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Crystallization and preliminary X-ray diffraction studies of the proteolytically engineered C-terminal half of buffalo lactoferrin in its iron-saturated form

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Abstract

The glycosylated functional monoferric C-terminal half (C lobe) ($M_r \simeq 40$ kDa) of buffalo lactoferrin has been produced by limited proteolysis using proteinase K. The iron-saturated C lobe has been crystallized by microdialysis. The crystals belong to the monoclinic system, space group $P2_1$ with unit-cell dimensions of $a = 44.4$, $b = 152.3$, $c = 38.8$ Å and $\beta = 105.5°$. There is one protein molecule of 40 kDa in the asymmetric unit. A data set at 2.8 Å has been collected on an imaging-plate scanner.

1. Introduction

Lactoferrin (also called lactotransferrin) is an iron-binding 80 kDa glycoprotein of the transferrin family (Aisen & Listowsky, 1980; Montreuil, Mazurier, Legrand & Spik, 1985). It serves to control the iron levels in body fluids by sequestering and solubilizing ferric iron. Its presence in leucocytes (Baggiolini, de Duve, Masson & Heremans, 1970) and in many exocrine secretions *e.g.* milk, saliva, tears, mucosal and genital secretions (Masson, Heremans & Dive, 1966) together with its ability to bind to a wide variety of cells (Birgens, Hansen, Karle & Ostergaard, 1983), has further been associated with other postulated functions. These include it roles in the immune and inflammatory responses as an antibacterial agent (Arnold, Russel, Champion, Brewer & Gauthier, 1982). It binds to DNA under stringent conditions with a distinct sequence specificity and the interaction between lactoferrin and specific DNA sequences intracellularly leads to transcriptional activation (He & Furmanski, 1995).

The lactoferrin chain folds into two globular lobes, representing the N- and C-terminal halves of the protein. Each lobe contains one of the two similar iron binding sites. The diferric structures of human lactoferrin at 2.2 Å resolution (Haridas, Anderson & Baker, 1995), buffalo lactoferrin at 3.4 Å resolution (Karthikeyan, 1996), mare lactoferrin form I at 3.8 Å resolution (Sharma, Karthikeyan, Kaur, Singh & Yadav, 1996) and form II at 2.9 Å resolution (Sharma *et al.*, 1996) have been described. The structure of human apolactoferrin has also been reported (Anderson, Baker, Norris, Rumball & Baker, 1990).

The two halves of the molecule have a high sequence homology and similar overall folding, probably as a result of gene duplication. The metal-binding properties of the two lobes are not very similar as one is acid stable and the other is acid labile (Mazurier & Spik, 1980; Sharma, Singh & Bhatia, 1997). The contents of secondary-structure elements of the two lobes are also not identical (Sharma *et al.*, 1997). Furthermore, the two fragments resemble each other strongly in certain functions while they differ greatly in others. Therefore, the isolated individual lobes are useful for uncoupling the properties of two molecular halves. Many fragments have been produced by limited proteolysis using various proteinases (Brock, Arbaze, Lampreane & Pineare, 1976; Bluard-Deconinck *et al.*, 1978; Legrande *et al.*, 1986; Legrande, Mazurier, Colavizza, Montreuil & Spik, 1990; Luis, Castillo, Sanchez, Puyol & Calvo, 1994; Sharma *et al.*, 1996) but their usefulness is limited in many cases by the fortuitous nature of the cleavage sites within the proteins. Thus, the efforts to prepare the monoferric functional N and C-terminal molecular halves by proteolytic methods for a detailed structure analyses have not been very successful. Therefore, the N lobe of human lactoferrin has been cloned and its structure has been reported recently (Day, Anderson, Tweedie & Baker, 1993). However, the cloned fragments lack the glycosylation and the impact of interlobe interactions on the folding of monoferric lobes. Therefore, the proteolytically engineered glycosylated 40 kDa N and C lobes may reveal interesting features of interlobe interactions and functions of two molecular halves. Here we describe the preparation of crystals of the C lobe of buffalo lactoferrin (Lf) in the iron-bound form. The crystals are of sufficiently high quality to allow the high-resolution X-ray crystallographic studies necessary to define the properties of the C-terminal half molecule.

2. Experimental procedure

2.1. *Hydrolysis of lactoferrin*

The usefulness of proteinase K in producing the true N and C lobes of buffalo lactoferrin has been demonstrated by us recently (Sharma *et al.*, 1996). The purified iron-saturated samples of lactoferrin were incubated with proteinase K in 0.05 *M* Tris–HCl/5 m*M* $CaCl_2$ (pH 7.8) for 1 h at an enzyme-to-lactoferrin molar ratio of 1:25 at 298 K. The hydrolyzed product was analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. The gel showed a major band at 40 kDa (Fig. 1, track *b*). The absence of a band at 80 kDa indicates the complete hydrolysis of lactoferrin while a band corresponding to very low molecular weight peptides can be seen at the bottom of track *b* (Fig. 1).

2.2. *Purification of N and C fragments*

The N and C fragments of lactoferrin were purified on an ion-exchange column (1.5 × 15 cm) of CM-Sephadex C-50 using a salt gradient of NaCl (0.0–0.5 *M*) in 0.05 *M* Tris–HCl (pH 8.0). Two major fractions in the elution profile (Fig. 2) were collected. The first major fraction did not bind to the gel, was acidic in nature and pink in colour. The second major

fraction, being basic in nature, bound to the gel, and was eluted by 0.4 *M* NaCl. The N-terminal sequences of these fractions and the intact buffalo lactoferrin were determined using an automated amino-acid sequencer (Biosystems). The comparison of these sequences indicated that fraction 1 was the C lobe and fraction 2 was the N lobe. The migration of fractions 1 and 2 on SDS–PAGE indicated that both fragments have a molecular weight of 40 kDa.

