Alternative Structural State of Transferrin

THE CRYSTALLOGRAPHIC ANALYSIS OF IRON-LOADED BUT DOMAIN-OPENED OVOTRANSFERRIN N-LOBE

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Transferrins bind Fe$^{3+}$ very tightly in a closed interdomain cleft by the coordination of four protein ligands (Asp$^{60}$, Tyr$^{92}$, Tyr$^{191}$, and His$^{250}$ in ovotransferrin N-lobe) and of a synergistic anion, physiologically bidentate CO$_3^{2-}$. Upon Fe$^{3+}$ uptake, transferrins undergo a large scale conformational transition: the apo structure with an opening of the interdomain cleft is transformed into the closed holo structure, implying initial Fe$^{3+}$ binding in the open form. To solve the Fe$^{3+}$-loaded, domain-opened structure, an ovotransferrin N-lobe crystal that had been grown as the apo form was soaked with Fe$^{3+}$-nitritotriacetate, and its structure was solved at 2.1 Å resolution. The Fe$^{3+}$-soaked form showed almost exactly the same overall open structure as the iron-free apo form. The electron density map unequivocally proved the presence of an iron atom with the coordination by the two protein ligands of Tyr$^{92}$-OH and Tyr$^{191}$-OH. Other Fe$^{3+}$ coordination sites are occupied by a nitritotriacetate anion, which is stabilized through the hydrogen bonds with the peptide NH groups of Ser$^{122}$, Ala$^{123}$, and Gly$^{124}$ and a side chain group of Thr$^{117}$. There is, however, no clear interaction between the nitritotriacetate anion and the synergistic anion binding site, Arg$^{121}$.

Transferrins are a group of iron-binding proteins which includes serum transferrin, lactoferrin, and ovotransferrin (1). The proteins serve to control the iron level in the body fluid of vertebrates by their ability to bind very tightly two Fe$^{3+}$ ions (1). They are ~80-kDa single-chain proteins and consist of two similarly sized homologous N- and C-lobes, which are further divided into two similarly sized domains (domains N1 and N2 in the N-lobe and domains C1 and C2 in the C-lobe). The two iron binding sites are located within the interdomain cleft of each lobe. Crystal structures of the diferric forms (2–10) and solution scattering (11–13) analyses have revealed that all of the transferrin lobes, except for the lactoferrin C-lobe in crystal, assume a conformation with an opening of the interdomain cleft. This implies that transferrin initially binds the Fe$^{3+}$ ion in the open form before being transformed into the closed holo form (18, 19). Differential domain and hinge locations of the four protein ligands (Asp$^{60}$ in the domain 1, Tyr$^{191}$ in the domain 2, and Tyr$^{92}$ and His$^{250}$ in different hinges) (2–10) inevitably require an alternative Fe$^{3+}$ coordination structure for the Fe$^{3+}$-loaded, domain-opened intermediate. Such an alternative structural state has been a central question to be solved for the understanding of the Fe$^{3+}$ binding pathway in transferrin.

A major difficulty encountered in the structural analysis for the intermediate is to prepare a stable protein form that reasonably mimics it. One of the most promising ways may be the site-directed mutagenesis approach for the amino acid residues that are implicated in the Fe$^{3+}$ coordination. An Fe$^{3+}$-loaded, domain-opened transferrin form, however, has not been obtained so far by site-directed mutagenesis; either the Asp- or His-ligand mutant of the lactoferrin N-lobe assumes the hololike closed conformation (20, 21). The mutant lactoferrin N-lobe in which the synergistic anion-binding residue, Arg$^{121}$, is replaced by the serine or glutamic acid residue also assumes the closed conformation (22).

In the present study, we employed an alternative strategy using an apo crystal: the Fe$^{3+}$ soaking conditions in which the colorless crystal turns red without any collapse were searched. As a successful condition, an apo crystal of ovotransferrin N-lobe was soaked with the Fe$^{3+}$-NTA$^1$ complex in the absence of CO$_3^{2-}$, and then its structure was solved at 2.1 Å resolution. We report here a novel structural state of transferrin: the Fe$^{3+}$-loaded structure of ovotransferrin N-lobe with essentially the same open conformation as the apo form. In this structure, the bound iron atom is coordinated by the two protein ligands of Tyr$^{92}$-OH and Tyr$^{191}$-OH. Other Fe$^{3+}$ coordination sites are occupied by a NTA anion, which is stabilized through the hydrogen bonds with protein groups. The observation strongly suggests that the two tyrosine residues are the initial Fe$^{3+}$-binding ligands in the open transferrin.

