Biochemical and Biophysical Research Communications **264,** 19–23 (1999)
Article ID bbrc.1999.1318, available online at http://www.idealibrary.com on IDEAL®

Bovine Lactoferrin Peptidic Fragments Involved in Inhibition of Herpes Simplex Virus Type 1 Infection

Rosa Siciliano,* Barbara Rega,* Magda Marchetti,† Lucilla Seganti,† Giovanni Antonini,‡ and Piera Valenti§

**Institute of Food Science and Technology, CNR, I-83100 Avellino Italy; †Institute of Microbiology, University of Rome "La Sapienza," I-00185 Rome, Italy; ‡Department of Basic and Applied Biology, University of L'Aquila, I-67010 L'Aquila, Italy; and §Institute of Microbiology, II University of Naples, I-80135 Naples, Italy*

Received July 27, 1999

Bovine lactoferrin (BLf) prevents the infection of some enveloped and naked viruses. To identify BLf sequences responsible for the antiviral activity, we tested 31 HPLC fractions, derived from tryptic digestion of BLf, toward herpes simplex virus type 1 (HSV-1). Only a few HPLC purified fragments were active against HSV-1, even if at lower extent than the native undigested BLf. Two large fragments, one corresponding to the C-lobe (amino acid sequence 345–689) and the other corresponding to a large portion of the N-lobe (1–280), were inhibitors of HSV-1 infection, while a smaller part of the N-lobe (86–258) was ineffective. Among the low-molecular-weight fragments, only two small peptides, which coeluted in a single chromatographic peak, were effective towards HSV-1. These peptides, both present in the N-lobe, were identified as peptides 222–230 (ADRDQYELL) and 264–269 (EDLIWK). The same peptides, chemically synthesised, were able to inhibit HSV-1 infection only when they were assayed in association.

Lactoferrin is an iron binding protein, belonging to the transferrin family, mainly found in external secretions, such as breast milk, tears, saliva and vaginal secretions, synthesised by polymorphonuclear leukocytes and glandular epithelial cells (1, 2). The bovine lactoferrin (BLf) sequence (689 amino acidic residues) is similar to that of human lactoferrin, and to that of lactoferrins isolated from other mammalian species (3, 4). BLf consists of a single polypeptide chain, folded in two symmetric globular lobes (N-lobe and C-lobe) and each lobe is subdivided in two structural domains (N1, N2 and C1,C2) delimiting a central cavity containing the metal binding site (5). The glycoprotein nature of bovine milk lactoferrin has been known since 1960 (6), but complete structures of the glycans of oligomannose-type and of the biantennary, partially fucosylated and sialylated, N-acetyllactosamine-type present in lactoferrins of different origins have been described only a few years ago (7, 8, 9). Matthews et al. reported in 1976 (10) the antiviral activity of milk proteins, but only recently lactoferrin has been recognised as a potent inhibitor towards different enveloped viruses such as herpes simplex virus (HSV) type 1 and 2 (11, 12, 13, 14), human cytomegalovirus (11, 15), human immunodeficiency virus (HIV) (15, 16, 17) and human hepatitis C virus (18). Recently the antiviral effect of lactoferrin on two naked viruses, the simian rotavirus SA-11 and poliovirus type 1, has also been demonstrated (19, 20). The antiviral activity of lactoferrin was mainly exerted towards the early phases of infection for all viruses investigated to date and is poorly influenced by lactoferrin saturation with different metal ions (14). This effect has been correlated to a competition for cell receptors since the N-terminus of lactoferrin binds to surface glycosaminoglycans (21, 22), which are initial binding sites for some viruses (23, 24) even though a direct lactoferrin interaction with viral particles cannot be ruled out (13, 16, 25).

In an attempt to identify lactoferrin amino acid sequences contributing to the antiviral activity, we have analysed the antiviral activity of peptide fragments, derived from the tryptic digestion of BLf, towards a susceptible enveloped virus (HSV-1). Fragments exhibiting the antiviral activity have been submitted to amino acid sequence characterisation integrating protein chemistry procedures and advanced mass spectrometric methodologies. The obtained results suggest that different BLf amino acid sequences together with defined conformational status are possibly involved in the inhibition of HSV-1 infection.

