Acta Cryst. (1996). D**52**, 1196–1198

Purification, crystallization and preliminary crystallographic analysis of mare lactoferrin

A. K. SHARMA,[a] S. KARTHIKEYAN,[a] P. KAUR,[a] T. P. SINGH[a] AND M. P. YADAV,[b] *at* [a]*Department of Biophysics, All India Institute of Medical Sciences, New Delhi-110 029 India, and* [b]*National Research Centre on Enquines, Hisar-125 001, India. E-mail: tps@tnedinst.ernet.in*

(*Received 15 March 1996; accepted 14 June 1996*)

Abstract

Lactoferrin is an iron-binding glycoprotein with a molecular weight of 80 kDa. The protein has two iron binding sites. It has two structural lobes, each housing one Fe^{3+} and the synergistic CO_3^{2-} ion. The protein was isolated from the colostrum/milk of mares maintained at National Research Centre on Equines, Hisar, India. The purified samples of the protein were crystallized using a microdialysis method. The protein was dialysed against low ionic strength buffer solution. Several crystal forms were obtained, out of which three were characterized which have cell dimensions as follows. Form I $a = 79.8$, $b = 103.5$, $c = 112.0$ Å, space group $P2_12_12_1$, with one protein molecule per asymmetric unit and a solvent content of 57%. Form II $a = 84.9$, $b = 99.7$, $c = 103.5$ Å, space group $P2_12_12_1$ with one molecule per asymmetric unit and a solvent content of 55%. Form III $a = 151.0$, $b = 151.0$, $c = 240.6$ Å, space group $P4_12_12$ with three molecules in the asymmetric unit and a solvent content of 57%. The intensity data up to 3.8 Å resolution for form I, 2.9 Å resolution data for form II and 6 Å resolution data for form III have been collected. Further calculations are in progress.

1. Introduction

Lactoferrin is an iron-binding glycoprotein present in most biological fluids of mammals including milk, saliva, tears and mucous secretions (Mason, Hermans & Dive, 1966) and released from activated neutrophils in the inflammatory response (Lash, Coates, Lafuze, Bachner & Boxer, 1983). The known biological functions of lactoferrin are host defense against bacterial infection (Bullen, Rogers & Leigh, 1972) and the protection of cells from free-radical damage (Baldwin, Jenny & Aisen, 1984), both of which appear to depend on the ability to bind iron tightly. In some cases, lactoferrin has been shown to have bactericidal as well as bacteriostatic properties (Arnold, Russel, Champion, Brewer & Ganthier, 1982) and it has been shown to contribute to several biological functions of white blood cells (Bennet, Merrit & Gabor, 1986). Its role in these processes is much less clear but may involve the interaction of lactoferrin with cell-surface receptors (Campbell, 1982). The function of the carbohydrate on the protein is as yet obscure, although recent experiments suggest that it may be involved in receptor binding (Davidson & Lonnerdal, 1988). A very recent study shows that the lactoferrin binds to DNA with a distinct sequence specificity and the interaction between lactoferrin and these sequences leads to transcriptional activation intracellularly (He & Furmanski, 1995). The primary sequences of human (Metz-Boutigue, Jollès, Mazurier, Schoentgen, Legrand, Spike, Montreuil & Jollès, 1984) and bovine (Pierce, Colavizza, Benaissa, Maes, Tartar, Montreiul & Spike, 1991) lactoferrins have been determined. The N-terminal sequence data on mare (Jollès, Donda, Amiguet & Jollès, 1984) and buffalo (Sharma, Singh & Bhatia, 1996) lactoferrins have become available recently. The comparison of the N-terminal sequences of lactoferrins from these four species indicates that the cluster of arginine residues found in human lactoferrin is no longer present in other species. The DNA binding of lactoferrin is attributed to the N-terminal region (He & Furmanski, 1995). The structure of human lactoferrin has been determined (Anderson, Baker, Norris, Rice & Baker, 1989) while the preliminary crystallographic data of bovine (Norris, Anderson, Baker, Baker, Gartner, Ward & Rumball, 1986) and buffalo (Raman, Bhatia, Singh, Srinivasan, Betzel & Malik, 1991) lactoferrins have been reported. In order to understand a wide range of specific functions of lactoferrins and the striking differences in primary sequences at the functional sites of lactoferrins from various species and closer similarities in the sequences of some of them with transferrins (Jollès, Donda, Amiguet & Jollès, 1984; Baker & Lindley, 1992), it is necessary to determine the three-dimensional structures of lactoferrins from different species. Here, we report the results of a preliminary crystallographic analysis of mare lactoferrin.

