

Crystal Structure of Hen Apo-ovotransferrin

BOTH LOBES ADOPT AN OPEN CONFORMATION UPON LOSS OF IRON*

(Received for publication, April 14, 1999, and in revised form, June 30, 1999)

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The three-dimensional crystal structure of hen apo-ovotransferrin has been solved by molecular replacement and refined by simulated annealing and restrained least squares to a 3.0-Å resolution. The final model, which comprises 5312 protein atoms (residues 1 to 686) and 28 carbohydrate atoms (from two monosaccharides attached to Asn⁴⁷³), gives an *R*-factor of 0.231 for the 11,989 observed reflections between 20.0- and 3.0-Å resolution. In the structure, both empty iron binding clefts are in the open conformation, lending weight to the theory that Fe³⁺ binding or release in transferrin proceeds via a mechanism that involves domain opening and closure. Upon opening, the domains rotate essentially as rigid bodies. The two domains of the N-lobe rotate away from one another by 53°, whereas the C-lobe domains rotate away each another by 35°. These rotations take place about an axis that passes through the two β-strands, linking the domains. The domains of each lobe make different contacts with one another in the open and closed forms. These contacts form two interdomain interfaces on either side of the rotation axis, and domain opening or closing produces a see-saw motion between these two alternative close-packed interfaces. The interdomain disulfide bridge (Cys⁴⁷⁸-Cys⁶⁷¹), found only in the C-lobe, may restrict domain opening but does not completely prevent it.

Transferrins are a group of iron-binding proteins that includes serum transferrin, ovotransferrin, and lactoferrin (1). These proteins serve to control the levels of iron in the body fluids of vertebrates by their ability to bind very tightly two Fe³⁺ ions together with two CO₃²⁻ ions. Serum transferrins and ovotransferrin can act as iron transporters, whereas the lactoferrins possess an antimicrobial activity (2) and a sequence-specific DNA binding capacity (3). The ~80-kDa transferrin molecule consists of two similarly sized homologous N- and C-lobes, which are further divided into two similarly sized

domains (N1 and N2 in the N-lobe; 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 fully iron-loaded diferric forms (4–7) and the monoferric N-lobes (8–11) of several transferrins reveal that the two domains of each lobe are closed over an Fe³⁺ ion. Four of the six Fe³⁺ coordination sites are occupied by protein ligands (2 Tyr residues, 1 Asp, and 1 His) and the other two by a bidentate carbonate anion. The recent x-ray structure of the *Hemophilus influenzae* Fe³⁺-binding protein has revealed that its overall structure, apart from consisting of a single lobe, is remarkably similar to that of the transferrin family (12). The ligands to iron are, however, a phosphate oxygen, a water molecule, and four protein ligands (2 Tyr residues, 1 Glu, and 1 His). Except for the carboxylate ligand, the other three protein ligands of the Fe³⁺-binding protein are all located in different parts of the protein fold from those of the transferrins, indicating convergent evolution for the development of the Fe³⁺ binding function (12).

It is generally believed that the two domains of each transferrin lobe must open to allow entry or release of Fe³⁺ (13–15); indeed, an x-ray solution scattering study has revealed iron-induced conformational changes in both lobes of several transferrins (16). The iron-loaded holo form shows a higher affinity for the transferrin receptor than does the apo form, indicating that iron-dependent conformational changes may be important in the cellular uptake of iron (17–20). Although the x-ray structures of the diferric forms of several transferrins are known (4–7), data for the bilobal apo form is limited. The crystal structure of the apo form of human lactoferrin has been determined at 2.8-Å resolution by Anderson *et al.* (14) and recently at 2.0-Å resolution by Jameson *et al.* (21). In that structure the iron binding cleft in the N-lobe is wide open when compared with the closed holo structure. Upon uptake or release of iron, domains N1 and N2 rotate 54° away from one another as rigid bodies around a rotation axis that passes through the two antiparallel β-strands linking the domains. By contrast, the C-lobe is found in the closed conformation in both the holo and apo forms. This unexpected lack of conformational change may be related to the presence of an interdomain disulfide bridge (Cys⁴⁸³-Cys⁶⁷⁷) in the C-lobe of lactoferrin. It is also possible that lactoferrin might lack, because of its non-iron-transporting nature, an open/close mechanism that might be important for the selective recognition of the holo form by the receptor. The absence in human (5) and bovine (22) lactoferrins of the N-lobe dilysine trigger, which has been proposed as a pH-sensitive molecular switch for the opening of that domain and subsequent iron release (9), is another structural point that differentiates the lactoferrins from the iron-transporting transferrins.

Ovotransferrin, a major egg white protein, should share the same structural characteristics as hen serum transferrin,

* This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan and the United States National Institutes of Health Grant GM45859. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates (code 1AIV) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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which functions as an iron transporter, since these proteins are derived from the same gene and differ only in their attached carbohydrate (23). Although an *in vivo* iron transport function for ovotransferrin has not been proved, specific transferrin receptor interactions have been demonstrated for the protein (18–20, 24). Hen ovotransferrin possesses the same six disulfide bridge pattern in the N-lobe as that observed in human lactoferrin: two bridges in the N1-domain and four in the N2-domain. There is, therefore, no interdomain disulfide bridge in the N-lobe of either protein (25–27). This six-disulfide motif is also observed in the C-lobes of ovotransferrin and lactoferrin. There are, however, three and four additional disulfides, respectively, in the ovotransferrin and lactoferrin C-lobes. One of the disulfides of the ovotransferrin C-lobe exists as the interdomain disulfide Cys⁴⁷⁸-Cys⁶⁷¹ (6) and is found in an equivalent site to the lactoferrin interdomain disulfide Cys⁴⁸³-Cys⁶⁷⁷ (5). This suggests that if a restriction of domain opening is caused by the interdomain cross-link, it may operate in a similar manner in the two proteins. We report here the x-ray crystal structure of hen apo-ovotransferrin determined at 3.0-Å resolution. Comparison of the current apo structure with the previous holo structure (6) shows that both lobes adopt an open conformation upon iron release.

EXPERIMENTAL PROCEDURES

Apo form of hen ovotransferrin was purified as described previously (28). Colorless apo-ovotransferrin crystals were grown at 4 °C by the hanging drop vapor diffusion method where 5 μ l of protein solution (85 mg/ml, pH 6.0, 0.02 M acetate buffer) was mixed with 5 μ l of precipitant solution (4–6% polyethylene glycol 6000, pH 6.0, 0.02 M acetate buffer) on a silanized coverslip that was inverted and sealed above 0.7 ml of the precipitant solution. The crystal used for data collection was the largest available and had approximate dimensions of 2.5 $\times$ 0.5 $\times$ 0.4 mm. Many crystals were examined, but the data set employed herein was the best obtained to date.

