The effect of salt concentration on the iron-binding properties of human transferrin

John WILLIAMS,* N. Dennis CHASTEEN† and Kathleen MORETON*

*Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K., and †Department of Chemistry, University of New Hampshire, Durham, NH 03824, U.S.A.

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The salt dependence of the iron-binding properties of transferrin was studied by urea/polyacrylamide-gel electrophoresis. The distribution of iron between the *N*-terminal and *C*-terminal binding sites under equilibrium conditions and the rates of release of iron from the two sites were studied. It was found that salt increases the thermodynamic stability of iron binding in the *N*-terminal site relative to the *C*-terminal site. Similar behaviour is observed for the kinetics of iron release, where salt retards the rate of removal of iron from the *N*-terminal site but facilitates removal from the *C*-terminal site.

The relative affinities of the two metal-binding sites of human transferrin for iron and the cooperativity of iron binding between the sites, under equilibrium conditions, appear to be influenced by environmental factors, since we recently showed that pH plays an important role (Chasteen & Williams, 1981). Earlier it was found that dialysis of serum markedly affects the distribution of iron between the two sites (Williams & Moreton, 1980). Thus, in undialysed serum incubated at 37°C, iron is bound mainly to the *N*-terminal site, but after dialysis against buffer at pH 7.4 iron is bound preferentially by the *C*-terminal site. Although the pH of the undialysed serum was not controlled in these experiments, the result suggested the possibility that the iron-binding properties of transferrin might be influenced by salt concentration. This possibility is confirmed by the experiments described in the present paper which show that salt affects the stabilities of the *N*-terminal and *C*-terminal iron complexes in opposite directions. Recent studies by Baldwin (1980, 1981) are in accord with these findings.

Materials and methods

Preparation of transferrin

Human transferrin was prepared from outdated plasma by ion-exchange chromatography or by

immuno-affinity chromatography (Chasteen & Williams, 1981).

Preparation of samples

To determine the distribution of iron between the two binding sites of transferrin at different salt concentrations, 50% iron-saturated transferrin (prepared by mixing equal amounts of iron-free transferrin and iron-saturated transferrin) was dissolved at 2 mg/ml in a solution with the following composition: 1 mM-trisodium citrate (to act as mediator), 1 mM-NaHCO₃, 0.05 M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer (pH 7.00 or 7.45 at 37°C) or 0.05 M-Hepps [4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid]/NaOH buffer (pH 8.4 at 37°C) and different salts at concentrations up to 1.0 M. The solutions were incubated at 37°C and samples were removed at 3-day intervals until there was no further change in the distribution of iron as judged by urea/polyacrylamide-gel electrophoresis (Chasteen & Williams, 1981). The pH of the solution at 37°C was checked with an Ag/AgCl microelectrode.

Electrophoresis of samples and estimation of the distribution of iron between the two binding sites was carried out as described by Chasteen & Williams (1981). The results are expressed as the site preference factor k'_{1N}/k'_{1C} ($= [\text{FeTf}]/[\text{TfFe}]$) and the co-operativity factor k'_{2N}/k'_{1N} ($= k'_{2C}/k'_{1C} = [\text{Tf}][\text{FeTfFe}]/[\text{FeTf}][\text{TfFe}]$) where k'_{1N} , k'_{2N} , k'_{1C} and k'_{2C} are the four conditional site constants for the stepwise binding of iron to the two sites of transferrin (Chasteen & Williams, 1981). In that paper the third term in the denominator of eqn. (A-14) should contain k'_{2C}

Abbreviations used; Tf, apotransferrin; FeTf, *N*-terminal monoferric transferrin; TfFe, *C*-terminal monoferric transferrin; FeTfFe, diferric transferrin.

instead of k'_{IC} and k_{IC} of eqn. (B-11) should be primed. In preliminary experiments the effect of salt concentration upon the conversion of TfFe into FeTf was studied, but it was later found that a mixture of Tf and FeTfFe attained equilibrium much more quickly than a mixture of Tf and TfFe.