2. Experimental

2.1. *Purification*

The mare milk was obtained from National Research Centre on Equines, Hisar, India. The purification was carried out using a locally modified procedure of Law & Reiter (Law & Reiter, 1977). Diluted colostrum/milk was defatted by skimming. Skimmed milk was diluted twice with 0.05 *M* Tris–HCl, pH 8.0. CM-Sephadex C-50 was added to it

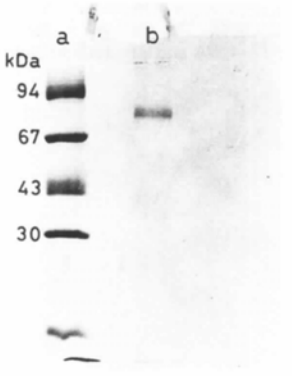
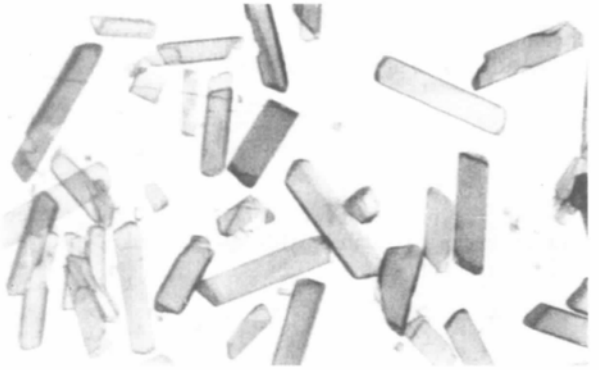


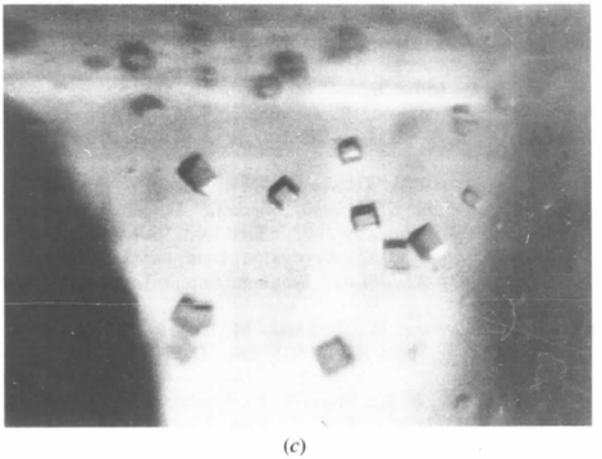
Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of mare lactoferrin with molecular-weight markers. (*a*) Protein molecular-mass standards: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa). (*b*) Purified mare lactoferrin (~ 80 kDa).

($7\,g\,l^{-1}$) and stirred slowly by mechanical stirrer for 1 h. The gel was allowed to settle and then milk was decanted. The gel was washed with excess of 0.05 *M* Tris–HCl, pH 8.0 and then packed in a column (25 × 2.5 cm) and washed with the same buffer containing 0.1 *M* NaCl which facilitated the removal of impurities. The lactoferrin was then eluted with the same buffer containing 0.25 *M* NaCl. The protein solution was dialysed against an excess of triple-distilled water. The protein was again passed through a CM-Sephadex C-50 column (10 × 2.5 cm) pre-equilibrated with 0.05 *M* 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 by an Amicon ultrafiltration cell. The concentrated protein was passed through a Sephadex G-100 column (100 × 2 cm) using 0.05 *M* Tris–HCl buffer, pH 8.0. The purity of lactoferrin was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Lammeli, 1970). The single band in Fig. 1, lane *b* corresponds to the purified lactoferrin.



(*a*)

(*b*)



(*c*)

Fig. 2. The crystals of mare lactoferrin. (*a*) form I grown by microdialysis from 10% ethanol at pH 8.0; (*b*) form II grown by microdialysis from 10% ethanol at pH 8.5; (*c*) form III grown by microdialysis from 9% PEG 4000 at pH 8.0.