X-ray data were collected at –150 °C (29) with CuK α radiation (λ = 1.5418 Å) using a Siemens Hi-Star area detector coupled to a MacScience M18XHF rotating-anode generator. A total of 31,370 reflections was collected and averaged (R_{symm} = 0.069), yielding 12,920 unique data (80% complete). The mean $I/\sigma(I)$ for the data set was 6.5. The structure was solved by molecular replacement using X-PLOR (30). The only search model to give the correct solution was the diferric ovotransferrin (6) C-lobe, with the domains opened by 40°. The N1-domain was then placed in the same relative orientation as domains N1/C1 of the diferric structure. After rigid body refinement, the N2-domain could be fitted into its electron density. Rigid body refinement, simulated annealing, and positional and B-factor refinement (30) have resulted in a final r = 0.231 (R_{free} = 0.265) for the 11,989 reflections (75% completeness) with $F > 2\sigma(F)$ between 20.0 and 3.0-Å resolution. A bulk solvent correction was applied to the data. The final model consists of 5312 non-hydrogen protein atoms (residues 1–686) and 28 carbohydrate atoms (two residues attached to Asn⁴⁷³). Further experimental details appear in Table I. Model building and superposition of the structures were performed with TURBO-FRODO (Biographics).

RESULTS

Quality of the Final Model—The final model is comprised of the entire polypeptide chain (residues 1 to 686). In the final $2F_o - F_c$ electron density map, there are no breaks in the main-chain density when contoured at the 1σ level. Two sugar residues are also included in the model attached to Asn⁴⁷³. The root mean square deviations from standard bond lengths and angles are 0.010 Å and 1.70°, respectively (Table I). A Ramachandran plot of the main-chain torsion angles shows that 60% of the residues are in the core regions, with 95% lying within the allowed regions as defined by the program PROCHECK (31). Two non-glycine residues (Leu²⁹⁹ and Leu⁶³⁶) lie outside the allowed regions of conformational space (ψ = 64°, ϕ = –48°, and ψ = 69°, ϕ = –55°, respectively); both are the central residues of two γ -turns in equivalent positions in each ovotransferrin lobe.

TABLE I
Statistics for data collection and refinement of hen apo-ovotransferrin

Crystal system	Tetragonal
Space group	P4 ₃ 2 ₁ 2
Unit cell (Å)	a = b = 92.26, c = 178.19
Molecules/asymmetric unit	1
Detector	Siemens Hi-Star
Crystal-detector distance (cm)	15
No. crystals	1
Resolution limit (Å)	3.0
Scan width (°/frame)	0.25
Scan speed (°/min)	0.25
Scan range	
ϕ -scan	0–45° (2 θ = 15°, χ = 0°)
ω -scan	0–45° (2 θ = 15°, χ = 30°)
Measured reflections	31,370
Unique reflections	12,920 [$ I > \sigma(I)$]
Completeness (%)	80.0
R_{symm} on I (%)	6.9
Final model	1–686 residues, 2 sugars
Resolution range (Å)	20.0–3.0
Reflections	11,989 [$ F > 2\sigma(F)$]
Completeness (%)	75.0
R -factor (%)	23.1
R_{free} (%)	26.5
Root mean square	
bond length (Å)	0.010
angle (°)	1.70

Overall Organization of the Structure—The apo-ovotransferrin structure, superimposed on that of diferric ovotransferrin (Protein Data Bank code 1OVT) (6), is shown as a ribbon trace in Fig. 1. There is no difference in the secondary structure assignment (β -strands a–k and α -helices 1–12) between the holo and apo forms (6). As in the diferric ovotransferrin structure, hen apo-ovotransferrin is folded into two homologous N- and C-lobes, and each of the lobes is divided into two distinct and similarly sized α/β domains (N-lobe: N1- and N2-domains; C-lobe: C1- and C2-domains). The two empty iron binding sites are located between the two domains in each lobe, and the two domains are linked by two antiparallel β -strands (e and j) that run behind the iron binding site of each lobe. Both iron binding clefts of apo-ovotransferrin are in the open conformation. The N2- and C2-domains move away from the N1- and C1-domains, respectively, almost as rigid bodies. Between the open and closed forms, the root mean square deviation of equivalent C- α atoms that are within a distance of 2.0 Å of one another is 1.02 Å for the N1-domain (186 C- α atoms) and 1.12 Å for the N2-domain (160 C- α atoms). For the C-lobe, these values are 1.06 Å for the C1-domain (204 C- α atoms) and 0.87 Å for the C2-domain (180 C- α atoms). The major conformational changes occur in the hinge regions on the two β -strands that link the domains of each lobe. In addition to the domain opening in the two lobes, there is a small rotation of the C1-domain relative to the N1-domain. The value of the rotation is 7°, suggesting that the N1- and C1-domains remain relatively fixed with respect to one another. Two GlcNAc residues attached to Asn⁴⁷³ reside between the C1- and C2-domains.

Rotation Axes for Domain Movement—To characterize the conformational changes that have occurred upon domain opening, the domain motion in each lobe was considered separately as a rotation and a translation (32). In the N-lobe the N1-domains of the open and closed forms were superimposed, and the rotation and translation required to superimpose the N2-domains were determined to be 53° and 1.8 Å, respectively (Fig. 2a). The rotation axis passes close to residues Ser⁹¹ and Val²⁴⁷ and is nearly parallel to a line through the C- α atoms of these two residues (Fig. 3). The same analysis was applied to the

FIG. 1. Stereo C- α plot of apo-ovo-transferrin (yellow) superimposed on diferric ovotransferrin (cyan). Domains N1/C1 of apo-ovotransferrin were superimposed on the same domains of the diferric molecule. Fe^{3+} bound to each lobe of the diferric ovotransferrin is shown as a purple sphere. Certain residues of apo-ovotransferrin have been labeled with their sequence number. The number of secondary structures (β -strands $a-k$ and α -helices 1–12) have been labeled with black color for apo ovotransferrin and with blue color for diferric ovotransferrin. The figure was produced with MOLSCRIPT (49) and RASTER3D (50).