The pH of serum which has been stored at -15°C was found to be 8.0–8.4. In order to determine the equilibrium distribution of iron in serum at physiological pH serum was incubated at 37°C under a vigorous flow of CO_2/O_2 (1:19). Growth of bacteria was prevented by the addition of rifampicin at $20\text{ }\mu\text{g/ml}$. The pH was monitored continuously and remained at 7.45 during incubation for 8 days. Alternatively, the pH of serum was maintained at 7.5 with 0.1 M-Mops (4-morpholinepropanesulphonic acid)/NaOH buffer.

Rate of iron removal from transferrin

In these experiments a 'mediating' substance was used to facilitate the removal of iron from iron-saturated transferrin and desferrioxamine was present to trap the dissociated iron. The mediators used were sodium citrate, 2,3-diphosphoglycerate, nitrilotriacetate, pyrophosphate and EDTA. Incubation mixtures contained FeTfFe (2 mg/ml), 1 mM-mediator, 1 mM-desferrioxamine ('Desferal', Ciba-Geigy, Basle, Switzerland), 0.05 M-Mops/NaOH buffer, pH 7.4, and NaCl at concentrations up to

where the concentrations as a function of time have the same meaning as before. $[\text{FeTf}]_0$ and $[\text{TfFe}]_0$ account for small amounts of monoferric species present initially. The ratio of rate constants is given by the above expression provided that significant amounts of the monoferric species do not react further to form apotransferrin. This was true for the early stage of the reaction and verified by the near constancy of the k_C/k_N ratio with time. The individual k_N and k_C values were calculated by combining the average value of k_C/k_N for different times with the value of k_{total} .

Results

Iron distribution in serum

In serum maintained at pH 7.5 with Mops buffer, the site preference factor $k'_{IN}/k'_{IC} = 0.76 \pm 0.16$, but when 5% CO_2 as the gas phase was used (pH 7.45), $k'_{IN}/k'_{IC} = 0.49 \pm 0.03$. When the pH of serum was allowed to rise to 8.0, there was marked preference for iron-binding to the N-terminal site ($k'_{IN}/k'_{IC} = 1.7$).

Effect of salt concentration on equilibria of iron distribution

Fig. 1 shows the effect of NaCl concentration on the site preference factor at three pH values. The equations of the regression lines are as follows.

$$\text{At pH 7.0, } k'_{IN}/k'_{IC} = 1.19 (\pm 0.21) [\text{NaCl}] + 0.246 (\pm 0.066)$$

$$\text{At pH 7.45, } k'_{IN}/k'_{IC} = 4.13 (\pm 0.69) [\text{NaCl}] + 0.454 (\pm 0.213)$$

$$\text{At pH 8.4, } k'_{IN}/k'_{IC} = 7.69 (\pm 1.28) [\text{NaCl}] + 1.85 (\pm 0.25)$$

0.5 M. In some experiments NaF was used instead of NaCl. Samples were taken for analysis for Tf, FeTfFe, TfFe and FeTf by urea/polyacrylamide-gel electrophoresis (Chasteen & Williams, 1981) at the starting time and after incubation at 37°C for 1, 2 and 3 h. In these experiments iron is removed effectively irreversibly as it dissociates from the transferrin complexes.

The rate constant k_{total} for the disappearance of FeTfFe was obtained from first-order plots of $\log[\text{FeTfFe}]_t/[\text{FeTfFe}]_0$ versus time, where $[\text{FeTfFe}]_0$ is the initial concentration of FeTfFe at time $t = 0$ and $[\text{FeTfFe}]_t$ is the concentration at time t . The slope ($-k_{\text{total}}$) of the regression line is equal to $-(k_N + k_C)$ where k_N and k_C are the rate constants for the removal of iron from the N- and C-terminal sites respectively. Correlation coefficients of greater than 0.97 were generally obtained from the regression analyses. The ratio of rate constants was determined from the relationship:

$$\frac{k_C}{k_N} = \frac{[\text{FeTf}]_t - [\text{FeTf}]_0}{[\text{TfFe}]_t - [\text{TfFe}]_0}$$

At pH 7.45, 0.125 M-NaCl, conditions which are near to those found in serum, $k'_{IN}/k'_{IC} = 0.95$. NaCl clearly favours the binding of iron to the N-terminal site and the magnitude of the salt dependence is a function of pH.