Table 1. *Crystallographic data*

	Form I	Form II	Form III
Crystallization conditions	10% ethanol pH 8.0	10% ethanol pH 8.5	9% PEG 4000 pH 8.0
System	Orthorhombic	Orthorhombic	Tetragonal
Space group	$P2_12_12_1$	$P2_12_12_1$	$P4_12_12$
a (Å)	79.8	84.9	151.0
b (Å)	103.3	99.7	151.0
c (Å)	112.0	103.5	240.6
V (Å^3)	9.3×10^5	8.8×10^5	5.5×10^6
V_m (Å^3 Da^{-1})	2.88	2.72	4.27
Total no. of reflections collected	90106	79652	—
Total no. of unique observed reflections	7974	13799	—
R_{sym}*	11.8	10.2	—
Solvent content (%)	57	55	71
Z	4	4	16
Resolution (Å)	4.0	2.9	6.0
No. of crystals used	4	3	—
Completeness (%)	95	92	—

* $R_{sym} = \sum_{hkl} \sum_i^N |\bar{I} - I_i| / \sum N\bar{I} \times 100$.

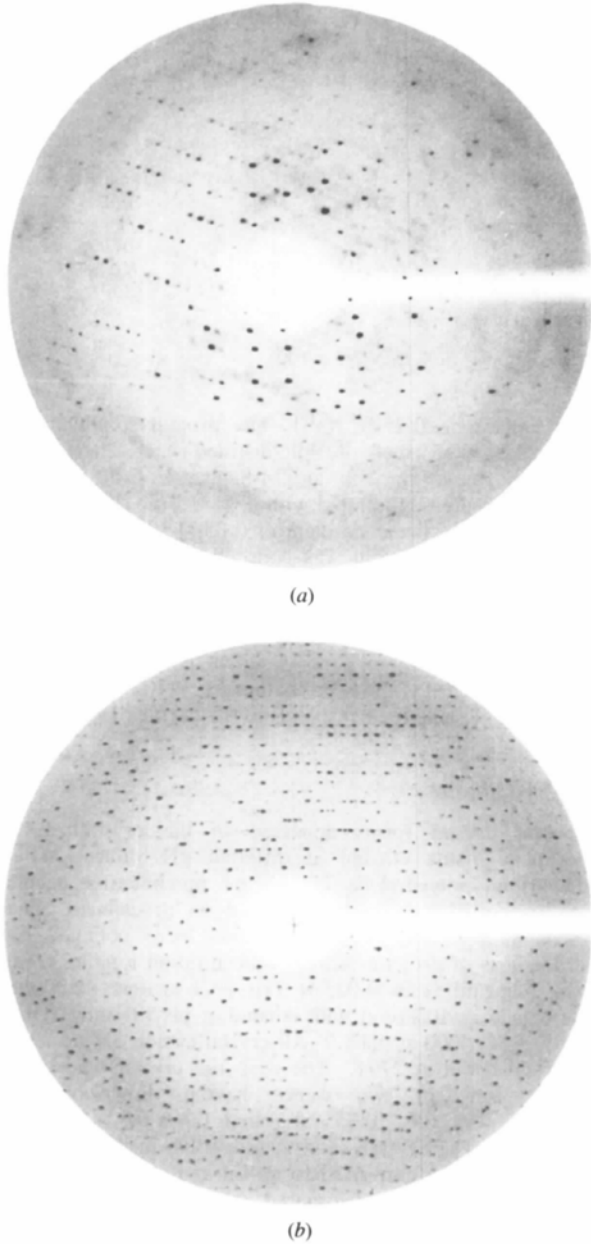
2.2. *Crystallization*

Several crystal forms appeared in the crystallization experiments using ethanol at different pH values, MPD and various polyethylene glycols as precipitating agents using vapour-diffusion and microdialysis procedures. The crystals suitable for X-ray diffraction were obtained by microdialysis of protein (protein concentration ranging from 20 to $30\,mg\,ml^{-1}$) in 0.025 *M* Tris–HCl against the same buffer made as 10%(*v*/*v*) with ethanol at pH 8.0 and pH 8.5 and 9% PEG 4000 at pH 8.0. All crystallization experiments were carried out at 279 K. The deep red crystals shown in Fig. 2 grew in two weeks having dimensions of 0.5 × 0.3 × 0.2, 0.6 × 0.35 × 0.3 and 0.4 × 0.3 × 0.18 mm respectively. The crystals tend to redissolve in their mother liquors or become converted to an oil at room temperature and sometimes become disordered soon after removal from the dialysis tubes in cold. The crystals grown from PEG 4000 diffracted poorly and contained three molecules in the asymmetric unit. The crystals grown from PEG 8000, PEG 3400 and PEG 400 were twinned and diffracted very poorly. The crystals grown from ethanol are stable in mother liquor with 20–25% MPD and last about 12 h in the X-ray beam. Further structure analysis work is being pursued on these two forms of ethanol grown crystals.