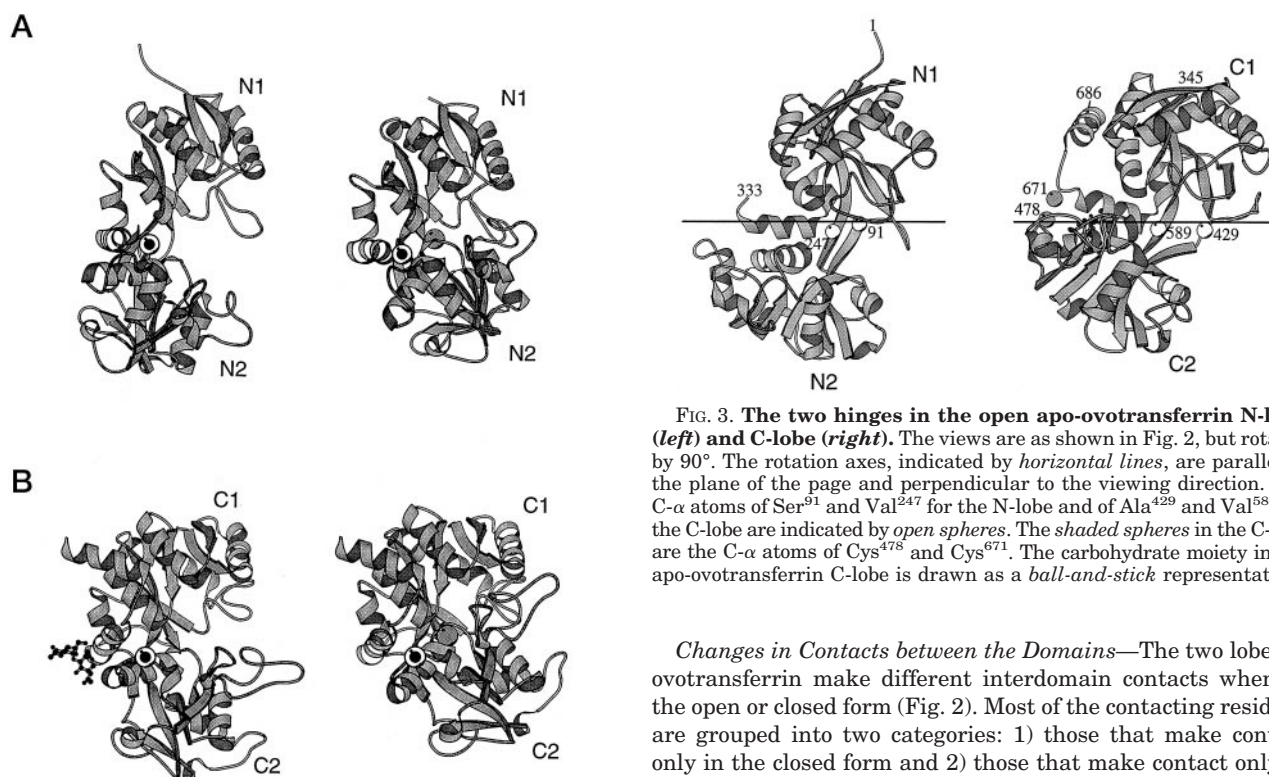
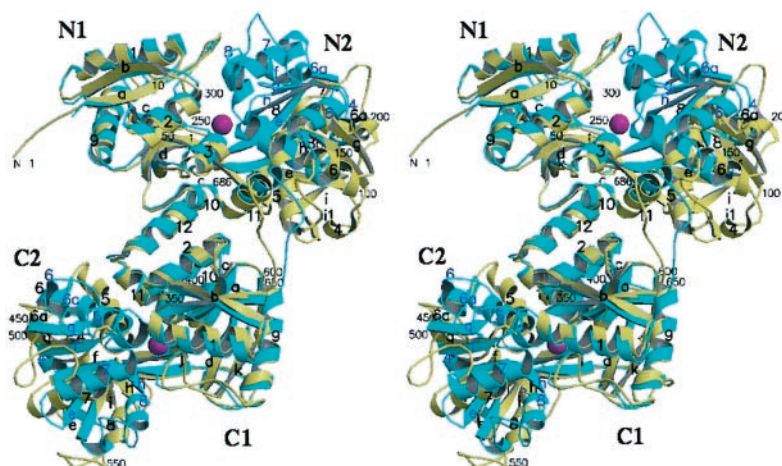


FIG. 2. Domain motions in the N- (A) and C-lobes (B) of ovotransferrin. Both lobes are shown in open (apo) and closed (diferric) forms, with domain 1 in the same orientation in each case. In both A and B the open conformation is on the left with the closed conformation on the right. The rotation axis lies near the center of each figure, and the view is down that axis, which is indicated by a circle with a dot in it. The carbohydrate moiety in the apo-ovotransferrin C-lobe is drawn as a ball-and-stick representation. The figure was produced with MOLSCRIPT (49).

motion in the C-lobe. A 35° rotation, with a $0.2\text{-}\text{\AA}$ translation, was required to superimpose the C2-domains of the open and closed forms (Fig. 2b). The rotation axis passes close to residues Ala^{429} and Val^{589} and is nearly parallel to a line through the two C- α atoms of these residues (Fig. 3). The C- α atoms of Cys^{478} and Cys^{671} , which form the C-lobe interdomain disulfide bridge, also lie close to this axis (Fig. 3). In either the N- or C-lobe, domain 2 translates only 1.8 or $0.2\text{ \AA}$ with respect to domain 1, and thus, the motions of the N2- or C2-domains relative to the N1- or C1-domains are almost a pure rotation about the two central residues in each hinge.

FIG. 3. The two hinges in the open apo-ovotransferrin N-lobe (left) and C-lobe (right). The views are as shown in Fig. 2, but rotated by 90° . The rotation axes, indicated by horizontal lines, are parallel to the plane of the page and perpendicular to the viewing direction. The C- α atoms of Ser^{91} and Val^{247} for the N-lobe and of Ala^{429} and Val^{589} for the C-lobe are indicated by open spheres. The shaded spheres in the C-lobe are the C- α atoms of Cys^{478} and Cys^{671} . The carbohydrate moiety in the apo-ovotransferrin C-lobe is drawn as a ball-and-stick representation.

Changes in Contacts between the Domains—The two lobes of ovotransferrin make different interdomain contacts when in the open or closed form (Fig. 2). Most of the contacting residues are grouped into two categories: 1) those that make contact only in the closed form and 2) those that make contact only in the open form. Fig. 4 shows the positions of the contacting residues in the open and closed forms. In the closed form there are 22 contacting residues observed for the N-lobe and 16 for the C-lobe. The differences in the accessible surface areas of these residues, between the open and closed forms, are listed in Table II. Almost all of the residues that make contact with the other domain in the closed form are exposed in the open form. In the open form there are 13 contacting residues for the N-lobe and 9 for the C-lobe, and most of them are exposed in the closed form (Table II). The contacting residues in the closed form are found mostly in the interdomain binding cleft and form the large interface. The contacting residues in the open form are located on the opposite side of the rotation axis and constitute the small interface. The alternating exposure and burying of the interfaces on either side of the rotation axis constitutes a “see-saw” motion that has been noted before by Gerstein *et al.* (32) in the case of lactoferrin.