Table 1 shows that the sodium salts of a variety of monovalent and divalent anions are effective at pH 7.4 in promoting preferential iron-binding at the N-terminal site, i.e. $k'_{IN}/k'_{IC} > 1.0$. Sodium salts were chosen for this study because Na^+ is the principal cation present in serum. In addition, Na^+ interacts only weakly with proteins (Record *et al.*, 1978). The chaotropic perchlorate ion (Table 1) is much more effective than the others tested ($P < 0.001$). Except for NaBr there appears to be little difference between the effectiveness of the remaining salts. There is no obvious trend.

The co-operativity factor k'_{2N}/k'_{IN} is difficult to determine with good precision since it depends on the ratio of four experimental quantities. Within experimental error, no effect of salt concentration on the small negative co-operativity of iron binding, $k'_{2N}/k'_{IN} \sim 0.7$, was observed for the protein samples

Fig. 1. Effect of NaCl concentration on the site preference factor k'_{1N}/k'_{1C} for equilibrated transferrin at three different values of pH

See the Materials and methods section for conditions.

used in this work. Previously we found that 0.5 M NaCl slightly increased k'_{2N}/k'_{1C} from ~ 0.7 to ~ 0.95 at pH 7.4 (Chasteen & Williams, 1981). In either case, iron binding to the two sites occurs largely independently of one another under these conditions.

Effect of salt on the rate of removal of iron from transferrin

Fig. 2 shows the rate constant (k_{total}) for the disappearance of FeTfFe at different NaCl concentrations for various mediators. Figs. 3 and 4 show the rate constants for the loss of iron from the N-terminal and C-terminal sites (k_N and k_C , respectively). Linear or near linear curves are obtained in most cases. Table 2 shows the equations of the linear regressions of the rate constants as functions of NaCl concentration for the different mediators. These lines serve as a basis for comparing the effect of salt in the presence of different mediators. It can be seen that the most effective mediators (i.e. large intercepts) are also the ones with the greatest dependence of k_N and k_C on NaCl concentration (i.e. large slopes). For k_C they follow the trend: pyrophosphate > nitrilotriacetate > citrate > 2,3-bisphos-

Table 1. Dependence of the site preference and cooperativity factors upon salt concentration

The experimental conditions are 1 mM NaHCO₃, 0.1 M-Hepes/NaOH pH 7.4, transferrin (equal parts of FeTfFe and Tf) 2 mg/ml, 1 mM-sodium citrate, varied salt concentrations as shown, 37°C. Samples marked (*) are controls.

Salt	Concn. (M)	k'_{1N}/k'_{1C}	k'_{2N}/k'_{1N}
NaF	0.0*	0.32	0.75
	0.1	0.83	0.67
	0.2	1.32	0.62
	0.3	1.50	0.68
	0.5	1.90	0.60
NaBr	0.0*	0.31	0.78
	0.1	0.79	0.48
	0.2	1.16	0.58
	0.3	1.34	0.56
	0.5	1.71	0.62
NaI	0.0*	0.31	0.78
	0.1	0.89	0.64
	0.2	1.30	0.70
	0.3	1.75	0.69
	0.5	2.53	0.55
NaNO ₃	0.0*	0.33	0.71
	0.1	1.19	0.84
	0.2	1.59	0.74
	0.3	1.84	0.65
	0.5	2.31	0.68
Na ₂ SO ₄	0.0*	0.32	0.75
	0.1	0.94	0.68
	0.2	1.33	0.76
	0.3	1.92	0.70
	0.5	2.69	0.62
NaClO ₄	0.0*	0.32	0.75
	0.1	2.28	0.59
	0.2	3.21	0.64
	0.3	3.92	0.75
	0.5	5.47	0.79

phoglycerate ~ EDTA and for k_N : pyrophosphate > 2,3-bisphosphoglycerate > nitrilotriacetate ~ citrate ~ EDTA.

