Site specificity of iron removal from transferrin by α-ketohydroxypyridine chelators

George J. Kontoghiorghes and Robert W. Evans[+]

Department of Haematology, Royal Free Hospital Medical School Hampstead, London NW3 2QG, and [+]Department of Biochemistry, Guy's Hospital Medical School (UMDS), London SE1 9RT, England

The site specificity of the removal of iron from diferric human transferrin, at pH 7.4, by two α-ketohydroxypyridine chelators, 1,2-dimethyl-3-hydroxypyrid-4-one (L_1) and mimosine, has been investigated using urea-polyacrylamide gel electrophoresis. Chelator L_1 removes iron preferentially from the C-terminal site whereas mimosine shows a small preference for iron in the N-terminal site. The removal of iron has also been followed spectrophotometrically and by monitoring the loss of ^{59}Fe from [^{59}Fe]transferrin. Both chelators are able to remove iron completely from diferric transferrin without additional mediators or reducing agents.

Transferrin   *Iron mobilisation*   *Site specificity*   *α-Ketohydroxypyridine chelators*   *Mimosine*

1. INTRODUCTION

Human serum transferrin belongs to a family of iron-binding proteins found in blood, egg-white and secretory fluids. The transferrins are all single-chain polypeptides of M_r ~80000 and possess 2 iron-binding sites which reside in separate domains. Studies on the physico-chemical properties of the metal-binding sites in a number of transferrins have revealed differences in the iron-binding constants [1], the uptake of iron from iron-chelator complexes [2,3] and the release of iron [4]

Abbreviations: Tf, transferrin; Fe_2-Tf, diferric transferrin; Fe-Tf, N-terminal monoferric transferrin; Tf-Fe, C-terminal monoferric transferrin; NTA, nitrilotriacetate; FeNTA, iron(III) nitrilotriacetate; LICAMS, 1,5,10,N',N'' - tris(5-sulfo - 2,3 - dihydroxybenzoyl)triazadecane; MECAM, 1,3,5-N,N',N''-tris(2,3-dihydroxybenzoyl)-aminomethylbenzene; EDTP, ethylenediamine - N,N' - tetra(methylenephosphonic acid); NTP, nitrilotri-(methylenephosphonic acid); HEDP, 1-hydroxyethane-1,1-diphosphonic acid

but it is still uncertain whether these observed differences are of physiological significance.

Iron mobilisation from transferrin at physiological pH is of great importance to those designing iron chelators intended for the treatment of iron overload and other diseases of iron imbalance [5]. Desferrioxamine, the chelator currently used subcutaneously for the treatment of transfusional iron overload, cannot mobilise significant amounts of iron from transferrin unless mediators such as citrate or NTA are present [6]. Several new α-ketohydroxy pyridine chelators (e.g., 1,2-dimethyl-3-hydroxypyrid-4-one, L_1) were shown to be orally effective in the removal of iron from animals [5,7]. In vitro experiments at physiological pH showed that these chelators removed iron from transferrin and ferritin. In the experiments with transferrin biphasis reactions were observed and each phase corresponded to the reaction of the chelator with one of the binding sites of transferrin [5]. Other chelators found to be effective in the mobilisation of iron from transferrin at physiological pH include the 2-hydroxy-pyridine *N*-oxide derivatives [5], the hexadentate

catechol-type chelators LICAMS and MECAM [8] and the amino alkyl phosphonic acid-derivatives EDTP, NTP and HEDP [9].

Mimosine is a naturally occurring plant product, the chemical structure of which is based on an α-ketohydroxy metal-chelating site on a pyridine ring, similar to L_1, but with an amino acid side-chain substituent. Although its chelating properties [10] and effects on some proteins [11] and animals [12] are well known, no correlation between all these has been established.

In an attempt to correlate the relationship between the chemical structure of the 2 chelators, L_1 and mimosine, and transferrin site specificity in iron removal, polyacrylamide gel electrophoresis in 6 M urea has been used here. This method resolved partially iron-saturated human transferrin into 4 bands [13] corresponding to the iron-free protein, the C-terminal monoferric protein, the N-terminal monoferric protein and the diferric protein in order of increasing mobility [2]. The removal of iron from transferrin by the 2 chelators has also been followed by monitoring the spectral changes which accompany the reactions and by measuring the release of ^{59}Fe from ^{59}Fe-labelled diferric protein.

2. MATERIALS AND METHODS

L_1 was prepared as previously described [5]. Mimosine (β-[N-(3-hydroxypyrid-4-one] α-aminopropionic acid) was purchased from Sigma.