MATERIALS AND METHODS

Chemicals. Bovine milk lactoferrin was supplied by Besnier Bridel (Laval, France). Two lactoferrin derived peptides, ADRDQYELL and EDLIWK, were synthesised by Tecnogen (Italy), and

0006-291X/99 $30.00

their purity (>95%) was checked by HPLC and electrospray mass spectroscopy (ESMS) (see below). All other chemicals, reagent grade, were purchased from Sigma Chemical Co. (St. Louis, MO).

Enzymatic hydrolysis and chemical modifications of lactoferrin. BLf (4 mg/ml) was dissolved in 50 mM ammonium bicarbonate, pH 8.5 and trypsin (from bovine pancreas TPCK treated, Sigma) digestion was performed at 37°C overnight using an enzyme to substrate ratio of 1:50 w/w. Deglycosilation was performed by treatment with PNGase F (Boehringer) in the same buffer at 37°C overnight using 0.1 enzyme unit per 300 μg of peptidic fragment. Reduction of disulphide bridges and carboxyethylation of cysteine residues of the fragments were performed as already reported (26), except that iodoacetamide has been used as alkylated agent, instead of iodoacetic acid.

HPLC separation of peptides. The BLf tryptic peptides were purified by RP-HPLC on a Vydac C18 column (250 × 10 mm, 5 μm) using a Waters HPLC System (Datasystem Millenium, HPLC pumps Waters 510, Detector Waters 486). Eluents were: 0.1% trifluoroacetic acid (solvent A) and 0.07% trifluoroacetic acid in 95% acetonitrile (solvent B). The elution was performed by means of a linear gradient from 10% to 55% solvent B over 45 min at a flow rate of 3.5 ml/min and monitored at 220 nm. The fractions were collected, dried in a Speed-Vac centrifuge (Savant), lyophilised twice and stored at −20°C. The amount of the large fragments was determined according to the method of Bradford (27).

Mass spectrometric analyses. HPLC fractions were submitted to electrospray mass spectrometric (ESMS) analyses using a Platform single quadrupole mass spectrometer (Micromass). Samples were dissolved in 1% acetic acid in 50% acetonitrile and 2–10 μl were injected into the mass spectrometer at a flow rate of 10 μl/min. The quadrupole was scanned from m/z 300 to 1600 at 10 s/scan and the spectra were acquired and elaborated using the MassLynx software. All mass values are reported as average masses.

Experiments of tandem mass spectrometry (MS/MS) were carried out on a LCQ ion-trap mass spectrometer (Finnigan Corporation) fitted with a Finnigan electrospray ion source.

Cells and viruses. Herpes simplex virus type 1 (HSV-1), strain F, was propagated in Vero cells (African green monkey kidney) as previously described (13). Virus titrations were performed by the plaque forming unit (p.f.u.) assay.

Cytotoxicity and antiviral assays of compounds. To establish noncytotoxic doses of compounds, two-fold serial dilutions of each substance in medium were incubated at 37°C for 48 h with confluent cell monolayers grown in 96-well tissue culture microplates (Flow Laboratories). Cell proliferation and viability were assessed by a nonradioactive quantitative colorimetric assay (Cell proliferation kit-MTT Boehringer Mannheim GmbH, Germany) and by neutral red uptake as previously described (17, 21). Confluent cell monolayers were infected with HSV-1, at a multiplicity of infection of 0.1 p.f.u./cell. Compounds, at non cytotoxic concentrations, were incubated at different concentrations with the cells during the viral adsorption step (1 h at 37°C) and newly added after the removal of virus inoculum. At 48 h post infection with HSV-1, cytopathic effect inhibition was measured by the neutral red uptake assay as previously described (17, 23), and results were evaluated as percentage of cytopathic effect inhibition by comparison with untreated cultures.

RESULTS

Bovine lactoferrin was treated with trypsin as above described. The pool of digested fragments, in which the amount of the intact protein was less than 1% by weight, retained the antiviral activity towards HSV-1, as already reported (28), indicating that the inhibition of viral infection was not exclusively linked to native, undigested BLf. HPLC purification of digested BLf fragments resulted in 31 different peaks that were individually tested for antiviral activity against HSV-1 by incubating the compounds with Vero cells during the entire viral infection cycle.