For all the mediators used, k_C increases with increasing salt concentration (Fig. 4) while k_N decreases (Fig. 3). With citrate as mediator the opposite effects of NaCl upon the two sites approximately cancel out, so that k_{total} shows little change with increasing NaCl concentration (Fig. 2). With pyrophosphate, nitrilotriacetate and EDTA the effect of NaCl on k_C was greater than on k_N so that k_{total} increased with increasing NaCl concentration. With 2,3-bisphosphoglycerate the greatest effect of NaCl was on k_N causing k_{total} to decrease with increasing NaCl concentration.

In the absence of added mediator both rate constants increase slightly with increasing NaCl concentration (lines *f* in Figs. 3 and 4) suggesting that

Fig. 2. Effect of NaCl concentration on the first-order rate constant k_{total} for the disappearance of FeTfFe. See the Materials and methods section for conditions. Straight lines are linear regressions. The mediators were (a) pyrophosphate, (b) nitrilotriacetate, (c) bisphosphoglycerate, (d) citrate, (e) EDTA, (f) no mediator. The concentration of mediators was 1 mM.

Fig. 3. Effect of NaCl concentration on the first-order rate constant k_N for removal of iron from the N-terminal binding site of FeTfFe.

See the Materials and methods section for conditions. Straight lines b, d, and e are linear regressions. The mediators were (a) pyrophosphate, (b) nitrilotriacetate, (c) bisphosphoglycerate, (d) citrate, (e) EDTA, (f) no mediator. The concentration of mediators was 1 mM.

Cl^- itself can act as a very poor mediator. When NaF was used in place of NaCl and in the absence of mediator, the rate constants likewise increased at both sites but much more than for Cl^- (Table 3) showing that fluoride is an effective mediator.

Discussion

The kinetic results described here for FeTfFe show that chloride facilitates the removal of iron from the C-terminal site of transferrin but retards its removal from the N-terminal site (Figs. 3 and 4). Baldwin (1981) has recently published

similar observations with the monoferric transferrins. The kinetic results are in complete agreement with influence of Cl^- and other anions on the equilibrium distribution of iron between the sites, since increasing salt concentration causes iron to be more tightly bound to the N-terminal site than to the C-terminal site (Fig. 1, Table 1). Thus the thermodynamic and kinetic properties of the two sites show parallel behaviour with respect to salts.

From the equilibrium distributions of iron at different pH values it is seen that salt has much more

Fig. 4. Effect of NaCl concentration on the first-order rate constant k_C for removal of iron from the C-terminal binding site of FeTfFe

See the Materials and methods section for conditions. Straight lines *b*, *c*, *d* and *e* are linear regressions. The mediators were (a) pyrophosphate, (b) nitrilotriacetate, (c) bisphosphoglycerate, (d) citrate, (e) EDTA, (f) no mediator. The concentration of mediators was 1 mM.

or more anions to groups bearing fixed positive charges or to amide dipoles in the 'high-pH' conformational state of each domain of the protein. Approx. 17 Cl^- ions are believed to bind to apo-ovotransferrin with association constants of approx. 10M^{-1} (Wishnia *et al.*, 1961) and the binding of Cl^- and other anions to FeTfFe has been demonstrated by e.p.r. studies (D. A. Folajtar & N. D. Chasteen, unpublished work). The binding of the anion may then cause a further conformational change which is associated with a change in the tightness with which iron is bound. This suggestion may be represented by the following scheme:

where S, S' and S'' are different conformations of the N-terminal and C-terminal domains and the relative affinities of the different conformations for iron vary in the sequence $\text{S}''_N/\text{S}'_N > \text{S}'_N/\text{S}_N > \text{S}_N/\text{S}_C$. Baldwin (1980) in his study of the effects of salts and detergents on the kinetics of iron release by FeTfFe to EDTA has likewise proposed that transitions between conformational states are important. The influence of perchlorate on the e.p.r. spectrum of the iron centres has been attributed to changes in conformation (Price & Gibson, 1972). In the absence of detailed information about the chemical structures of the metal-binding domains it is not possible to explain why anion binding exerts opposite effects at the two sites.

