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Biochemical and Biophysical Research Communications 295 (2002) 125–128

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Purification and preliminary X-ray studies on hen serotransferrin in apo- and holo-forms

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Received 9 May 2002

Abstract

Serum transferrins are monomeric glycoproteins with a molecular mass of around 80 kDa, that transport iron to cells via receptor-mediated endocytosis. Although both serum transferrins (STfs) and ovotransferrins (OTfs) are derived from the same gene in aves, the ovotransferrins do not transport iron in vivo. Crystal structures of OTf have been solved, in contrast no three-dimensional structure of avian STf have been determined as yet. Here we report the purification, crystallization, and preliminary crystallographic studies of the hen STf both in apo- (iron free) and holo- (iron loaded) forms. The hen STf has been purified to homogeneity by hydrophobic interaction chromatography. Both the apo- and holo-forms were crystallized by hanging drop vapor diffusion method at 277 K. The apo-crystals diffract to a resolution of 3.0 Å and belong to the space group $P4_32_12$ with unit cell parameters $a = b = 90.5$ and $c = 177.9$ Å. The holo-crystals diffract to a resolution of 2.8 Å and belong to space group $P2_1$ with $a = 72.8$, $b = 59.6$, $c = 88.2$ Å, and $\beta = 95.7°$. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Avian serum transferrin; Purification; Crystallization; X-ray crystallographic studies

Serum transferrin is a bilobal ~80 kDa protein that transports iron from sites of storage to regions of iron metabolism. It belongs to a family of monomeric iron-binding glycoproteins, the other principal members of which are ovotransferrin (OTf) found in avian egg whites and lactoferrin (LTf) found in numerous extracellular fluids and in the specific granules of polymorphonuclear lymphocytes. They have a characteristic ability to bind, tightly but reversibly, two Fe^{3+} ions, together with two CO^{3-} anions [1,2] and they also have high sequence homology amongst themselves. The transferrins sequester and solubilize iron, thereby controlling "available" iron levels with attendant bacteriostatic and conservation effects. Although originally thought to be restricted to vertebrates, transferrin-like proteins have also been identified in several invertebrate species where they occur in the hemolymph [3]. Serum transferrin is responsible for the transport of iron from sites of absorption and degradation to those of storage and utilization, primarily by the hemopoietic system. The iron-loaded transferrin molecule is bound by the dimeric transferrin receptor and is internalized via receptor-mediated endocytosis. The bound-iron is released in a pH dependent manner, as the pH of the vesicle is lowered from 7.4 to 5.5 in presence of the transferrin bound to the receptor molecule. The isoelectric points of serum transferrin and ovotransferrin are on the acidic side of neutrality, while that of lactoferrin is strongly basic.

The structures of diferric form of ovotransferrin have been determined at 2.35 Å for duck [4] and at 2.4 Å for hen [5]. Crystal structures of the apo–ovotransferrin have been solved at 3.0 Å for hen [6] and at 4.0 Å for duck [7]. High resolution crystal structures are available for N-lobe of hen apo–ovotransferrin at 2.1 and 1.9 Å, respectively [8,9] and N2 domain of iron-bound duck ovotransferrin at 2.3 Å [10,11].

Relatively few structural data are available for the vertebrate serum transferrins, [12–18], as a result of which, the differences in iron binding and release between species and classes of serum transferrins are still poorly

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PII: S0006-291X(02)00626-5

understood. In addition to this, no structure of the full or part of avian serum transferrin in either apo- or holo-form has been reported yet. Despite the fact that both ovo- and serum transferrins have same amino acid sequence and differ only in their attached carbohydrate moiety [19], an in vivo iron-transport function, characteristic of serum transferrin has not been proved for ovotransferrin [20]. In vitro studies indicate another difference between serum transferrin and ovotransferrin—while serum transferrin can utilize other mammalian receptors, ovotransferrin cannot do so [21].

Hence to establish a structural basis for the physiological and functional differences between ovotransferrin and serum transferrin, comparison of detailed crystallographic structures of these transferrins is crucial. Our aim is to study the three-dimensional structure of both apo- and holo-forms of the full hen serotransferrin molecule and its related iron-transport function.

