Iron-Dependent Binding of 8-Anilidonaphthalene-1-sulphonate by both Lactoferrin and Transferrin

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Fluorescence of 8-anilidonaphthalene-1-sulphonate is enhanced by both lactoferrin and transferrin. The enhancement is relatively low between pH 4 and 8, and high at below pH 4. At physiological pH the fluorescence enhancement is higher with the iron-deprived than with the iron-saturated proteins. Binding of iron by lactoferrin is associated with lowering affinity for the dye.

Lactoferrin resembles the transferrins of blood serum and egg white in regard to molecular weights, single chain nature, almost identical amino acid composition and the two metal-binding sites (Querineau *et al.*, 1971). Much attention has been given to the mechanism of metal binding by these proteins. They both incorporate one CO_3^{2-} ion for each metal ion bound (Masson & Heremans, 1968). However, iron is bound by lactoferrin at lower pH and with a few hundred times larger binding constant than by transferrin (Aisen & Leibman, 1972). When saturated with iron, the absorbance of lactoferrin in the ultraviolet and visible regions is lower than that of transferrin (Teuwissen *et al.*, 1974). The optical and electron-paramagnetic-resonance spectroscopic parameters indicate that the two metal-binding sites of lactoferrin are strikingly similar to those of transferrin (Aisen & Leibman, 1972). Binding of iron to both the proteins appears to involve equivalent and non-interacting sites (Aisen & Leibman, 1972), which in transferrin are about 4 nm (40 Å) apart (Luk, 1971).

In the present study it is shown that fluorescence of 8-anilidonaphthalene-1-sulphonate is enhanced by both lactoferrin and transferrin. The effect is pH-dependent. At physiological pH the fluorescence enhancement is, however, higher with the iron-deprived than with the iron-saturated proteins. Fluorescence titrations show that binding of iron by lactoferrin is associated with lowering affinity for the dye.

Materials and Methods

Chemicals were of analytical grade. Bovine transferrin and bovine albumin were B-grade preparations from Calbiochem A.-G., Lucerne, Switzerland. Goat lactoferrin was isolated in our laboratory by using the following procedure. Dry CM-Sephadex C-50 (3 g) was added to 1 litre of goat colostrum diluted with 2 vol. of water. After 2 h magnetic stirring the gel was allowed to settle. The supernatant was removed by

aspiration, and the reddish gel was packed into a 250 ml Sartorius filtration apparatus and washed exhaustively with 0.25 M-NaCl/10 mM-Tris/HCl, pH 8. When the effluent became clear, lactoferrin was eluted with 0.35 M-NaCl/10 mM Tris/HCl, pH 8. The protein solution was desalted by using a column (2.5 cm × 40 cm) of Sephadex G-25 and freeze-dried. The yield was about 200 mg. Lactoferrin was further purified by gradient chromatography at pH 5.5 by using a column (1.5 cm × 30 cm) of CM-Sephadex C-50, and by gel filtration on agarose (Bio-Gel A-0.5).

In order to prepare both the iron-deprived and the iron-saturated proteins, samples of both lactoferrin and transferrin were weighed and dissolved in 10 mM-HCl. Iron was removed by passing the solutions through columns (0.6 cm × 2 cm) of Dowex 50 (X12; H^+ form), equilibrated with 10 mM-HCl. The effluents were adjusted to pH 7.5 with 1 M-Tris base. Further dilutions were made as required with 10 mM-Tris/HCl, pH 7.5. To a sample of each protein 1 M- NaHCO_3 (final concentration 10 mM) and 1 mM-ferric nitrilotriacetate (to saturate the protein with iron) were added (Schlabach & Bates, 1975). In calculating amounts of iron needed for saturation, a mol.wt. of 75000 was assumed for both lactoferrin and transferrin.

The magnesium salt of 8-anilidonaphthalene-1-sulphonic acid was from Serva, Heidelberg, Germany. Before use, the salt was dissolved in water and passed through a column (0.6 cm × 10 cm) of Dowex 50 (X8; H^+ form). The effluent was adjusted to pH 7.5 with 1 M-Tris base. The dye concentrations were determined by using 4.95×10^3 for the molar absorption coefficient at 350 nm (Daniel & Weber, 1966).

Fluorescence measurements were made at room temperature (21–24°C) in a Perkin-Elmer model MPF-2A fluorescence spectrophotometer equipped with a high-pressure 100 W xenon lamp. Excitation and emission were at 380 and 480 nm respectively,

with a bandwidth of 10nm in each case. Fluram, a fluorogenic reagent for the assay of primary amines, was obtained from Hoffmann-LaRoche, Basle, Switzerland.

Results

It was observed that iron preparations of both lactoferrin and transferrin enhance fluorescence of 8-anilino-naphthalene-1-sulphonate with the emission maximum at about 480nm. The fluorescence effect of these proteins is relatively low between pH4 and 8, and high at below pH4, when compared with the effect of serum albumin. Results are given in Fig. 1.