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1 The abbreviations used are: NTA, nitritotriacetate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; Trf, transferrin.

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Iron-loaded, Domain-opened Transferrin Structure

Crystal data

| Parameter | Value |
|-----------|-------|
| Space group | P6_32 |
| a (Å) | 125.29 |
| b (Å) | 125.29 |
| c (Å) | 87.53 |
| V, (Å³/Da) | 2.7 |
| Molecules/asymmetric unit | 1 |

Data Collection and Refinement

| Parameter | Value |
|-----------|-------|
| Resolution (Å) | 2.10 |
| Independent reflections | 100 |
| Rsym (%) | 7.9 |
| No. of protein atoms | 2,543 |
| No. of solvent molecules | 153 |
| Ions | 3SO_{4}/1Fe/1NTA |
| Final R factor | 0.189 |
| Free R value | 0.256 |
| Average B factor (Å²) | 22.2 |

Model Building and Refinement

Quality of the Final Model

Overall Organization of the Structure

RESULTS

Iron-loaded transferrin comprises 332 amino acid residues (23). Residues 1–3, however, are not included in the final model because no clearly interpretable electron density could be seen for these residues. In the final 2Fo – Fc electron density map, there is no break in the main chain density when contoured at the 1σ level. Relevant refinement statistics are given in Table I. The overall completeness, R factor, and free-R value were 88.3%, 0.189, and 0.256, respectively, for the data more than 2σ (Fo). For the highest resolution bin (2.10–2.19 Å), the completeness was 75.5%, and the R factor and free-R value were, respectively, 0.262 and 0.274. From a Luzzati plot, the mean absolute error in atomic position is estimated to be 0.24 Å.

A Ramachandran plot (25) of the main chain torsion angles is shown in Fig. 1; 88.3% of the residues are in the core regions, with 99.3% of the residues lying within the allowed regions as defined in the program PROCHECK (26). As a non-glycine residue, Leu^{299} lies outside the allowed regions (ϕ = 75.0°, ψ = −52.2°). This leucine residue is the central residue in a γ-turn. The γ-turn of the equivalent leucine residue is the one conserved in all of the N- and C-lobes of serum transferrin (2), ovotransferrin (4), and lactoferrin (6).

Overall Structure of the N-lobe

The overall structure of the Fe^{3+}-soaked form was almost exactly the same as that of the apo form. The root mean square deviation for 329 Cα atoms was only 0.19 Å. These structures, when compared with the holo (the Fe^{3+}- and CO_{2}^{2-}-loaded form) structure of ovotransferrin N-lobe (8), comprise a domain-opened conformation (Fig. 2). The extent and mode of the opening were almost the same as in the N-lobe of the whole molecules of lactoferrin (11) and duck (12) and hen^{3} ovotransferrin: as calculated by the rigid body motion method (27), the domains move 49.7° around a rotation axis passing through the

EXP. PROCEDURES

Crystalization—The isolated N-lobe (N-terminal half-molecule) of hen ovotransferrin was purified as described (23). The apo form of the protein was crystallized using the hanging drop vapor diffusion method. A solution of a crystallization droplet was prepared on a siliconized coverslip by mixing 5 μl of protein solution (44.4 mg/ml in 0.05 M BisTris-HCl buffer, pH 6.0, 52% ammonium sulfate). The colorless crystals were incubated at 20 °C and 0.256, respectively, for the data more than 2σ (Fo). For the highest resolution bin (2.10–2.19 Å, the completeness was 75.5%, and the R factor and free-R value were, respectively, 0.262 and 0.274. From a Luzzati plot, the mean absolute error in atomic position is estimated to be 0.24 Å.

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Overall Organization of the Structure—Fig. 2 displays the overall structure of ovotransferrin N-lobe as a Cα trace. The overall structure of the Fe^{3+}-soaked form was almost exactly the same as that of the apo form. The root mean square deviation for 329 Cα atoms was only 0.19 Å. These structures, when compared with the holo (the Fe^{3+}- and CO_{2}^{2-}-loaded form) structure of ovotransferrin N-lobe (8), comprise a domain-opened conformation (Fig. 2). The extent and mode of the opening were almost the same as in the N-lobe of the whole molecules of lactoferrin (11) and duck (12) and hen^{3} ovotransferrin: as calculated by the rigid body motion method (27), the domains move 49.7° around a rotation axis passing through the

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Model Building and Refinement—As the model structure, we employed the apo structure of ovotransferrin N-lobe[^2] that had been solved at 1.9 Å resolution by the isomorphous replacement method using the hexagonal apo crystals. Using the apo structure model and the diffraction data of the Fe^{3+}-soaked form, refinement calculations were carried out by X-PLOR (24). One NTA molecule and one iron atom, which were identified from a clear difference density (Fo – Fc) map of the first refinement round, were included in the model, followed by more than 10 rounds of refinements and manual model buildings. The parameter and topology files of NTA for X-PLOR (24) were prepared after building and energy minimization of NTA by QUANTA and CHARMM (Molecular Simulations Inc., San Diego, CA).