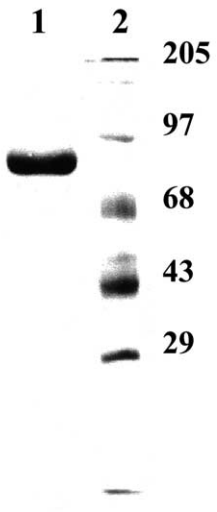

Fig. 1. SDS–PAGE of hen serum transferrin. Lane 1—transferrin eluted from the phenyl-Sepharose CL-4B column and lane 2—molecular weight markers (205, 97.4, 68, 43, and 29 kDa).

Materials and methods

Isolation and purification. Serum was extracted from the blood of ovulating White Leghorn birds and kept frozen at −20 °C. Serum transferrin in its apo-form was isolated and purified from the blood serum on a hydrophobic interaction chromatography column (phenyl-Sepharose CL 4B, Sigma) according to the previously reported method [22] with some modifications. For the purification procedure, a 3-ml aliquot was thawed and mixed with 12 ml of buffer A (1.2 M ammonium sulphate, 0.5 M sodium citrate, pH 6.0). The diluted serum was filtered through 0.8 μ filter units (Sartorius, Germany) and loaded onto a phenyl-Sepharose column (1.0 cm diameter, 18 ml bed volume) that had been equilibrated with buffer A. Chromatography was performed at a flow rate of 18 ml/h at room temperature. The protein was eluted by a two-step gradient. After loading the sample, the column was washed with 6 bed volume of buffer A. Following which, 3 bed volumes of buffer B (0.6 M ammonium sulphate, 0.25 M sodium citrate, pH 6.0) was allowed to pass through the column. Transferrin in the sample was eluted with buffer C (0.3 M ammonium sulphate, 0.125 M sodium citrate, pH 6.0). Protein absorption at 280 nm (A_{280}) was measured for each fraction and 40 μl of each was analyzed by SDS–PAGE to check the purity of the protein (Fig. 1). The fractions containing pure transferrin were pooled, dialyzed exhaustively against phosphate buffered saline (PBS), and then concentrated using Ultrafree-MC filter units (Sigma). To confirm the absence of iron, the absorbance of the purified protein was monitored at 465 nm. The protein was used for crystallization experiments only if it showed zero absorbance at 465 nm. To obtain the holo-form, the purified protein was first dialyzed exhaustively against 100 mM citrate–bicarbonate buffer, pH 8.0. The protein was then concentrated and saturated with iron by incubating it with $FeCl_3$, in a protein:$FeCl_3$ ratio of 1:2. Excess iron was removed by dialyzing the iron-saturated protein against water. This protein was then concentrated to ~40 mg/ml using Ultrafree-MC filter units (Sigma) for crystallization experiments.

Crystallization. All crystallization experiments were performed by the hanging drop vapor diffusion method in 24-well tissue culture plates. Initial attempts to crystallize the apo- and holo-forms of the protein with commercially available sparse-matrix screening kits, Crystal Screen I and II of Hampton Research [23], were unsuccessful. The apo-transferrin crystals were obtained under two conditions; 18–23% PEG 6000 in 0.02 M acetate buffer, pH 6.0, and 23–27% PEG 6000 in 50 mM disodium piparazine-*N*-*N*-bis-(2-ethanesulphonate) buffer, pH 6.0. Similarly, the holo-crystals were also obtained under two conditions, 17–20% PEG 6000 in 0.02 M acetate buffer, pH 6.0, and 18–21% PEG 4000 in 20 mM $NaHCO_3$. The protein concentrations used for crystallization of apo- and holo-forms were ~80 mg/ml (in PBS) and ~40 mg/ml (in 40 mM Na-cacodylate buffer, pH 5.9), respectively. The hanging drops were set by mixing equal volumes of protein and precipitant on siliconized coverslips and equilibrating