2.3. *Crystallization*

The purified sample of the C fragment was prepared for crystallization by filtration through a 0.2 µm membrane and then concentrated to about 60 mg ml^{-1} in a microconcentrator (Amicon) which had a molecular weight cutoff of 10 kDa. The crystals of the C lobe were obtained by microdialysis after about two months at 279 K against 10 m*M* Tris–HCl, pH 8.0 containing 10% ethanol. 25 µl of 60 mg ml^{-1} protein in 10 m*M* Tris–HCl, pH 8.0 were inserted into a glass capillary of 3 mm diameter. The protein sample was separated from a reservoir by a 10 kDa dialysis membrane. The reservoir contained 10 m*M* Tris–HCl pH 8.0 with 10% ethanol. The complete setup was placed at 279 K. The dark red single crystals of irregular shape grew to dimensions of 0.40 × 0.28 × 0.20 mm (Fig. 3). The crystals were unstable at room temperature and tended to redissolve in their mother liquor. The crystals were transferred to a new solution containing 45% 2-methyl-2,4-pentanediol (MPD) in which they were stable for some days in the X-ray beam.

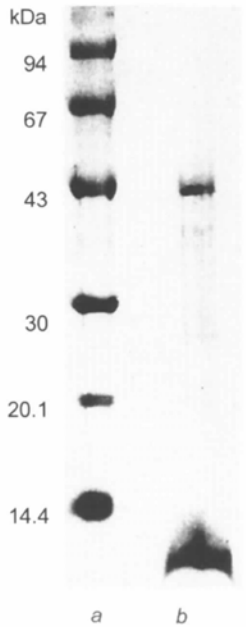

Fig. 1. SDS–PAGE of buffalo lactoferrin under reducing conditions after mild hydrolysis by proteinase K. Track *a*, molecular weight markers (Pharmacia) from top: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). Track *b*: lactoferrin hydrolyzed by proteinase K showing a major band at about 40 kDa along with very low molecular weight peptides at the bottom of track *b*.

3. Results and discussion

The X-ray intensities were measured at 283 K using a MAR Research imaging-plate scanner with a diameter of 300 mm. The crystal-to-detector distance was kept at 250 mm. Monochromatic Cu $K\alpha$ radiation was produced with a graphite crystal monochromator mounted on a Rigaku RU-200 rotating-anode X-ray generator operating at 40 kV and 100 mA with a focal point of 0.3 × 3 mm. The crystals diffracted beyond 2.8 Å resolution. The intensity data were processed using *DENZO* and *SCALEPACK* packages (Otwinowski, 1993; Minor, 1993). The crystals were found to be monoclinic, space group $P2_1$ with cell dimensions $a = 44.4$, $b = 152.3$, $c = 38.8$ Å, $\beta = 105.5°$. Assuming a molecular weight of about 40 kDa and one molecule per asymmetric unit, a value of $V_m = 3.16$ Å^3 Da^{-1} was obtained which is comparable with values obtained for other protein crystals (Matthews, 1968). It gave a solvent content of 61%. The data showed a completeness of 91% up to 2.90 Å resolution. In the last cell (3.04–2.90 Å), the completeness was 56.2%. Attempts are in progress to solve the crystal structure of the C lobe by molecular replacement using the models of uncleaved lactoferrins available (Haridas *et al.*, 1995; Anderson *et al.*, 1990; Sharma *et al.*, 1996; Karthikeyan, 1996).

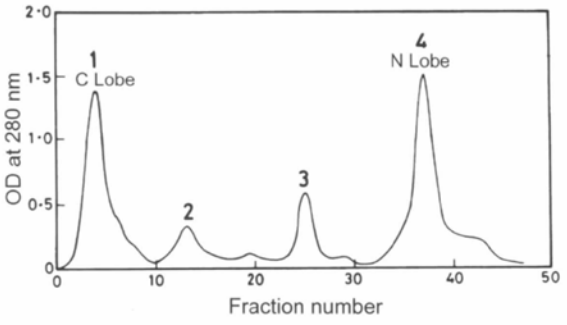

Fig. 2. Elution profile of the purification of N and C fragments of buffalo lactoferrin by iron-exchange chromatography on CM-Sephadex C-50 in 0.05 *M* Tris–HCl (pH 8.0) with an NaCl gradient (0.0–0.5 *M*).

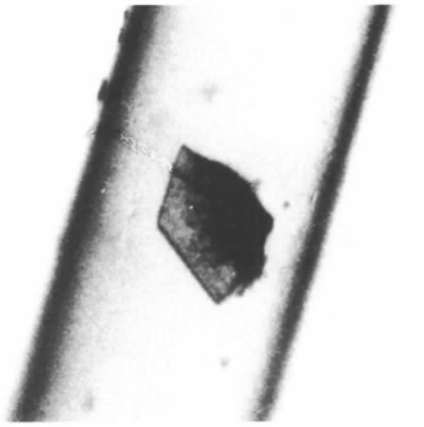

Fig. 3. One of the crystals (0.40 × 0.28 × 0.20 mm) mounted in a capillary and used for data collection.

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