Morgan *et al.* (1978) found that 2,3-bisphosphoglycerate released iron more rapidly from the N-terminal site of human transferrin than from the C-terminal site. The present work confirms this result for 0.15 M-NaCl but shows that the relative rates of iron removal are not constant but are salt dependent. Depending on the salt concentration, the relative rates can be reversed.

Pyrophosphate is one of the most effective substances known for mediating iron removal from transferrin *in vitro* (Morgan, 1979). We find this also. The rate of iron removal follows saturation kinetics with respect to pyrophosphate and other mediators, for which values of K_m and $V_{\max}$ have been obtained (Morgan, 1979). Morgan's experiments suggest that an intermediate complex(es) between the mediator and transferrin is formed in the reaction pathway. The stabilization or destabilization of such a complex relative to the reactants by the binding of Cl^- to the protein would be expected to lead to an increase or decrease in the 'first-order' rate constants measured in our experiments. This we observe for k_C and k_N respectively. Furthermore, if the concentration of such a complex governs the overall rate of iron removal, then a salt effect on complex formation would be expected to

effect at pH 8.4 than at pH 7.0 (Fig. 1). Chasteen & Williams (1981) have suggested that pH changes alone induce conformational changes in the iron-binding sites so that in the absence of salt at pH 7.0 $k'_{1N}/k'_{1C} = 0.1$ but at pH 8.5 $k'_{1N}/k'_{1C} = 1.0$. It seems likely that the salt effects described here are due largely to the binding of one

Table 2. Equations of linear regressions of rate constants (h^{-1}) for the removal of iron from transferrin as functions of NaCl concentration at pH 7.4

The table gives the values of the parameters m and b in the equation:

$$k = m[\text{NaCl}] + b$$

Values show the 95% confidence interval.

Mediator	Parameter	k_{total}	k_N	k_C
Citrate	m	0.006 ± 0.011	-0.245 ± 0.169	0.233 ± 0.044
	b	0.189 ± 0.012	0.181 ± 0.029	0.023 ± 0.007
2,3-Bisphosphoglycerate	m	-0.460 ± 0.373	-0.626 ± 0.346	0.167 ± 0.055
	b	0.371 ± 0.064	0.348 ± 0.059	0.024 ± 0.010
Pyrophosphate	m	0.748 ± 0.557	-2.19 ± 1.80	3.11 ± 1.74
	b	1.60 ± 0.09	0.999 ± 0.255	0.580 ± 0.247
Nitrilotriacetate	m	0.997 ± 0.247	-0.08 ± 0.13	1.08 ± 0.20
	b	0.409 ± 0.042	0.212 ± 0.022	0.196 ± 0.034
EDTA	m	0.186 ± 0.103	0.062 ± 0.080	0.124 ± 0.03
	b	0.066 ± 0.018	0.049 ± 0.014	0.017 ± 0.005
None	m	0.171 ± 0.014	0.076 ± 0.011	0.094 ± 0.022
	b	0.008 ± 0.002	0.008 ± 0.002	-0.001 ± 0.004

Table 3. Rate constants (h^{-1}) for removal of iron from transferrin in presence of NaF and with no added mediator

[NaF] (M)	k_{total}	k_N	k_C
0	0.067	0.029	0.038
0.1	0.101	0.042	0.059
0.2	0.246	0.113	0.133
0.3	0.474	0.214	0.260

have a larger effect on the better mediators, as is observed. There are, however, other possible explanations of our observations. A fuller understanding of the mechanism of iron release from transferrin and the role that salts play in this process will require additional experimentation.

The fact that F^- is an effective mediator demonstrates that mediator substances need not be bi-functional reagents. The far superior mediating ability of F^- over Cl^- is consistent with the much higher affinity of F^- for Fe(III) compared with Cl^- (Smith & Martell, 1976).

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