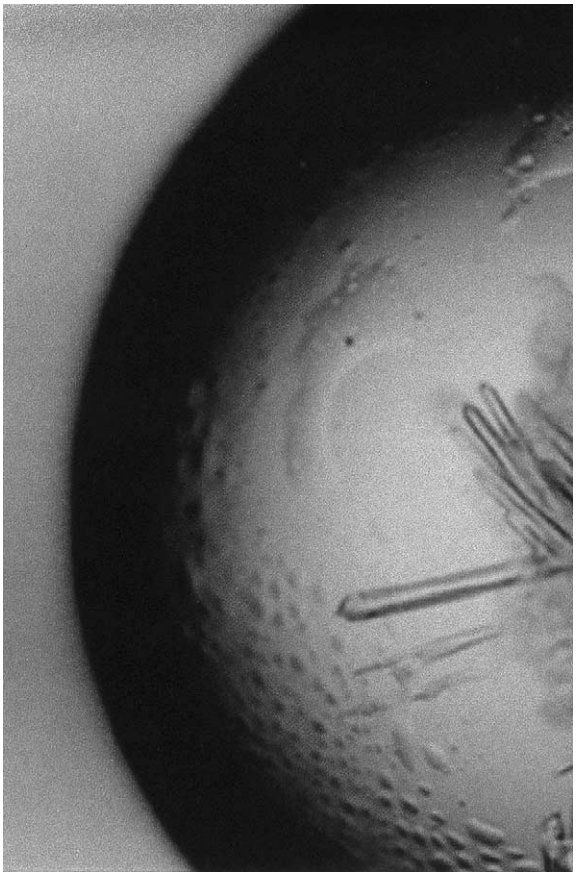

Fig. 2. Crystals of hen serum transferrin (apo-form).

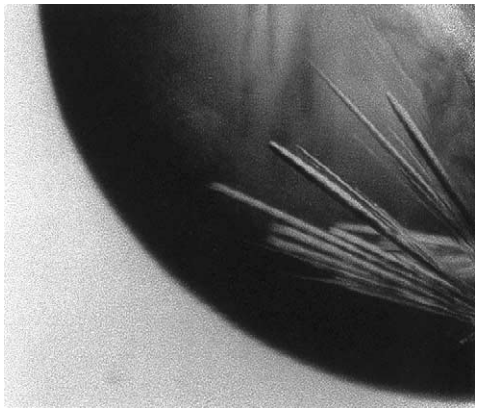

Fig. 3. Crystals of hen serum transferrin (holo-form).

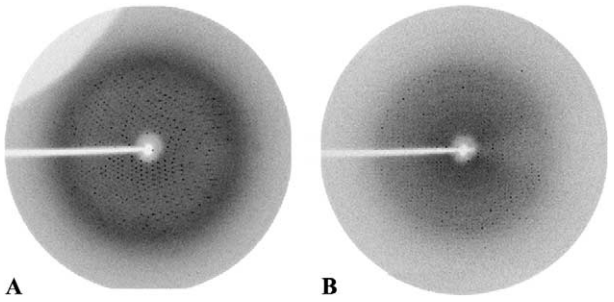

Fig. 4. Diffraction images of (A) apo- and (B) holo-form of hen serum transferrin. The resolution of the outer edge of the images: (A) 3.03 Å and (B) 2.8 Å, respectively.

them against 700 μl of precipitant solution at 277 K. The rod shaped colorless apo-crystals and thin needle shaped deep red colored holo-crystals grew within two weeks (Figs. 2 and 3).

Diffraction data collection and processing. X-ray diffraction data were collected on a MAR-300 image plate system using CuK_α radiation from a Rigaku RU 200B X-ray generator equipped with Osmic MaxFlux confocal optics, operated at 50 kV and 90 mA. Prior to data collection, individual crystals were scooped up from the drop with a rayon cryoloop (Hampton Research) and cryoprotected by equilibrating them for a few seconds in 25–30% MPD solution prepared in mother liquor. The crystal was then directly flash-frozen and maintained at 100 K under a stream of liquid nitrogen during the entire data collection using an Oxford Cryosystem cooling unit. A total of 73 and 152 frames were recorded with an oscillation angle of 1.0° with crystal to detector distance of 200 and 180 mm for apo- and holo-transferrin crystals, respectively. The exposure time for each image was 8 min. Although the apo-transferrin crystal diffracted upto 3.0 Å, the R_{merge} of the last resolution shell was high and so the data were processed upto 3.45 Å. For the same reason, although the holo-transferrin crystal diffracted upto 2.8 Å, the data were processed up to 3.25 Å (Fig. 4).