2.3. *X-ray diffraction analysis*

X-ray intensities were measured at 288 K using a MAR Research imaging-plate scanner with a diameter of 300 mm, and the crystal-to-detector distances of 350 mm for form I and 250 mm for form II were used. Monochromatic Cu $K\alpha$ radiation was produced with a graphite crystal monochromator mounted on a Rigaku RU-200 rotating-anode generator operating at 40 KV and 100 mA with a focal point of 0.3×3 mm. The details of crystal data and reduction statistics are given in Table 1. The diffraction patterns from crystal forms I and II are shown in Fig. 3. The crystals of form II diffracted better (Fig. 3*b*) than those of form I (Fig. 3*a*). The reflections in the form II can be seen beyond 2.9 Å resolution (Fig. 3*b*). The *DENZO* and *SCALEPACK* packages (Otwinowski, 1993; Minor, 1993) were used for the determination of unit cell parameters, data processing and scaling.

Further calculations, sequence determination of the protein and the collection of high-resolution data are in progress.



(*a*)

(*b*)

Fig. 3. 1.0° rotation images of the diffraction patterns of crystals of, (*a*) form I with a crystal-to-detector distance of 350 mm; (*b*) form II with a crystal-to-detector distance of 250 mm.

The authors thank the Department of Biotechnology, New Delhi for financial assistance. AKS and SK thank CSIR, New Delhi for fellowships.

References

Anderson, B. F., Baker, H. M., Norris, G. E., Rice, D. W. & Baker, E. N. (1989). *J. Mol. Biol.* **209**, 711–734.

Arnold, R. R., Russel, J. E., Champion, W. J., Brewer, M. & Ganthier, J. J. (1982). *Infect. Immun.* **35**, 792–799.

Baker, E. N. & Lindley, P. F. (1992). *J. Inorg. Biochem.* **47**, 147–160.

Baldwin, D. A., Jenny, E. R. & Aisen, P. (1984). *J. Biol. Chem.* **257**, 13391–13394.

Bennet, R. M., Merrit, M. M. & Gabor, G. (1986). *Br. J. Haematol.* **63**, 105–117.

Bullen, J. J., Rogers, H. J. & Leigh, L. (1972). *Br. Med. J.* **3**, 69–75.

Campbell, E. J. (1982). *Proc. Natl Acad. Sci. USA*, **79**, 6941–6915.

Davidson, L. A. & Lonnerdal, B. (1988). *Am. J. Physiol.* **254**, G580–G585.

He, J. & Furmanski, P. (1995). *Nature (London)*, **373**, 721–724.

Jollès, J., Donda, A., Amiguet, P. & Jollès, P. (1984). *FEBS Lett.* **176**(1), 185–188.

Lammeli, U. K. (1970). *Nature (London)*. **227**, 680–685.

Lash, J. A., Coates, T. D., Lafuze, J., Bachner, R. L. & Boxer, L. A. (1983). *Blood*, **61**, 885–882.

Law, B. A. & Reiter, B. (1977). *J. Dairy Res.* **44**, 595–599.

Mason, P. L., Hermans, J. F. & Dive, C. (1966). *Clin. Chim. Acta*, **13**, 735–739.

Metz-Boutigue, M. H., Jollès, J., Mazurier, J., Schoentgen, F., Legrand, D., Spike, G., Montreuil, J. & Jollès, P. (1984). *Eur. J. Biochem.* **145**, 659–676.

Norris, G. E., Anderson, B. F., Baker, E. N., Baker, H. M., Gartner, A. L., Ward, J. & Rumball, A. L. (1986). *J. Mol. Biol.* **191**, 143–145.

Otwinowski, Z. (1993). *Oscillation Data Reduction Program*, in *Proceedings of the CCP4 Study Weekend: Data Collection and Processing, 29–30 January 1993*, edited by L. Sawyer, N. Issacs and S. Bailey, pp. 56–62, Warrington: Daresbury Laboratory.

Minor, W. (1993). *XDISPLAYF* Program, Purdue University, West Lafayette, USA.

Pierce, A., Colavizza, D., Benaissa, M., Maes, P., Tartar, A., Montreuil, J. & Spike, G. (1991). *Eur. J. Biochem.* **196**, 177–184.

Raman, A., Bhatia, K. L., Singh, T. P., Srinivasan, A., Betzel, C. & Malik, R. C. (1991). *Arch. Biochem. Biophys.* **294**, 319–321.

Sharma, S., Singh, T. P. & Bhatia, K. L. (1996). In preparation.