The different interdomain contacts in the apo and holo forms play a role in Fe^{3+} binding stability. For example, helix 11 (residues 321–332 in the N-lobe; 658–669 in the C-lobe) is located in the small interface, and 6 and 4 residues of the helix,

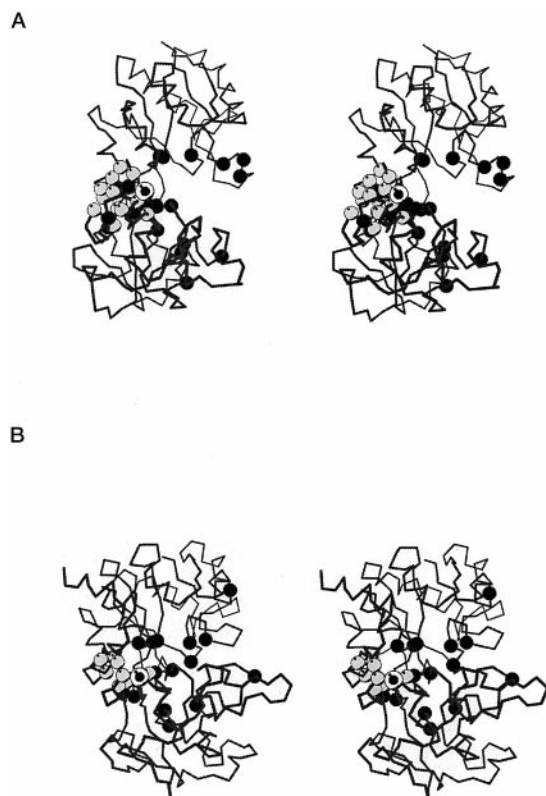


FIG. 4. Stereo views of the large and small interfaces in the N- (A) and C-lobes (B) of apo-ovotransferrin. Filled spheres show the C- α positions of residues that close pack in the closed form only. Shaded spheres show residues that close pack in the open form only. The view for both parts of the figure is down the rotation axis as in Fig. 2. The rotation axis lies near the center of each figure and is indicated by a circle with a dot in it.

respectively, in the N- and C-lobes participate in interdomain contacts (Table II). Previous observations have stressed the importance of the helix in Fe^{3+} binding stability. The removal of residues 280 to 332 from the ovotransferrin N-lobe results in a greatly reduced Fe^{3+} binding stability (33). Likewise, helix 11 occurs in both lobes of lactoferrin and is disordered in the isolated N-lobe crystal structure. This half molecule displays a much lower Fe^{3+} binding affinity (10).

Orientation of Domain Opening—Fig. 5 shows the superposition, on domain 1 in each case, of three open apo-transferrin lobes, namely the N- and C-lobes of apo-ovotransferrin and the apo-lactoferrin N-lobe (Protein Data Bank code 1LFH). The root mean square deviation of equivalent C- α atoms that are within 2 Å of one another is 1.09 Å between the N1-domain of apo-ovotransferrin and the N1-domain of apo-lactoferrin (199 C- α atoms) and 1.10 Å between the N1-domain and C1-domain of apo-ovotransferrin (161 C- α atoms). The extent and orientation of the opening in each lobe, however, are all different. When the different modes of domain opening are expressed as the relative orientations of the rotation axes of domain, the axis of the apo-ovotransferrin N-lobe intersects those of the apo-ovotransferrin C-lobe and of opening, the apo-lactoferrin N-lobe with angles of 17.5° and 12.3°, respectively. The rotation axes of the apo-ovotransferrin C-lobe and the apo-lactoferrin N-lobe can be related to one another by a rotation of 12.4° and a 1.0-Å translation. Such differences may be accounted for, at least in part, by different hinge sequences.

Table III shows a sequence alignment of the hinge regions (part of β -strands e and j) in vertebrate transferrins. Other than the iron-coordinating ligands (Tyr⁹², His²⁵⁰, Tyr⁴³¹, and His⁵⁹² in the ovotransferrin sequence), several highly con-

served residues are observed. Ala⁹⁴ (Ala⁴³³ in the C-lobe) and Ala²⁴⁵ (Ala⁵⁸⁷ in the C-lobe) are conserved in both the N- and C-lobes of all the vertebrate transferrins except for the substitution of a Ser residue in the rabbit transferrin C-lobe. Thr⁹⁰, Tyr⁹³, and Val²⁴⁷ are also conserved in all the N-lobes of the transferrins. Ser⁸⁸ (Arg⁴²⁷ in the C-lobe), Ser⁹¹ (Ser⁴³⁰ in the C-lobe), and Ala²⁴⁹ (Thr⁵⁹¹ in the C-lobe) are, however, unique residues in the avian transferrin as compared with the mammalian transferrins. Another unique residue to the avian protein is Val⁵⁸⁹ in the C-lobe; this residue is located at the equivalent position of Val²⁴⁷, that is conserved in the N-lobes of all the transferrins. The hinge residues that are unique to the ovotransferrin N-lobe are Trp²⁴⁴ and Ala²⁴⁸; at positions 244 and 248 (positions 586 and 590 in the C-lobe) Leu and Pro residues are, respectively, conserved in both lobes of all the other transferrins, including the C-lobe of ovotransferrin.

Although the three open lobes in Fig. 5 show some differences, there are common features among them: 1) each domain behaves essentially as a rigid body; 2) major conformational changes occur mainly in the hinge regions located on the two β -strands that connect the two domains of each lobe; and 3) the domain movement produces a see-saw motion between the two alternative interfaces on either side of the rotation axis.

DISCUSSION

Comparison of the current apo and the previous holo (6) structures demonstrates that both the N- and C-lobes of ovotransferrin assume an open conformation upon iron release. Upon opening, the domains of the N-lobe rotate away from one another by 53°, and there is a corresponding 35° rotation in the C-lobe. Each domain rotates essentially as a rigid body. As well as these rotations, the two domains of each lobe also undergo small translations with respect to one another. In the N-lobe, this translation amounts to 1.8 Å, whereas in the C-lobe it is 0.2 Å. The rotation axis in each lobe passes through the two β -strands linking the domains, and the major conformational changes occur in these hinge regions of each lobe. The results of the current crystallographic analysis are consistent with those from an x-ray solution scattering study (16) that demonstrates large scale conformational changes in both lobes of several transferrins upon Fe^{3+} binding or release. In addition, the x-ray scattering data suggest that the extent of opening of the ovotransferrin C-lobe is equivalent to a 75% opening of the N-lobe; in the current crystal structure the opening of the C-lobe corresponds to a 66% opening of the N-lobe.

