The ability of salts to inhibit the reaction between periodate anions and ovotransferrin

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The reaction between periodate anions and apo-ovotransferrin results in the rapid abolition of the iron-binding ability of the protein and the loss of approximately 4 mol of tyrosine/mol of protein. The degree of inhibition exerted by a variety of salts on the rate of this reaction is found to be inconsistent with the lyotropic series and suggests the existence of a complex anion-binding site in the apoprotein. The existence of this site may explain the action of periodate anions on ovotransferrin.

INTRODUCTION

The transferrins are composed of two very similar domains that can each bind one Fe(III) ion and an anion with an extremely low dissociation constant at physiological pH. A variety of different studies has shown the iron ligands to be two or three tyrosine residues, one to three histidine residues, a water molecule or hydroxy ion, and the anion itself (Brock, 1985).

Azari & Phillips (1970) reported that the modification of hen apo-ovotransferrin by periodate anions (IO_4^-) led to the loss of iron-binding ability with the concomitant loss of one tryptophan and three to five tyrosine residues. There were no significant changes in the physical properties of the whole protein, and complete protection was afforded by iron binding. This highly localized modification of tyrosine residues was confirmed by Geoghegan *et al.* (1980), but the loss of a tryptophan residue was not found. The reaction can also be inhibited by concentrations of urea that perturb the native structure of ovotransferrin as seen by optical rotation (Geoghegan *et al.*, 1980). More recently it has been shown that periodate also oxidizes methionine residues, but that this is not a factor in the loss of iron-binding ability, and that other transferrins react similarly with periodate anions (Penner *et al.*, 1983). Geoghegan *et al.* (1980) attributed this rapid modification of tyrosine residues to the location of positive charge at the iron-binding sites of ovotransferrin which leads to an electrostatic attraction of periodate anions. This causes periodate anions to come near to the tyrosine residues at each site and their chemical modification is thereby accelerated.

Geoghegan *et al.* (1980) suggested that the periodate anion is an affinity reagent for the iron-binding sites of transferrins, and their hypothesis may be tested by comparing the inhibitory properties of various anions on the rate of the reaction; if electrostatic attraction alone is important, the relative degree of inhibition exerted by an anion should follow from its position in the lyotropic series (Record *et al.*, 1978).

The reaction between periodate anions and transferrins is of considerable interest because of its high level of specificity; the periodate anion is shown to be a member of the small group of reagents that react only with the

folded protein and may thus be used to detect a specific structural property. It is the identification of this property that is the aim of the present study. The reaction may also provide evidence for the identity of the iron-binding tyrosine residues.

EXPERIMENTAL

Materials

All chemicals were of reagent grade. Ovotransferrin was prepared by the method of Williams (1968) and made free of iron by the method of Evans & Williams (1978). Hepes and Mes were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Methods

Determination of the reaction rates. This was achieved by using 6 M-urea/polyacrylamide-gel electrophoresis to fractionate samples removed at intervals from a reaction mixture; the gels were run and analysed as described by Chasteen & Williams (1981).

The reaction mixture was prepared from a stock apo-ovotransferrin solution (4 mg/ml) and stock Hepes buffer (0.133 M, pH 7.4). The latter was diluted to 0.1 M and the pH checked. Diluted buffer (5 ml) was mixed with stock protein solution (5 ml) and the pH checked again.

In the dark, 0.1 M-sodium metaperiodate solution (50 μl) was added to the buffered protein solution. Samples (0.5 ml) were withdrawn at intervals and immediately mixed with aq. 10% (v/v) ethan-1,2-diol (5 μl) and 0.1 M-FeNTA (5 μl) to quench the reaction and load all unmodified protein sites with iron. Saturated NaHCO_3 solution (5 μl) was added to each sample, followed by urea/gel application buffer (0.5 ml). Samples (10 μl) were applied to a gel in 1 cm slots.

Effects of salts. A range of sodium salts was used. The above method was followed except that the required amount of salt was dissolved in the stock Hepes buffer.

Effect of pH. Three buffers were used containing 0.1 M-Hepes (pK 7.4 at 25 °C), 0.1 M-Mes (pK 6.1 at 25 °C), and 0.05 M-Hepes + 0.05 M-Mes. For each buffer

the pH was adjusted with concentrated NaOH solution and two samples (0.5 ml) removed at each pH required. Stock apo-ovotransferrin (0.5 ml) was mixed with each sample. Periodate oxidation and gel electrophoresis were carried out as described above, except that the pH was adjusted to 8.0 by the addition of 0.2 M-NaHCO₃, pH 8.0, before loading with iron.