TABLE 1

Antiviral Activity toward HSV-1 of Peptidic Fragments Deriving from Tryptic Digestion of Bovine Lactoferrin

Compounds	EC_{50} μg/ml	EC_{50} μM
Undigested BLf	10	0.125
Fraction 30 (345–689)	320	7.1
Fraction 28 (1–280)	25	0.725
Fraction 31 (86–258)	>10,000	>480
Chemically synthesised fragments with aminoacid sequences 222–230 and 264–269	850	440

Note. In parentheses are indicated the corresponding aminoacid sequences in undigested BLf. Compounds were incubated with Vero cells during the herpes simplex virus type 1 (0.1 p.f.u./cell) adsorption step (1 h at 37°C) and newly added after the removal of virus inoculum. EC_{50} corresponds to the effective concentration required to inhibit viral cytopathic effect by 50% after 48 h from infection. Experiments were carried out in triplicate and standard deviations never exceeded 7% of the mean values.

Among the 31 fractions, only three exhibited antiviral activity: two large fragments (fractions 30 and 28), and one low molecular weight fragment (fractions 19) (Table 1).

In order to achieve a structural characterisation, fraction 30 was analysed by ESMS; the spectrum showed the presence of two main peaks with molecular weights (MW) of 33016.8 ± 0.8 Da and 33178.6 ± 0.6 Da (Fig. 1A). The 162 mass difference between them (corresponding to a monosaccharide residue) suggested that the two peaks were originated from different glycoforms of the same fragment. The HPLC fraction was, then, submitted to deglycosylation, reduction and alkylation of the cysteine residues. ES/MS spectra were recorded after each reaction. The molecular mass of the deglycosylated and alkylated peptide fragment was 32125.3 ± 0.6 Da which was attributed to the fragment 1–280 on the basis of the MW and the specificity of trypsin, by means of a dedicated computer program. This fragment contained a single glycosylation site on the Asn 233. The mass difference between the two glycoforms (MW = 33016.8 ± 0.8 and 33178.6 ± 0.6 Da) and the deglycosylated form (MW = 31313.3 ± 0.8 Da; ΔM = 1703.5 Da and 1865.6 Da respectively) indicated that high-mannose structures (composition: $Man_9GlucNAc_2$ and $Man_8GlucNAc_2$) were linked to Asn 233.

Fraction 28, whose molecular weight was about 45 kDa, (as estimated by SDS–PAGE), was attributed to the C-lobe (345–689), on the basis of mass spectrometric analyses performed on the reduced and alkylated