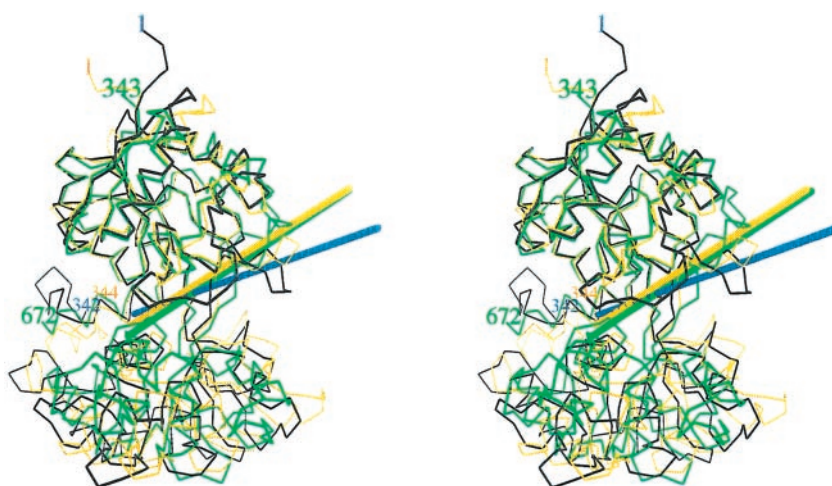
The open structure for both lobes of apo-ovotransferrin exposes all four iron-coordinating amino acid residues to solvent, showing that either lobe is accessible for iron entry. Taken together with gel electrophoretic evidence (34, 35), it is likely that there are two monoferric forms of transferrin in solution in which either lobe assumes an empty open conformation with the other lobe closed over a bound iron atom. Indeed, the structure of C-monoferric human serum transferrin (36) is consistent with such an idea; the domains are closed over Fe^{3+} bound in the C-lobe, but the empty N-lobe assumes an open conformation. The occurrence of such monoferric forms along with the closed (diferric) and open (apo) forms may be related to the observed modes of transferrin receptor interactions. Several lines of evidence have demonstrated that the diferric form is essential for the full binding of transferrin to the receptor (17–20, 23), indicating that the closed conformation is that recognized by the receptor. The values of the association constants of the monoferric transferrins for the receptor fall between those of the diferric and apo forms: the values are $1.1 \times 10^8 \text{ M}^{-1}$ for diferric, $2.8 \times 10^7 \text{ M}^{-1}$ for N-monoferric, $2.5 \times 10^7 \text{ M}^{-1}$ for C-monoferric, and $4.6 \times 10^6 \text{ M}^{-1}$ for the apo form (17). These data are consistent with the view that the receptor

TABLE II
Residues making interdomain contacts and their accessible surface areas

Each residue listed makes at least one contact between the domains. The interfaces L/N1, L/N2, L/C1, and L/C2 represent the large interfaces of the N1, N2, C1, and C2 domains, respectively, whereas S/N1, S/N2, S/C1, and S/C2 represent the small interfaces of the N1, N2, C1, and C2 domains, respectively. Contacts in the closed (C), open (O), or closed and open forms (C/O) are defined as atoms separated by less than the sum of their van der Waals radii. Diff. is the difference in the accessible surface area for each contacting residue calculated for the closed form minus the open form. Surface areas were calculated by NACCESS (51).

Interface	Residue	Contact in	Surface area (Å ²)			Interface	Residue	Contact in	Surface area (Å ²)		
			Open	Closed	Diff.				Open	Closed	Diff.
L/N1	D60	C	51.6	3.0	-48.6	L/C1	K352	C	154.3	150.3	-4.0
	I184	C	93.7	17.3	-76.4		D395	C	41.4	7.8	-33.6
	T85	C	126.0	100.2	-25.8		G397	C	6.3	3.7	-2.6
	Y92	C	58.4	0.3	-58.1		S430	C	53.1	48.9	-4.2
	E215	C	143.1	7.5	-135.6		V589	C/O	1.8	2.7	0.9
	V247	C/O	25.8	6.6	-19.2		H592	C	34.6	13.7	-20.9
	H250	C	39.6	14.7	-24.9		D634	C	65.1	36.1	-29.0
	P293	C	109.6	23.2	-86.4		K638	C	39.3	4.5	-34.8
	V294	C	104.6	58.8	-45.8		L640	C	88.0	27.9	-60.1
	K296	C	95.3	12.8	-82.5		L668	C	17.1	5.3	-11.8
	K301	C	27.5	6.7	-20.8		C671	C/O	32.6	7.8	-24.8
	Y324	C	17.5	22.2	4.7	L/C2	R460	C	97.4	28.6	-68.8
L/N2	Y93	C	29.2	11.4	-17.8		T461	C	10.7	0.1	-10.6
	R121	C	132.1	39.2	-92.9		G469	C	14.8	0.5	-14.3
	S122	C	16.0	0.5	-15.5		H472	C/O	33.4	34.7	1.3
	N126	C	31.5	10.3	-21.2		H518	C	121.8	37.0	-84.8
	I127	C	1.6	19.0	17.4		Y524	C	54.3	5.5	-48.8
	L133	C/O	20.3	28.9	8.6		Q541	C	35.3	3.4	-31.9
	W140	C/O	31.6	25.5	-6.1		S543	C	33.0	11.2	-21.8
	P187	C	130.6	41.6	-89.0	S/C1	F661	C/O	0.8	9.6	8.8
	Y191	C	95.4	4.7	-90.7		I665	O	1.2	2.7	1.5
	H196	C	61.8	22.0	-39.8		S667	O	35.4	47.6	12.2
	K209	C	46.2	1.3	-44.9		L668	O	17.1	5.3	-11.8
	E215	C	143.1	7.5	-135.6	S/C2	F432	O	10.0	15.2	5.2
	K240	C	109.0	69.7	-39.3		T461	O	69.4	74.4	5.0
S/N1	L320	O	1.7	8.5	6.8		V465	O	18.2	1.2	-17.0
	E323	O	30.3	128.8	98.5		G469	O	0.5	14.8	14.3
	S326	O	18.3	43.7	25.4		H472	O	34.7	33.4	-1.0
	Q329	O	25.3	48.5	23.2		T477	O	73.7	57.6	-16.1
	S330	O	12.7	40.6	27.9						
	M331	C/O	22.7	23.2	0.5						
	R332	O	149.1	124.1	-25.0						
S/N2	G130	O	0	13.3	13.3						
	H134	O	92.6	156.2	63.6						
	I1e43	O	63.9	100.3	36.5						
	V148	O	18.9	19.8	0.9						
	W244	O	7.8	18.7	10.9						
	A245	O	0	24.0	24.0						
	R246	O	78.6	103.8	25.2						

FIG. 5. **Different domain opening in the transferrins.** Stereo views of a C- α plot showing the superposition of the apo-ovotransferrin N-lobe (blue), apo-ovotransferrin C-lobe (green), and lactoferrin N-lobe (yellow). These lobes have been superimposed on domain 1 in each case. The rotation axes of domain opening are indicated by straight lines with the corresponding colors.



recognizes the four forms of transferrin in different ways.