RESULTS AND DISCUSSION

A large excess of periodate anions over protein was used and the results are presented (Table 1) in terms of a pseudo-first-order rate constant, k . This Table shows the effects of various anions on the rate of modification of apo-ovotransferrin as k is the sum of the rate constants for the attack by periodate on the *N*- and C-domain iron-binding sites.

The lyotropic series represents a purely electrostatic ranking of ions and does not consider any other properties, such as size and shape. In spite of this, the series is consistent with the interactions between simple inorganic anions, complex proteins and aqueous solvent that result in the 'salting-in' and 'salting-out' effects for example (Hatefi & Hanstein, 1969). Furthermore it has been shown that the series matches the relative strengths of interaction between anions and individual arginine residues in proteins (Pande & McMenamy, 1970; Jonas & Weber, 1971; Norne *et al.*, 1975a,b). The lyotropic number, N , is an empirical evaluation of lyotropic activity based upon several different phenomena, including salting-out, swelling and gelation of lyophilic colloids, viscosity of salt solutions, rates of reactions, and flocculation of lyophobic colloids (Voet, 1932).

The electrostatic attraction of anions to the iron-

Fig. 1. Dependence of k on lyotropic activity

The graph shows the marked deviation of complex anions from the lyotropic series, resulting in a reduced reaction rate. Simple anions, exerting a purely lyotropic effect, give a near linear correlation of k with N as shown (---). Values of k were determined as described in the text and the letters refer to the anions labelled in Table 1.

Table 1. Effect of anions on k

The amount of unmodified transferrin present during the reaction with periodate anions in the presence of various salts was estimated as described in the text and the rate constants were determined from a semi-logarithmic analysis of the data. Errors are given as ± 1 S.D., derived from performing each experiment three times. Bond lengths and angles are taken from crystal-structure data given by Mitchell & Cross (1958, 1965) and the inter-oxygen distances are calculated from these values. The alphabetical labelling of the anions is used in Fig. 1.

Anion	Concn. (M)	$10^3 k$ (s ⁻¹)	Inter-oxygen distance (nm)
a. Mes	0.05	17.8 ± 1.62	—
b. Hepes	0.05	14.0 ± 0.59	—
c. F ⁻	0.10	11.9 ± 0.53	—
d. Cl ⁻	0.10	10.9 ± 0.32	—
e. Ac ⁻	0.10	9.53 ± 1.65	2.32
f. Br ⁻	0.10	7.61 ± 0.57	—
g. ClO ₄ ⁻	0.10	4.93 ± 0.46	2.34
h. SCN ⁻	0.10	3.13 ± 0.18	—
i. HCO ₃ ⁻	0.10	3.11 ± 0.11	2.27
j. NO ₃ ⁻	0.10	2.58 ± 0.19	2.11
k. IO ₃ ⁻	0.10	1.78 ± 0.13	2.77
l. SO ₄ ²⁻	0.10	0.80 ± 0.09	2.47
m. HPO ₄ ²⁻	0.10	0.73 ± 0.06	2.52
n. 2,3-DPG	0.001	1.71 ± 0.11	—
o. PP _i	0.001	1.40 ± 0.10	—

binding sites of apo-ovotransferrin, as proposed by Geoghegan *et al.* (1980) for the attraction of periodate, leads to inhibition of the periodate reaction.

Fig. 1 shows that the inhibitory effects of halide anions do follow the lyotropic series, allowing a direct correlation of k with the lyotropic number, N ; this result is consistent with a pure electrostatic attraction. In addition Williams *et al.* (1982) have suggested that the binding of salts by diferric human transferrin induces a conformational change in the iron-binding sites, which is far greater at pH 8.5 than at pH 7.0. This may alter the relative positions of basic residues and tyrosine residues thereby changing the reaction rate.