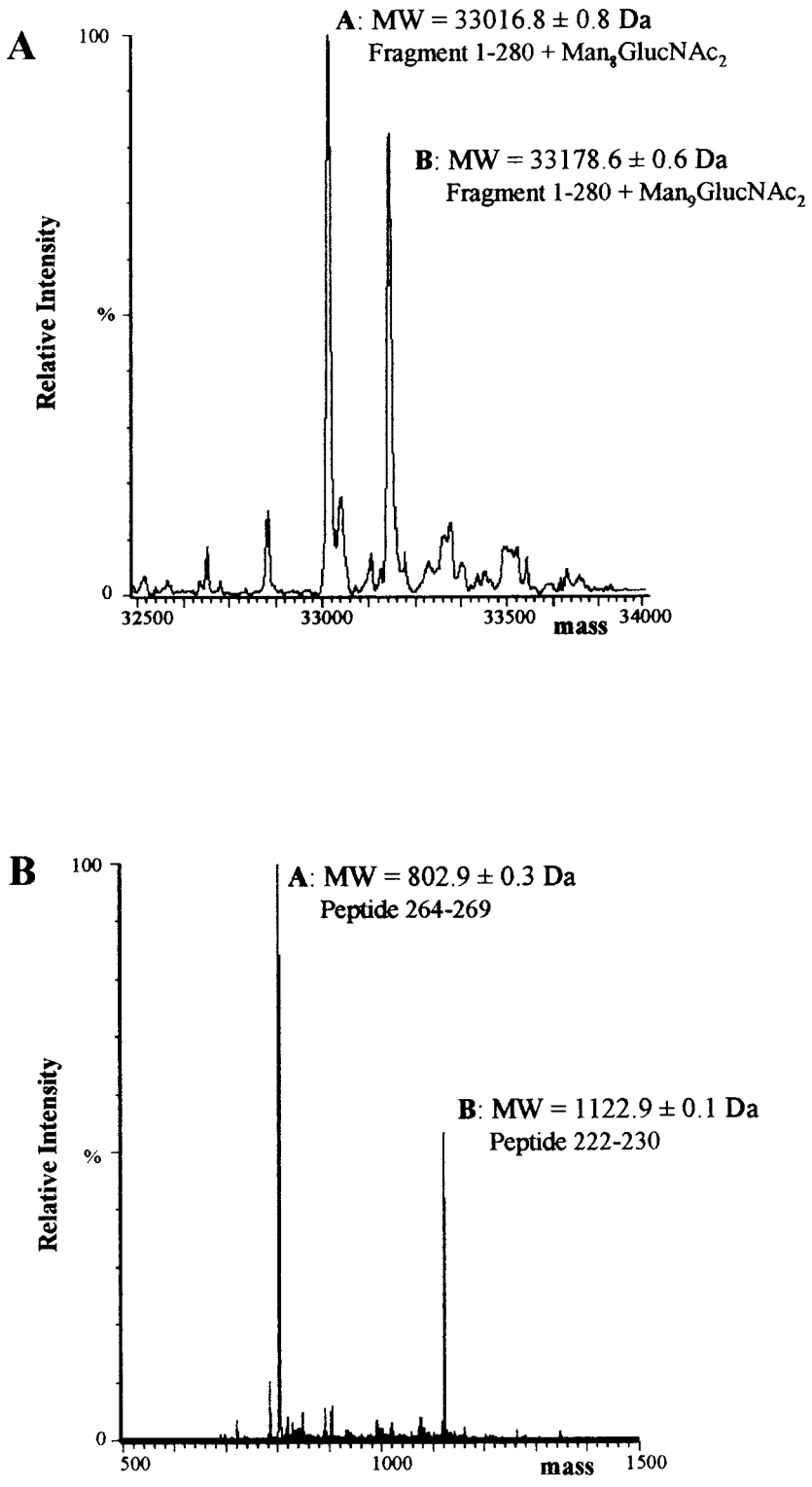

FIG. 1. Transformed ES/MS spectra of two fractions exhibiting antiviral activity: Fraction 30 (A) and fraction 19 (B). The measured mass values and the corresponding attributions are reported. For details see text.

fraction. Moreover, the presence of partial tryptic cleavages, leading to large peptides linked by disulphide bridges, was also detected (data not shown).

Fraction 28, belonging to the C-lobe, exerted an antiviral activity lower than the other large peptide (fraction 30), belonging to the N-lobe. However, both large

fragments showed an anti-HSV-1 effective concentration (50%) about 60- or 6-fold lower than untreated BLf, respectively (Table 1).

Similar structural analyses were also carried out on the ineffective fraction 31 which resulted to be fragment 86–258, corresponding to the N2 domain. It is relevant to highlight that, although this fragment is included in 1–280, it was deprived of any antiviral effect (Table 1).

Among the low-molecular-weight fragments, only the HPLC fraction 19 was active against HSV-1. The fraction contains two peptides, 19a (MW = 802.9 ± 0.3 Da) and 19b (MW = 1122.9 ± 0.1 Da), which were attributed to the amino acid sequences 222–230 (EDLIWK) and 264–269 (ADRDQYELL) respectively (Fig. 1B). The amino acidic sequences of these peptides were confirmed by MS/MS experiments. To quantify and separately evaluate the antiviral activity, these two small peptides were chemically synthesised. Interestingly, neither peptide 222–230 nor peptide 264–269, obtained by chemical synthesis, displayed inhibitory effect when separately tested. The antiviral activity towards HSV-1 was observed when peptide 222–230 was tested in a 1:1 stoichiometric association with peptide 264–269 (Table 1), suggesting that the presence of both peptides was required to prevent HSV-1 infection. However, the specific activity of this peptide association, considering the molecular mass, was significantly lower than that possessed by the two effective large fragments or by the whole BLf molecule.

DISCUSSION

Bovine lactoferrin, like human lactoferrin, is folded in two symmetrical lobes (C-lobe and N-lobe) that have high sequence homology, possibly resulting from an ancestral gene duplication (29, 30). In the three dimensional structure of iron saturated bovine lactoferrin, each lobe is subdivided in two structural domains (N1, N2 and C1, C2) delimiting a central cavity containing the metal binding site. In BLf, the N1 and C1 domains are formed by amino acid sequences 1–90 together with 253–316 and 344–423 together with 595–689, respectively; while N2 and C2 domains are formed by amino acid sequences 91–252 and 424–594, respectively (5).