The omit maps (2Fo – Fc, contoured at 1σ and Fo – Fc, contoured at 3σ) were obtained using the reflection data of the Fe^{3+}-soaked form at 7.0–2.1 Å resolution after refinement of the model in which the NTA molecule was excluded. An anomalous difference Fourier density map contoured at 3σ was calculated with a separate data set of the Fe^{3+}-soaked form at 7.0–2.1 Å resolution with pair completeness of 95.8% by the program PHASES. The phases were calculated from the final model

[^2]: K. Mizutani, H. Yamashita, B. Mikami, and M. Hirose, manuscript in preparation.

[^3]: H. Kurokawa, J. C. Dewan, B. Mikami, J. C. Sacchettini, and M. Hirose, manuscript in preparation.
two β-strands linking the domains.

Another important observation in Fig. 2 is that an iron atom exists in the opened interdomain cleft of the Fe³⁺-soaked form. The apo structure of ovotransferrin N-lobe is the one employed as the model for the current structural determination of the Fe³⁺-soaked form (see “Experimental Procedures”). The residue numbers are labeled for the Fe³⁺-soaked form. The iron atom (green sphere) and the side chains (blue) of His²⁵⁰, Asp⁶⁰, Tyr⁸², and Tyr¹⁹¹ (from top to bottom in this order) for the Fe³⁺-soaked form are also displayed.

FIG. 3. Stereo views depicting the iron binding site in the Fe³⁺-soaked form. a, electron density maps (green: 2Fo – Fc, contoured at 1 σ; blue: Fo – Fc, contoured at 3 σ) obtained using the reflection data of the Fe³⁺-soaked form after refinement of the model in which the NTA molecule was omitted. b, anomalous difference Fourier density map contoured at 3 σ (purple) calculated with exclusion of an iron atom using the reflection data of the Fe³⁺-soaked form at 7.0–2.1 Å. The final model is superimposed in stick presentation with atoms in standard colors.

The Structure of the Fe³⁺-Binding Site—The Fe³⁺ binding structure was investigated in more details for the Fe³⁺-soaked form. Fig. 3a is a stereo diagram of the electron density map calculated with the exclusion of the NTA model (green, 2Fo – Fc; blue, Fo – Fc) for the Fe³⁺-soaked form. The figure clearly demonstrates the existence of iron and NTA close to the Tyr⁹² and Tyr¹⁹¹ ligands.

To evaluate the existence of an iron atom by an alternative way, we calculated the anomalous difference Fourier density map with exclusion of an iron atom. As shown in Fig. 3b (purple), the existence of an iron atom is clearly confirmed by the highest anomalous difference Fourier peak in the density map.

Fig. 4a is a diagram displaying the iron coordination and hydrogen bonding structure in the Fe³⁺-soaked form. As summarized in Table II, the distances from the iron of Tyr⁹²·OH and Tyr¹⁹¹·OH are 1.90 Å and 1.76 Å, respectively, indicating the Fe³⁺ coordination by these two tyrosine residues. The other
The binding of NTA is stabilized through the interactions with the protein chains: NTA-O12 is hydrogen bonded to Ala122-N, and NTA-O13, to Thr117-OG1 and Gly124-N. These protein groups are the ones that form the hydrogen bonds with CO3\(^-\) anion in the holo form (Fig. 4b). As a surprising observation, however, NTA has no direct interaction with the synergistic anion-binding residue, Arg121. Another difference in the protein-anion interactions is that the hydrogen bond of NTA-N is not clear because of a thick solid line.

Previous x-ray crystallographic (2–13) and solution scattering (14–17) analyses have revealed that upon Fe\(^{3+}\) uptake, transferrins undergo a large scale conformational transition from the domain-opened apo structure into the closed holo structure. The structural pathway for the large conformational transition, however, has not been known. The current Fe\(^{3+}\)-soaked structure provides crucial information about the structural mechanism for the Fe\(^{3+}\) binding.