Hen ovotransferrin contains six disulfide bridges in the N-lobe: two in the N1-domain, three in the kringle of domain 2, and one in the N2-domain (25, 26). There is no interdomain disulfide in the N-lobe. This six-disulfide bridge motif is conserved in the ovotransferrin C-lobe and also in the N- and

C-lobes of serum transferrin and lactoferrin (25–27). There are three additional disulfides in the ovotransferrin C-lobe, and these are also observed in the C-lobes of lactoferrin and serum transferrin. Some of the three conserved disulfides appear to be related to the asymmetric structural and functional characteristics of the N- and C-lobes of the transferrins. Cys⁴⁷⁸-Cys⁶⁷¹

forms the only interdomain cross-link in the C-lobe of ovotransferrin. In the structure of apo-lactoferrin the N-lobe is wide open; upon release of iron, domains N1 and N2 rotate away from one another by 54° as rigid bodies (14, 21). By contrast, the C-lobe of apo-lactoferrin remains closed as in the holo form (14, 21). As a possible explanation for this unexpected observation, a restriction on domain opening by the interdomain disulfide (Cys⁴⁸³-Cys⁶⁷⁷ in lactoferrin) was examined. Anderson *et al.* (14) estimate that a 53° rotation of the C-lobe domains would stretch the C-α . . . C-α distance of the two cysteine residues from 5.2 to 8.5 Å, more than the maximum value of about 7 Å for a disulfide bridge. This led to the prediction of a disulfide-dependent restriction on domain opening, and the prediction appears to be correct in the case of the ovotransferrin C-lobe. The interdomain cross-link is located in an equivalent structural site in the hen ovotransferrin (6) and lactoferrin C-lobes (5). The C-α . . . C-α distances for Cys⁴⁷⁸-Cys⁶⁷¹ are 4.8 and 5.9 Å in the holo and apo forms of ovotransferrin, respectively. The significant domain opening observed in the ovotransferrin C-lobe (35°), accompanied by this small disulfide stretch, although still only a partial (66%) opening when compared with that in the N-lobe, is probably related to the close proximity of the interdomain disulfide bridge to the rotation axis (Fig. 3). Also, the domain opening observed here in the ovotransferrin C-lobe makes it unlikely that the interdomain disulfide is the major force preventing domain opening in the lactoferrin C-lobe. A similar conclusion is also reached in the recent report of the crystal structure of the apo form of the N-terminal half-molecule of human serum transferrin (37), which contains an equivalent interdomain disulfide.

It has been suggested previously that the dilysine trigger

(Lys²⁰⁹-Lys³⁰¹) is central to the release of Fe³⁺ from the N-lobes of iron-transporting transferrins (9). The distance between the N-ζ atoms of Lys²⁰⁹ and Lys³⁰¹ is 8.5 Å in the current apo structure, whereas it is 2.3–2.6 Å in the holo structure (7, 9). The greater iron binding stability of the ovotransferrin C-lobe, when compared with that of the N-lobe, can be explained by the substitution of the dilysine arrangement by Gln⁵⁴¹-Lys⁶³⁸ in the C-lobe (7, 9). In addition to the absence of the dilysine trigger, we hypothesize that the interdomain disulfide Cys⁴⁷⁸-Cys⁶⁷¹ might also contribute to the greater iron binding stability of the C-lobe. The hypothesis stems from the observation that the two domains are covalently linked by the two closely spaced hinge strands that cross almost perpendicular to the rotation axis (Fig. 3). The interdomain disulfide is located close to this axis but significantly distant from the two hinge strands (Fig. 3); the presence of such a disulfide would protect the protein from an aberrant domain opening, including a rotation around an axis perpendicular to the true axis. A previous report that the selective reduction and alkylation of Cys⁴⁷⁸ and Cys⁶⁷¹ in ovotransferrin results in lower iron binding stability (38) supports this hypothesis.

Another disulfide, Cys⁴⁰⁵-Cys⁶⁸⁰, might play a role in the cooperativity of iron binding between the two ovotransferrin iron binding sites. Cooperativity for iron binding to the N- and C-lobes, whether positive or negative, has been detected by iron stability analyses for the two sites of human serum transferrin (39) and those of ovotransferrin (35, 40). Also, Ward *et al.* (41) show that the iron binding stability of lactoferrin is due primarily to the C-lobe, which functions cooperatively to stabilize iron binding in the N-lobe. Lactoferrin mutants that selectively lack the iron binding function in either lobe were used in this study. The cooperative interactions upon iron release at acidic pH are modulated by binding to the receptor (42–44). The cooperativity between the two iron sites, therefore, plays an important functional role in the uptake and release of iron.

According to titration calorimetric analyses of ovotransferrin (45), the cooperativity operates in such a way that the thermodynamic iron binding constants increase for the N-lobe site but decrease for the C-lobe site. The two bound iron atoms are separated by 43.7 Å in diferric ovotransferrin (6), leading to the question of how the two binding sites communicate with one another. It has been proposed that carbonate anion and Fe³⁺ bind initially to domain 2 of each open lobe (14–16), followed by domain closure, which is triggered by the Asp ligand binding to iron (46). The carboxylate ligands, Asp⁶⁰ in the N-lobe and Asp³⁹⁵ in the C-lobe, both belong to domain 1 of their respective lobes. Structural comparison of the current apo form and previous holo form (6) of hen ovotransferrin reveals that the dimer,

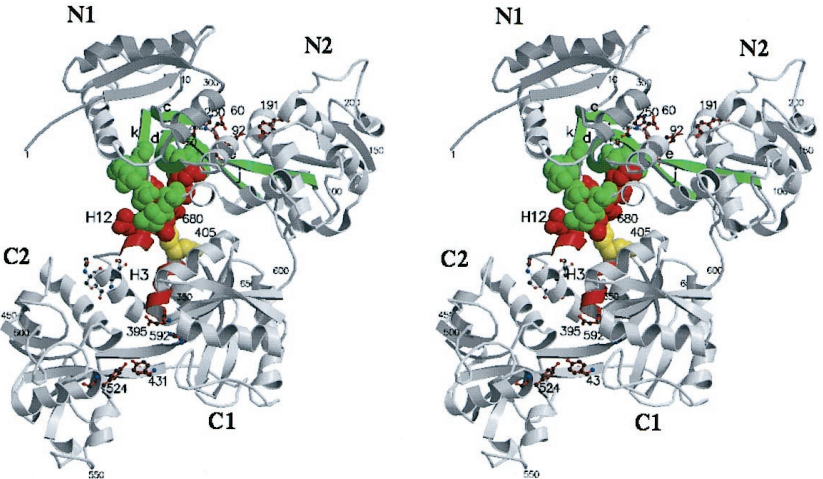
TABLE III

Sequence alignment of vertebrate transferrins in the hinge regions

The residue numbers are according to the ovotransferrin sequence.