Although the antiviral activity of lactoferrin has been well described, its mechanism of action is poorly characterised. In a previous study, we have already reported the antiviral activity toward HSV-1 of the pool of fragments deriving from tryptic digestion of BLf, suggesting that the inhibition of viral infection could not be exclusively linked to native, undigested BLf (28).

In this report, we tried to identify BLf structural domains responsible for antiviral activity by analysing the protective effect towards HSV-1 infection possessed by low and high molecular weight peptides deriving from tryptic digestion of BLf. Among high molecular weight peptides, the fraction 30 (amino acid sequence 1–280), belonging to the N-lobe, was ten-fold more effective towards HSV-1 infection than fraction 28, representing the whole C-lobe being effective concentration 50% (EC 50) 0.750 μM versus 7.1 μM, respectively. On the other hand, fraction 30 was still six-fold less active than native BLf, which exerted the maximal antiviral activity (EC50 0.125 μM).

The different antiviral activity of the C-lobe and N-lobe toward HSV-1 cannot be explained on the basis of their different glycosylation sites (three glycosylation sites present in C-lobe while only one in N-lobe) since their removal from undigested BLf did not affect anti HSV-1 activity, as reported in a previous research (31). Therefore, we could tentatively explain these differences on the basis of their different amino acid sequences and/or of their topographic localisation in BLf. It is likely that the absence of antiviral activity of the large fraction 31 (amino acid sequence 86–258), which corresponds to the N2 domain, is correlated to the lack of amino acid sequences 1–85 and/or 259–280, present in the effective fraction 30 (amino acid sequence 1–280) which contains the N2 domain together with part of the N1 domain.

Furthermore, we have observed that, among the low-molecular weight fragments, only the association of two small peptides (amino acid sequences 222–230 and 264–269) was effective. Considering their molecular mass, these peptides showed a much lower antiviral activity (EC_{50} μM 850) than that displayed by undigested BLf and by the fraction 28 and 30. Interestingly, these small peptides did not display any antiviral activity when they are separately tested. It is important to note that effective fraction 30 (amino acid sequence 1–280) contains both amino acid sequences of the two small co-purified peptides (amino acid sequences 222–230 and 264–269), while ineffective fraction 31 (amino acid sequence 86–258), does not contain the amino acid sequence 264–269. In the three-dimensional structure of iron-saturated BLf, these two small peptides are exposed to the solvent at the BLf surface and are located at opposite sites of the N-lobe (belonging to N2 and N1 domains respectively) (5). The markedly reduced antiviral activity displayed by the two associated peptides (amino acid sequences 222–230 and 264–269) could therefore be correlated with the lack of the correct folding when they are separated from the protein.

All together, these results suggest that different amino acid sequences of bovine lactoferrin, possibly depending on their conformations, can be involved in HSV-1 infection.

ACKNOWLEDGMENTS

This work was supported by grants from MURST (PRIN Role of transition metal ions in the intracellular infections) and CNR target project on “Biotechnology.”

