrins. Residues 121–124(463–466) are part of the positively charged anion binding pocket. Although it has gained a hydrogen bond through a water molecule in each lobe, but overall the anions remain slightly loosely bound in the structure of intermediate.

**DISCUSSION**

The current crystal structure corresponds to intermediate III, LF-2 FeIII⋅2 CO3– complex (Fig. 1), with an open conformation in which only four of the six FeIII coordination sites are occupied by the side chains of protein ligands Tyr-92(433) and Tyr-192(526) and bidentate CO3–. This is the first lactoferrin structure that demonstrates that the two tyrosine residues are the protein ligands for the FeIII entry in the intact lactoferrin with the open domain conformation. In this arrangement the other two ligands, Asp and His, seem to move toward the iron atom after two tyrosines have joined the iron. The positive macro-dipole at the N terminus of helix 5 (11) and Arg-121 are oriented toward the metal binding site, making an unfavorable environment to receive a ferric cation. This configuration readily explains the requirement for the synergistic carbonate anion, which effectively neutralizes the positive charges and confers site specificity for binding ferric ions. In the intact protein the cation charge is then balanced by the two tyrosine residues, Tyr-92(433) and Tyr-192(526). Therefore, the critical steps in the mechanism of iron binding involve an early entry of CO3– ions to interact with Arg-121(463) in the positively charged anion binding site of domain 2. The binding of the carbonate ion induces a conformational change in the side chain of Arg-121(463); this is the first conformational change in the protein in the mechanism of iron binding. The observed conformational change in Arg-121(463) corresponds to intermediate II (Fig. 1) but is extremely short-lived, as the iron binding followed by almost instantaneously. These events occur in the proximity of two tyrosine residues, 92(433) and 192(526), thus preparing the cleft for iron binding. As a result, the iron binds to the combination of two tyrosines and a carbonate ion first to form a tetrahedral intermediate. This is seemingly a relatively stable intermediate (III) (Fig. 1). It is noteworthy that upon binding to FeIII, Tyr-92(433) and Tyr-192(526) shift in the present structure from their original positions in camel apo-lactoferrin. The largest displacement between any two corresponding atoms in the N-lobe was 0.7 Å, whereas it was 1.9 Å in the C-lobe (Fig. 5). Yet another striking difference between the structures of the present intermediate and those of the iron-saturated forms of lactoferrin pertains to the positioning of the FeIII and CO3– ions. Although the positioning of FeIII is only slightly different in ULFI, the CO3– occupies a drastically displaced position. Because the lobes are in open conformation, the FeIII forms only four coordination links. These four bonds although weaker are geometrically identical to those observed in the structures of iron-saturated lactoferrins (4–5, 11–13). On the other hand, the hydrogen-bonding interactions of CO3– anion with the protein are substantially different. Although the two key interactions with Arg-121(463) are similar, the other links are different. As seen in Fig. 6, A–D, in HLF and BLF, the remaining three hydrogen bonds involve Thr-117(459) Oγ and backbone nitrogen atoms of Ala-123(465) and Gly-124(466). In contrast, in the present structure, there are a total of only four hydrogen bonds. Two of them are with Arg-121(463), and of the remaining two, one is with Ser-122 Oγ in the N-lobe and Thr-464 Oγ in the C-lobe as well as one each with the solvent water molecule. Overall, the hydrogen binding pattern of CO3– in the intermediate is different from derrifer lactoferrins. The CO3– anion is also less tightly held in the intermediate, leaving scope for an adjustment on final folding. The domain closure occurs with the introduction of aspartic acid, Asp-60(395), from domain 1, which is attracted toward cation. The environment of ferric cation is completed by the neutral His-253(595) residue sited on the second interdomain-connecting strand as the polypeptide chain traverses back from domain 2 to domain 1. This mechanism is consistent with experiments on iron-binding kinetics that show a conformational change to be the limiting step in iron binding (36, 37).

Of the four protein ligands at the iron-binding site in lactoferrins, the two tyrosine residues, Tyr-92(433) and Tyr-192(526), play a fundamental role in the initial stages of iron-binding, whereas Asp-60(395) appears to facilitate domain closure as well as provide a negatively charged ligand to interact with iron. The role of His-253(595) in the mechanism of iron binding is less well defined, although its presence as a neutral ligand completing the octahedral environment of the iron is chemically relevant. Further studies of lactoferrin intermediates from other species may provide additional information on the mechanism of iron uptake and release.

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Protein Intermediate Trapped by the Simultaneous Crystallization Process: CRYSTAL STRUCTURE OF AN IRON-SATURATED INTERMEDIATE IN THE Fe3+ BINDING PATHWAY OF CAMEL LACTOFERRIN AT 2.7 Å RESOLUTION

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