For loading Fe\(^{3+}\) to apotransferrin (apo-Trf), a ferric chelate, most widely ferric NTA (Fe\(^{3+}\)-NTA), is employed (1). The binding reaction yields a ternary complex consisting of transferrin, Fe\(^{3+}\), and NTA molecules (TrfFe\(^{3+}\)-NTA) (1, 28).

The TrfFe\(^{3+}\)-NTA complex is a stable form in the absence of other synergistic anions. In the presence of a high concentration of bicarbonate, however, NTA is replaced by CO3\(^-\); this reaction yields the physiological holo form consisting of transferrin, Fe\(^{3+}\), and CO3\(^-\) (TrfFe\(^{3+}\)-CO3\(^-\)) (1, 28).

### DISCUSSION

The current crystal structure of the Fe\(^{3+}\)-soaked form demonstrates essentially the same open conformation as apo-Trf, whereas TrfFe\(^{3+}\)-CO3\(^-\) assumes the closed one (Fig. 2). About the implications of the Fe\(^{3+}\)-soaked structure for the iron binding pathway, two different mechanisms may be possible.

In the first mechanism, TrfFe\(^{3+}\)-NTA assumes the same conformation in solution as the Fe\(^{3+}\)-soaked structure, and the total domain closure occurs in Reaction 2. In this mechanism, the domain closure should depend on the anion replacement. The TrfFe\(^{3+}\)-NTA complex shares the two protein ligands (Tyr92 and Tyr191 residues) with TrfFe\(^{3+}\)-CO3\(^-\) (Fig. 4). Nev-
Nevertheless, some structural modulations in the iron binding site, other than the protein ligand structures, appear to be highly relevant to the structural mechanism in Reaction 2. As displayed in Fig. 4b, CO$_3^-$ forms hydrogen bonds with Thr$_{117}$, OG1, Ala$_{123}$-N, and Gly$_{124}$-N in the holo form; these protein groups are all hydrogen bonded to carboxylic groups of NTA in the Fe$_3^+$-soaked form (Fig. 4a). The protein group Ser$_{122}$-O6a in the holo form also forms a hydrogen bond with NTA-O4 in the Fe$_3^+$-soaked form. However, Arg$_{121}$-NE and $\cdot$NH$_2$, which are the anchor sites for CO$_3^-$ in the holo form, are both vacant in the Fe$_3^+$-soaked form. Such an open situation would be suitable for the subsequent entry of CO$_3^-$ in Reaction 2. Our putative pathway for Reaction 2 includes an initial entry of CO$_3^-$ into the Arg$_{121}$ anchor sites and then the total replacement of NTA by CO$_3^-$.

This reaction should yield a short lived TrfFe$_3^+$-CO$_3^-$ complex with the open conformation, in which only four of the six Fe$_3^+$ coordination sites are occupied by the protein side chains (Tyr$_{92}$ and Tyr$_{191}$) and bidentate CO$_3^-$. As a structural counterpart, the crystal structure of a domain 2 fragment complex, in which all parts of domain 1 as well as the aspartic acid and histidine ligands are deleted by proteolysis, demonstrates the presence of an equivalent Fe$_3^+$ coordination structure by the two tyrosine residues and CO$_3^-$ (29). The formation of the holo structure is then completed by the coordination of Asp$_{60}$ and His$_{250}$ ligands to the two vacant Fe$_3^+$ sites and hence by the domain closure.

In the second mechanism, the Fe$_3^+$-soaked form is initially transformed into a closed conformation by the Fe$_3^+$ coordination of Asp$_{60}$ and His$_{250}$ ligands, before the bound NTA molecule is replaced by a carbonate anion. This mechanism, however, requires as a prerequisite the occurrence, in solution, of the differential ternary TrfFe$_3^+$-NTA complex with the closed conformation in a carbonate-free condition; the maintenance of the open conformation in the Fe$_3^+$-soaked form is accounted for by the arrest due to a crystal packing force from otherwise (free in solution) induced transformation into the closed conformation. The mechanism would also require a rearranged coordination and hydrogen bonding structure for NTA in the TrfFe$_3^+$-NTA complex because the Asp$_{60}$ and His$_{250}$ coordination sites are occupied by NTA in the Fe$_3^+$-soaked form (Figs. 3 and 4). As a related observation to the second mechanism, no clear diffraction has been detected for the apo crystal when it is soaked with Fe$_3^+$-soaked form is almost indistinguishable from that of the apo crystal (Fig. 2) is consistent with the view that the two tyrosine residues are the protein ligands for the Fe$_3^+$ entry in the intact transferrin lobe with the domain-opened conformation.

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