REFERENCES

1. Levay, P. F., and Viljoen, M. (1995) *Haematologica* **80,** 252–267.
2. Chaasteen, N. D., and Woodworth, R. C. (1990) *in* Iron Transport and Storage (Ponk, P., Mshulman, H., and Woodworth, R. C., Eds.), pp. 67–69, CRC Press, Boston.
3. Metz-Boutigue, M. H., Jolles, J., Mazurier, J., Schoentgen, F., Legrand, D., Spik, G., Montreuil, J., and Jolles, P. (1984) *Eur. J. Biochem.* **145,** 659–676.
4. Pierce, A., Colavizza, D., Benaissa, M., Maes, P., Tartar, A., Montreuil, J., and Spik, G. (1991) *Eur. J. Biochem.* **196,** 177–184.
5. Moore, S. A., Anderson, B. F., Groom, C. R., Haridas, M., and Baker, E. N. (1997) *J. Mol. Biol.* **274,** 222–236.
6. Groves, M. L. (1960) *J. Am. Chem. Soc.* **82,** 3345–3350.
7. Spik, G., Coddeville, B., and Montreuil, J. (1988) *Biochimie* **70,** 1459–1469.
8. Coddeville, B., Strecker, G., Wieruszeski, J. M., Vliegenthart, J. F. G., van Halbeek, H., Peter-Katalinic, J., Egge, H., and Spik, G. (1992) *Carbohydrate Res.* **236,** 145–164.
9. Spik, G., Coddeville, B., Mazurier, J., Bourne, Y., Cambillaut, C., and Montreuil, J. (1994) *Adv. Exp. Med. Biol.* **357,** 21–32.
10. Matthews, T. H. J., Nir, C. D. G., Lawrence, M. K., and Tyrrell, D. A. J. (1976) *Lancet* **2,** 1387–1389.
11. Hasegawa, K., Motsuchi, W., Tanaka, S., and Dosako, S. (1994) *Jpn. J. Med. Sci. Biol.* **47,** 73–85.
12. Fujihara, T., and Hayashi, K. (1995) *Arch. Virol.* **140,** 1469–1472.
13. Marchetti, M., Longhi, C., Conte, M. P., Pisani, S., Valenti, P., and Seganti, L. (1996) *Antiviral Res.* **29,** 221–231.
14. Marchetti, M., Pisani, S., Antonini, G., Valenti, P., Seganti, L., and Orsi, N. (1998) *BioMetals* **11,** 89–94.
15. Harmsen, M. C., Swart, P. J., de Béthune, M. P., Pawels, R., De Clercq, E., The, T. H., and Meijer, D. K. F. (1995) *J. Infect. Dis.* **172,** 280–388.
16. Swart, P. J., Kuipers, M. E., Smith, C., Pawels, R., De Béthune, M. P., De Clerck, E., Meijer, D. K. F., and Huisman, J. G. (1996) *AIDS Res. Human Retrov.* **12,** 769–775.
17. Puddu, P., Borghi, P., Gessani, S., Valenti, P., Belardelli, F., and Seganti, L. (1998). *Int. J. Biochem. Cell Biol.* **30,** 1055–1062.
18. Ikeda, M., Sugiyama, K., Tanaka, T., Tanaka, K., Sekihara, H., Shimotohno, K., and Kato, N. (1998) *Biochem. Biophys. Res. Commun.* **215,** 744–749.
19. Superti, F., Ammendolia, M. G., Valenti, P., and Seganti, L. (1997) *Med. Microbiol. Immunol.* **186,** 83–91.
20. Marchetti, M., Superti, F., Ammendolia, M. G., Rossi, P., Valenti, P., and Seganti, L. (1999) *Med. Microbiol. Immunol.* **187,** 199–204.
21. Mann, D. M., Romm, E., and Migliorini, M. (1994) *J. Biol. Chem.* **269,** 23661–23667.
22. Wu, H. F., Monroe, D. M., and Church, F. C. (1995) *Arch. Biochem. Biophys.* **317,** 85–92.
23. WuDunn, D., and Spear, P. G. (1989) *J. Virol.* **69,** 2233–2239.
24. Roderiquez, G., Oravecz, T., Yanagishita, M., Bou-Habib, D. C., Mostowski, H., and Nocross, M. A. (1995) *J. Virol.* **357,** 393–399.
25. Yi,M., Kaneko, S., Yu, D. Y., and Murakami, S. (1997) *J. Virol.* **71,** 5997–6002.
26. Nitti, G., Orrù, S., Bloch, C., Mohry, L., Marino, G., and Pucci, P. (1995) *Eur. J. Biochem.* **228,** 250–256.
27. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–251.
28. Valenti, P., Antonini, G., Siciliano, R., Rega B., Superti, F., Marchetti, M., Ammendolia M. G., and Seganti, L. (1999) *in* Lactoferrin: Structure, Function and Applications (Shimazaki, K., Ed.), Elsevier Science, in press.
29. Norris, G. E., Gartner, A. L., Anderson, B. F., Ward, J., Baker, E. N., Rumball, S. V., and Baker, H. M. (1986) *J. Mol. Biol.* **191,** 143–145.
30. Anderson, B. F., Baker, H. M., Dodson, E. J., Norris, G. E., Rumball, S. V., Waters, J. M., and Baker, E. N. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1769–1773.
31. Antonini, G., Rossi, P., Pitari, G., Marchetti, M., Superti, F., and Valenti, P. (1999) *in* Lactoferrin: Structure, Function and Applications (Shimazaki, K., Ed.), Elsevier Science, in press.