The weak fluorescence produced by both lactoferrin and transferrin at physiological pH received our attention, for it could be amplified to the full scale and be used in observing possible effects of iron on its intensity. Therefore, solutions of both the iron-deprived and iron-saturated proteins were prepared at a $1\mu\text{M}$ concentration, and transferred to 3ml fluorimeter cells. The dye ($30\mu\text{l}$) was added to each cell to give a final concentration of $10\mu\text{M}$. Relative fluorescence readings were 98 and 100 for iron-deprived lactoferrin and iron-deprived transferrin respectively, and 48 and 51 for iron-saturated lactoferrin and iron-saturated transferrin respectively. Then both NaHCO_3 and iron were added to cells containing the iron-deprived proteins. Fluorescence readings were made as soon as possible again. However, these were decreasing with time, but stabilized after a minute or so. The final fluorescence

Fig. 1. Fluorescence enhancement of 8-anilino-naphthalene-1-sulphonate by lactoferrin (○), transferrin (●) and serum albumin (■)

The proteins and the dye were at $1\mu\text{M}$ and $30\mu\text{M}$ concentration respectively. Solutions were made up in sodium citrate/phosphate buffer (pH3–8), 0.01 M-HCl (pH2) and 0.1 M-HCl (pH1).

readings approached those of the iron-saturated proteins. When the iron addition step was repeated twice, no significant change in fluorescence intensity was observed.

A possibility of quenching of the fluorescence by the proteins' own absorption when saturated with iron was considered. In a separate experiment, iron-deprived and iron-saturated proteins as well as the dye were transferred to fluorimeter cells to give 1 and $10\mu\text{M}$ concentration respectively. Fluorescence readings were made. Then a $10\mu\text{l}$ volume containing 2.5 nM of the Fluram derivative of diglycine, showing excitation and emission at 380 and 480nm respectively, was added to each cell. An increase in the fluorescence due to the Fluram derivative was identical irrespective of the form of the protein. This indicates no quenching of emission light. Absorption of excitation light by $1\mu\text{M}$ -protein solutions is insignificant to contribute to the effect of iron on fluorescence enhancement.

To define the effect of iron on the fluorescence enhancement in more detail, fluorescence titrations with both iron-deprived and iron-saturated lactoferrin were performed, with some advantage in our case, by adding portions of the protein to a fixed dye concentration ($1\mu\text{M}$). A binding plot was made of $\Delta F/P$ versus ΔF , where ΔF is fluorescence of protein-dye solutions after subtraction of the due fluorescence in the absence of protein and P is the total protein concentration (Fig. 2). The dissociation constant, K_d , was determined from the slope of the plot, $-1/K_d$; the fluorescence when all the dye is bound by protein, F_{max} , was given by the intercept on the abscissa. The number of binding sites, n , was determined from the initial slope of the plot of the bound dye, X , versus protein concentrations, P , where $X/P = n/D + K_d$, where D is the total concentration of the dye (Weber & Young, 1964). Values for n and K_d were 0.90 and $51 \pm 3\mu\text{M}$ respectively for iron-deprived lactoferrin, and 1.04 and $74 \pm 2\mu\text{M}$ respectively for iron-saturated lactoferrin.

Fig. 2. Binding plot from 8-anilino-naphthalene-1-sulphonate binding to iron-deprived lactoferrin (○) and iron-saturated lactoferrin (●)

Discussion

Both lactoferrin and transferrin, either iron-deprived or iron-saturated, enhance fluorescence of 8-anilino-naphthalene-1-sulphonate with the shift of the emission maximum from 515 to 480 nm. This fluorescence between pH 7 and 8 is low in intensity and at longer wavelengths in comparison with that of serum albumin. Such findings are indicative of adsorption of the dye by a protein surface of weak hydrophobicity (Daniel & Weber, 1966; Weber & Daniel, 1966).

At pH 7.5 intensities of the enhancement are higher with the iron-deprived than with the iron-saturated proteins. Reasons for these differences were investigated in lactoferrin. It is shown that binding of iron by this protein does not change the number of binding sites, which remains at 1, but affects affinity of the protein for the dye.

These observations are consistent with the conclusion that the dye-binding site is not identical with the two metal-binding sites. Affinity of the protein for the dye seems to be related to the presence of iron in the protein molecule. This effect is possibly associated with iron-dependent changes in surface topography.

In support of this conclusion are observations by other investigators that iron makes both lactoferrin and transferrin more resistant to denaturation by urea (Teuwissen *et al.*, 1974) as well as making them more elongated and more compact in ultracentrifuga-

tion studies (Bezkorovainy, 1966; Ulmer, 1969; Querinjean *et al.*, 1971) and changing permeation properties of transferrin in gel-filtration studies (Charlwood, 1971).

The present observations do not explain the differences in tightness of metal binding between lactoferrin and transferrin. Both the pattern of the fluorescence enhancement and the effect of iron seem to be identical for these proteins.

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