N-Lobe	88	91	94	244	247	250								
Hen ovotransferrin	S	T	T	S	Y	Y	A	W	A	R	V	A	A	H
Human lactoferrin	P	R	T	H	Y	Y	A	L	A	R	V	P	S	H
Bovine lactoferrin	P	Q	T	H	Y	Y	A	L	A	Q	V	P	S	H
Human transferrin	P	Q	T	F	Y	Y	A	L	A	Q	V	P	S	H
Rabbit transferrin	P	K	T	F	Y	Y	A	L	A	R	V	P	S	H
Frog transferrin	T	D	T	C	Y	Y	A	L	A	K	V	P	A	H
C-Lobe	427	430	433	586	589	592								
Hen ovotransferrin	R	P	A	S	Y	F	A	L	A	E	V	P	T	H
Human lactoferrin	P	V	E	G	Y	L	A	L	A	M	A	P	N	H
Bovine lactoferrin	P	T	E	G	Y	L	A	L	A	V	A	P	N	H
Human transferrin	P	E	A	G	Y	F	A	L	A	R	A	P	N	H
Rabbit transferrin	P	E	E	G	Y	L	S	L	A	K	A	P	N	H
Frog transferrin	A	K	G	V	Y	Y	A	L	A	E	V	P	A	H

FIG. 6. Stereo views of the interlobe interaction found in apo-ovotransferrin. Filled spheres show residues that contact each other between helix 12 (red) and strands e and k (green). The disulfide bridge Cys⁴⁰⁵-Cys⁶⁸⁰ is also drawn as yellow spheres. The iron ligand residues in the holo ovotransferrin (Asp⁶⁰, Tyr⁹², Tyr¹⁹¹, His²⁵⁰ in N-lobe and Asp³⁹⁵, Tyr⁴³¹, Tyr⁵²⁴, and His⁵⁹² in C-lobe), and attached carbohydrate residues are drawn as ball-and-sticks. The ribbon representations of helices 3 and 12 are colored red, and those of strands c, d, e, and k are colored green. The figure was produced with MOLSCRIPT (49) and RASTER3D (50).



consisting of domains N1 and C1, behaves essentially as a rigid body upon uptake or release of iron (Fig. 1). The two Asp ligands are located at different sites on that rigid body. Asp³⁹⁵ in the C-lobe, however, is linked to certain ligating and hinge residues in the N-lobe. Helix 12 (residues 674–686), in the C-lobe, interacts with the hinge residues Thr⁸⁹, Thr⁹⁰, and Ser⁹¹ in the N-lobe (6); an iron ligand, Tyr⁹², is the next residue after Ser⁹¹, and both are involved in the β -strand e hinge (residues 91–100). Helix 12 in the C-lobe also interacts with strand k (residues 305–310) in the N-lobe. Strand k is part of a β -sheet that includes strands k, d (residues 74–82), j (residues 244–255), and c (residues 54–59). Strand j is the second inter-domain hinge in the N-lobe and includes the iron ligand, His²⁵⁰. Another iron ligand, Asp⁶⁰, is one residue removed from the C terminus of strand c (6). Asp³⁹⁵ is located at the N terminus of helix 3 (residues 395–405), whereas Cys⁴⁰⁵ is at the C terminus. The disulfide bridge Cys⁴⁰⁵–Cys⁶⁸⁰ makes a covalent cross-link between helix 3 and helix 12 in the C-lobe. Taken together, therefore, Asp³⁹⁵ forms a structural network through the disulfide bridge with several hinge residues and the iron ligands, Asp⁶⁰, Tyr⁹², and His²⁵⁰, in the N-lobe. Fig. 6 illustrates the interlobe interactions found in the apo-ovotransferrin. This interlobe network is retained in both the holo and current apo structures. Upon iron binding, the distances between Aps³⁹⁵ and the hinge residues are reduced: the C- α . . . C- α distances are reduced from 33.7 to 30.4 Å for Asp³⁹⁵ to Thr⁸⁹, from 32.4 to 30.7 Å for Asp³⁹⁵ to Thr⁹⁰, and from 32.7 to 31.9 Å for Asp³⁹⁵ to Ser⁹¹. These movements would require a minor structural adjustment in the N1/C1 dimer, as might be reflected in the small rotation (7°) of the C1-domain relative to the N1-domain upon uptake or release of iron (Fig. 1). The quantitative calorimetric analysis reveals that binding site cooperativity largely arises from differential interlobe association constants for the iron-loaded and iron-free proteins (44). It is likely that the minor structural alteration in the N1/C1 dimer upon iron binding is related to these differential interlobe interactions.

In addition to the interactions described above, there is a possible involvement of the covalent carbohydrate component in stabilizing the open conformation in the current structure. Ovotransferrin is glycosylated at Asn⁴⁷³ in the C-lobe (27); the carbohydrate moiety is not seen in the holo structure, probably because of disorder (6), but the two core GlcNAc residues can be clearly detected in the current apo structure (Fig. 2b). In the apo structure, the second carbohydrate residue appears to form hydrogen bonds with two amino acid residues: GlcNAc⁶⁸⁹O6 with Thr⁶⁶³O and Ser⁶⁶⁷O γ . These hydrogen bonds are inter-domain and are located in the small interface of the C-lobe, suggesting an involvement in stabilizing the open conformation. The carbohydrate in human lactoferrin was enzymatically removed before crystallization (14, 47), and this might be related, at least in part, to the observation of the closed C-lobe in that structure. As an alternative, it has been postulated that an equilibrium exists between the open and closed forms of an apo transferrin in solution and that the observed structure is selected by crystal packing (14, 21). Such an equilibrium might be inclined to favor the open conformation of the apo form of an iron-transporting transferrin, since extensive transferrin receptor interaction studies have been consistent, as discussed above, with a mechanism in which the closed holo form is selectively recognized by the receptor. The possibility, however, that crystal packing affects the extent of domain opening (14, 21) cannot be ruled out. This possibility might be related to the difference in the extent of domain opening in the hen and duck apo-ovotransferrin structures. Rawas *et al.* (48) recently report the structure of duck apo-ovotransferrin at 4.0 Å resolution.

Although their resolution is rather low for a discussion of detailed molecular structure, the extent of domain opening is reported to be 51.6° for the N-lobe and 49.9° for the C-lobe. The latter value is some 15° larger than the 35° observed in the C-lobe of the current hen apo-ovotransferrin structure. It remains to be determined whether this difference in C-lobe opening is a consequence of protein sequence, crystallization conditions, or crystal packing.

Acknowledgments—Computational time was provided by the Super Computer Laboratory, Institute for Chemical Research, Kyoto University.