Inhibition: PP_i > 2,3-DPG > HPO₄²⁻ > SO₄²⁻ > IO₃⁻ > NO₃⁻ > HCO₃⁻ > SCN⁻ > ClO₄⁻ > Br⁻ > Ac⁻ > Cl⁻ > F⁻

Lyotropic series: SCN⁻ > ClO₄⁻ > NO₃⁻ > PP_i, Br⁻ > Cl⁻ > HPO₄²⁻ > SO₄²⁻, HCO₃⁻, Ac⁻ > IO₃⁻ > F⁻

The low value of k for complex anions indicates that stereochemical factors may be involved, such as the ability to bind more than one basic residue. Anions similar to periodate in structure demonstrate the strongest inhibition (Table 1), which supports the hypothesis that the periodate anion is an affinity reagent for the iron-binding sites (Geoghegan *et al.*, 1980), and the degree of inhibition can be understood as a function of both lyotropic activity and structural similarity to the periodate anion.

Fig. 2. Effect of pH on the rate of modification of apo-ovotransferrin

The concentration of unmodified ovotransferrin was estimated by scanning urea/polyacrylamide gels of samples stained with Coomassie Brilliant Blue R as the A_{560} is directly proportional to the concentration of protein. Samples were removed from a reaction mixture containing 2 mg of apo-ovotransferrin/ml, 0.5 mM- NaIO_4 , 25 mM-Hepes and 25 mM-Mes 2 min after the addition of NaIO_4 solution, and quenched with ethan-1,2-diol. Iron was loaded and samples fractionated as described in the text.

The interaction between ovotransferrin and various complex anions may therefore require more than a single basic residue. The work of Norne *et al.* (1975a, b) led to the conclusion that anion-binding sites in proteins are often complex and may involve several positively charged residues. In such cases the stereochemical properties of the anions are important in addition to their charge, so the relative inhibition shown by complex anions is a reflection of the tertiary structure of the protein.

The relationship of pH to reaction rate was studied in order to see if histidine residues, known to reside at or near the iron-binding sites (Bezkorovainy & Grohlich, 1971; Krysteva *et al.*, 1975; Mazurier *et al.*, 1977; Zweier & Aisen, 1977; Zweier *et al.*, 1979), are important. Azari & Phillips (1970) found that 3 mol of tyrosine/mol of ovotransferrin were modified by periodate anions at pH 8.5 in bicarbonate buffer, but this was raised to 5 mol of tyrosine at pH 5.0 in acetate buffer. Geoghegan *et al.* (1980) suggest that this pH-dependence is due to an increased protonation of histidine residues at the lower pH, which enhances the electrostatic attraction of periodate anions to the iron-binding sites, but the results presented here show that carbonate is a stronger inhibitor of the reaction than acetate at pH 7.4. It is therefore necessary to study the pH-dependence using the same buffer at each pH. Hepes and Mes are shown to inhibit very weakly (Table 1) and the three buffers used gave similar results, showing little contribution from the ionization of the buffer itself.

Over the pH range studied, ovotransferrin retains its iron-binding ability and the rate maximum at pH 7–8 (Fig. 2) implies that there are at least two pH-dependent effects. The increase in rate from pH 8.5 to pH 7.5 may be due to the hydration and ionization of the periodate anion to an unreactive, octahedral form, $\text{H}_3\text{IO}_6^{2-}$ ($\text{p}K = 8.36$; Crouthamel *et al.*, 1949) and the increasing positive charge on histidine residues at the iron-binding sites. Alsaadi *et al.* (1981) have assigned $\text{p}K$ values of 7.50 and 7.70 to histidine residues involved in liganding iron and the anion respectively, the former being lowered to 7.30–7.40 in the presence of complex anions. The opposing effect may be a consequence of a pH-dependent conformational change in the iron-binding sites (Chasteen & Williams, 1981; Baldwin *et al.*, 1982).

In summary, there may be a strong binding site for complex anions on ovotransferrin that involves histidine residues and is near to a pair of tyrosine residues important in iron binding. These features are present in a model of the iron-binding site given by Windle *et al.* (1963). The identity of the reactive tyrosine residues has not yet been established, but it appears that the modification is specific to four tyrosine residues, as periodate appears to selectively cross-link only two tyrosine residues in the C-domain (J. J. Hsuan, unpublished work). If periodate does selectively modify four tyrosine residues, it remains possible that their reactivity is not merely a consequence of proximal basic residues; for example, their relative configuration may allow an intramolecular cross-linking reaction to occur, which is impossible for distant tyrosine residues, but is an established reaction of periodate with free tyrosine (Tashiro, 1963, 1966) and with vicinal thiols in proteins (Rippa *et al.*, 1981).

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Received 4 November 1985/11 July 1986; accepted 15 July 1986