The X-ray diffraction data were indexed, integrated, and subsequently scaled using DENZO and SCALEPACK from the HKL program package [24].

Results

The apo-crystals were tetragonal, with unit cell parameters $a = b = 90.4$ Å, $c = 177.8$ Å, and the holo-crystals were monoclinic, with unit cell parameters $a = 72.8$, $b = 59.6$, $c = 88.2$ Å, and $\beta = 95.7°$. Evaluation of the crystal packing parameter [25] indicated that the lattice of both the forms can accommodate one molecule per asymmetric unit (V_M (Å^3/Da) = 2.29 and 2.4 for apo- and holo-forms) with solvent content of 46.2% and 48.8%.

A summary of crystal and experimental data is presented in Table 1.

The structure solution for both the forms were carried out by molecular replacement using the atomic coordinates of hen apo–ovotransferrin (PDB code: 1AIV) for the apo-form and hen diferric transferrin (PDB code: 1OVT) for the holo-form, as a starting model, using the program AMoRe [26] implemented in the CCP4 Suite (Collaborative Computational Project, No. 4, 1994). Clear peaks were found with correlation coefficients of 61.3% and 60.3% and *R*-factors of 37.5% and 37.7% for apo- and holo-forms, respectively, using reflections in the range of 10.0–4.0 Å. Rigid-body refinement (10.0–4.0 Å) resulted in correlation coefficients of 60.4% and

Table 1
Data processing parameters

	Apo-form	Holo-form
X-ray source and wavelength	CuK_α, 1.54 Å	CuK_α, 1.54 Å
Space group	$P4_32_12$	$P2_1$
Unit cell parameters (Å, °)	$a = b = 90.4$, $c = 177.8$	$a = 72.8$, $b = 59.6$, $c = 88.2$, $\beta = 95.7$
Oscillation range (°)	1.0	1.0
Mathews coefficient, V_M (Å^3/Da)	2.29	2.4
Solvent content (%)	46.2	48.8
Resolution (Å)	3.45	3.25
No. of observations	145,598	125,735
No. of unique reflections	10,337	13,951
Temperature (K)	100	100
Average $I/\sigma(I)$	11.6 (3.1)	6.6 (4.0)
Completeness (%)	98.6 (99.8)	97.0 (96.3)
R_{merge}	0.101 (0.484)	0.165 (0.314)

$R_{merge} = \sum((I - \langle I\rangle)^2)/\sum(I^2)$, where I is the integrated intensity of a given reflection and $\langle I\rangle$ is average intensity of the reflection.
Values in the parentheses refer to the highest resolution shell (3.55–3.45 Å) and (3.33–3.25 Å) for apo- and holo-forms, respectively.

68.6% and *R*-factors of 36.9% and 33.9% for apo- and holo-forms. The molecular replacement models were subjected to further refinement as six rigid bodies (1–95, 96–245, 246–335, 336–435, 436–585, and 586–686 with ovotransferrin numbering) delineating the domain organization of serum transferrin and using the resolution range of 20.0–3.45 Å for the apo-protein and 20–3.25 Å for the holo-protein. This cycle of refinement yielded a *R*-factor of 40.8% ($R_{free} = 40.5\%$) for the apo-form and 29.9% ($R_{free} = 29.8\%$) for the holo-form. Model building of the protein in both forms by manual fitting to the electron density map using program O [27] is underway.

Acknowledgments

We acknowledge the help of the State Poultry Farm, Government of West Bengal, India, for providing us with the blood samples and of Bikram Nath in the protein purification process.

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