REFERENCES

1. Aisen, P., and Listowsky, I. (1980) *Annu. Rev. Biochem.* **49**, 357–393
2. Arnold, R. R., Cole, M. F., and McGhee, J. R. (1977) *Science* **197**, 263–265
3. He, J., and Furmanski, P. (1995) *Nature* **373**, 721–724
4. Bailey, S., Evans, R. W., Garratt, R. C., Gorinsky, B., Hasnain, S., Horsburgh, C., Jhoti, H., Lindley, P. F., Mydin, A., Sarra, R., and Watson, J. L. (1988) *Biochemistry* **27**, 5804–5812
5. Anderson, B. F., Baker, H. M., Norris, G. E., Rice, D. W., and Baker, E. N. (1989) *J. Mol. Biol.* **209**, 711–734
6. Kurokawa, H., Mikami, B., and Hirose, M. (1995) *J. Mol. Biol.* **254**, 196–207
7. Rawas, A., Muirhead, H., and Williams, J. (1996) *Acta Crystallogr. Sec. D* **52**, 631–640
8. Sarra, R., Garratt, R., Gorinsky, B., Jhoti, H., and Lindley, P. (1990) *Acta Crystallogr. Sec. B* **46**, 763–771
9. Dewan, J. C., Mikami, B., Hirose, M., and Sacchettini, J. C. (1993) *Biochemistry* **32**, 11963–11968
10. Day, C. L., Anderson, B. F., Tweedie, J. W., and Baker, E. N. (1993) *J. Mol. Biol.* **232**, 1084–1100
11. MacGillivray, R. T. A., Moore, S. A., Chen, J., Anderson, B. F., Baker, H., Luo, Y., Bewley, M., Smith, C. A., Murphy, M. E. P., Wang, Y., Mason, A. B., Woodworth, R. C., Brayer, G. D., and Baker, E. N. (1998) *Biochemistry* **37**, 7919–7928
12. Bruns, C. M., Nowalk, A. J., Arvai, A. S., McTigue, M. A., Vaughan, K. G., Mietzner, T. A., and McRee, D. E. (1997) *Nat. Struct. Biol.* **4**, 919–924
13. Baker, E. N., Rumball, S. V., and Anderson, B. F. (1987) *Trends Biochem. Sci.* **12**, 350–353
14. Anderson, B. F., Baker, H. M., Norris, G. E., Rumball, S. V., and Baker, E. N. (1990) *Nature* **344**, 784–787
15. Lindley, P. F., Bajaj, M., Evans, R. W., Garratt, R. C., Hasnain, S. S., Jhoti, H., Kuser, P., Neu, M., Patel, K., Sarra, R., Strange, R., and Walton, A. (1993) *Acta Crystallogr. Sec. D* **49**, 292–304
16. Grossmann, J. G., Neu, M., Pantos, E., Schwab, F. J., Evans, R. W., Townes-Andrews, E., Lindley, P. F., Appel, H., Thies, W.-G., and Hasnain, S. S. (1992) *J. Mol. Biol.* **225**, 811–819
17. Young, S. P., Bomford, A., and Williams, R. (1984) *Biochem. J.* **219**, 505–510
18. Mason, A. B., Brown, S. A., and Church, W. R. (1987) *J. Biol. Chem.* **262**, 9011–9015
19. Mason, A. B., Woodworth, R. C., Oliver, R. W. A., Green, B. N., Lin, L.-N., Brandts, J. F., Savage, K. J., Tam, B. M., and MacGillivray, R. T. A. (1996) *Biochem. J.* **319**, 361–368
20. Mason, A. B., Tam, B. M., Woodworth, R. C., Oliver, R. W. A., Green, B. N., Lin, L.-N., Brandts, J. F., Savage, K. J., Lineback, J. A., and MacGillivray, R. T. A. (1997) *Biochem. J.* **326**, 77–85
21. Jameson, G. B., Anderson, B. F., Norris, G. E., Thomas, D. H., and Baker, E. N. (1998) *Acta Crystallogr. Sec. D* **54**, 1319–1335
22. Moore, S. A., Anderson, B. F., Groom, C. R., Haridas, M., and Baker, E. N. (1997) *J. Mol. Biol.* **274**, 222–236
23. Thibodeau, S. N., Lee, D. C., and Palmiter, R. D. (1978) *J. Biol. Chem.* **253**, 3771–3774
24. Brown-Mason, A., and Woodworth, R. C. (1984) *J. Biol. Chem.* **259**, 1866–1873
25. Williams, J., Elleman, T. C., Kingston, I. B., Wilkins, A. G., and Kuhn, K. A. (1982) *Eur. J. Biochem.* **122**, 297–303
26. Williams, J. (1982) *Trends Biochem. Sci.* **7**, 394–400
27. Crichton, R. R., and Charleaux-Wauters, M. (1987) *Eur. J. Biochem.* **164**, 485–506
28. Hirose, M., Oe, H., and Doi, E. (1986) *Agric. Biol. Chem.* **50**, 59–64
29. Dewan, J. C., and Tilton, R. F. (1987) *J. Appl. Crystallogr.* **20**, 130–132
30. Brünger, A. T. (1996) *X-PLOR*, Version 3.85, Yale University, New Haven, CT
31. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Crystallogr.* **26**, 283–291
32. Gerstein, M., Anderson, B. F., Norris, G. E., Baker, E. N., Lesk, A. M., and Chothia, C. (1993) *J. Mol. Biol.* **234**, 357–372
33. Kurokawa, H., Mikami, B., and Hirose, M. (1994) *J. Biol. Chem.* **269**, 6671–6676
34. Makey, D. G., and Seal, U. S. (1976) *Biochim. Biophys. Acta* **453**, 250–256
35. Williams, J., Evans, R. W., and Moreton, K. (1978) *Biochem. J.* **173**, 535–542
36. Mecklenburg, S. L., Donohoe, R. J., Olah, G. A. (1977) *J. Mol. Biol.* **270**, 739–750
37. Jeffrey, P. D., Bewley, M. C., MacGillivray, R. T. A., Mason, A. B., Woodworth, R. C., and Baker, E. N. (1998) *Biochemistry* **37**, 13978–13986
38. Williams, J., Moreton, K., and Goodearl, A. D. (1985) *Biochem. J.* **228**, 661–665
39. Aisen, P., Leibman, A., and Zweier, J. (1978) *J. Biol. Chem.* **253**, 1930–1937
40. Chasteen, N. D., and Williams, J. (1981) *Biochem. J.* **193**, 717–727
41. Ward, P. P., Zhou, X., and Conneely, O. M. (1996) *J. Biol. Chem.* **271**, 12790–12794
42. Bali, P. K., Zak, O., and Aisen, P. (1991) *Biochemistry* **30**, 324–328

43. Bali, P. K., and Aisen, P. (1992) *Biochemistry* **30**, 9947–9952
44. Bali, P. K., and Aisen, P. (1992) *Biochemistry* **31**, 3963–3967
45. Lin, L.-N., Mason, A. B., Woodworth, R. C., and Brandts, J. F. (1991) *Biochemistry* **30**, 11660–11669
46. Grossmann, J. G., Mason, A. B., Woodworth, R. C., Neu, M., Lindley, P. F., and Hasnain, S. S. (1993) *J. Mol. Biol.* **231**, 554–558
47. Norris, G. E., Baker, H. M., and Baker, E. N. (1989) *J. Mol. Biol.* **209**, 329–331
48. Rawas, A., Muirhead, H., and Williams, J. (1997) *Acta Crystallogr. Sec. D* **53**, 464–468
49. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950
50. Merrit, E. A., and Bacon, D. J. (1997) *Methods Enzymol.* **277**, 505–524
51. Hubbard, S. J., and Thornton, J. M. (1993) *NACCESS Computer Program*, Department of Biochemistry and Molecular Biology, University College, London