Human serum transferrin was isolated from fresh plasma by immunoaffinity chromatography [14]. The protein, in 0.1 M NaHCO$_3$, was made iron-saturated by addition of excess iron nitrilotriacetate. Unbound iron was removed by gel filtration on Sephadex G-25 equilibrated with 50 mM NH$_4$HCO$_3$ and the protein was then freeze-dried.

Diferric human transferrin (32.5 μM) was taken up in 50 mM Tris-HCl, pH 7.4, and incubated at 20°C with iron-free chelators (4 mM). The removal of iron from the protein was followed spectrophotometrically using a Pye Unicam SP 1800 spectrophotometer. Samples were taken over a period of about 4 h and applied to a polyacrylamide gel containing 6 M urea. Polyacrylamide gels were stained with PAGE blue 83 (BDH) and after destaining were scanned at

570 nm on a Camlab EC 910 Transmission Densitometer. The relative amounts of the 4 transferrin bands were determined by cutting out and weighing the peaks from the densitometric traces and these values were then used to calculate the iron-saturation of each sample.

[^{59}Fe]Transferrin was prepared as described [5] by mixing [^{59}Fe]NTA (5 μCi) in 0.1 M Tris-HCl, pH 7.4, with apotransferrin (Sigma) in the same buffer which also contained 10 mM NaHCO$_3$, and in the ratio of 1.4 μg : 1 mg apotransferrin, for 100% iron saturation. The mobilisation of ^{59}Fe from [^{59}Fe]transferrin was studid by dialysing a fresh solution of the labelled protein (1.5 ml) against 15 ml of the chelator (4 mM) in 0.1 M Tris-HCl, pH 7.4, in a stoppered glass tube containing a magnetic flea. The amount of ^{59}Fe in the dialysate and the dialysis bag was determined using an LKB Wallac 1280 Ultragamma counter.

3. RESULTS AND DISCUSSION

Figs 1 and 2 show that under the conditions of the experiments both L_1 and mimosine are able to remove iron completely from diferric human transferrin, at pH 7.4, within 4 h. It is apparent
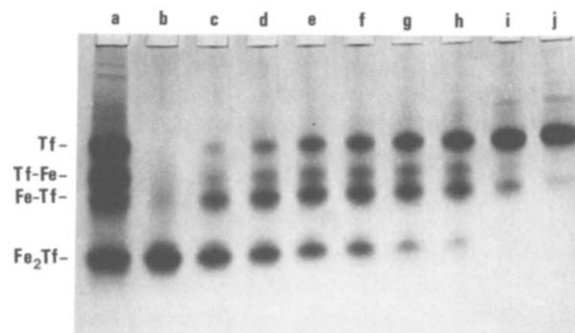

Fig.1. Urea-polyacrylamide gel electrophoresis of diferric human transferrin incubated with chelator L_1 (1,2-dimethyl-3-hydroxypyrid-4-one). (a) Partially iron-saturated sample of transferrin containing iron-free transferrin (Tf), C-terminal monoferric transferrin (Tf-Fe), N-terminal monoferric transferrin (Fe-Tf), and diferric transferrin (Fe$_2$Tf). (b–j) Samples taken from reaction mixture at (b) 0, (c) 5, (d) 10, (e) 16, (f) 20, (g) 30, (h) 40, (i) 88, (j) 200 min.
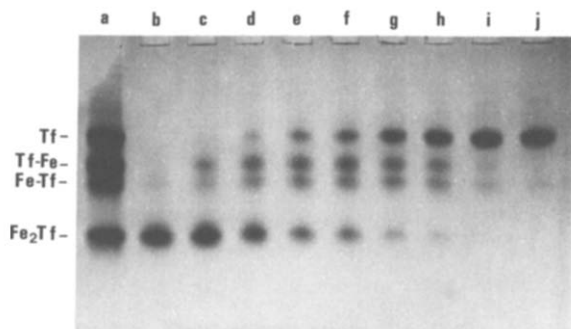
Fig.2. Urea-polyacrylamide gel electrophoresis of diferric human transferrin incubated with mimosine. (a) Partially iron-saturated sample of transferrin containing iron-free transferrin (Tf), C-terminal monoferric transferrin (Tf-Fe), N-terminal monoferric transferrin (Fe-Tf) and diferric transferrin (Fe_2Tf). (b–j) Samples taken from reaction mixture at (b) 0, (c) 5, (d) 10, (e) 16, (f) 20, (g) 30, (h) 40, (i) 80, (j) 110 min.

Fig.3. Time dependence of removal of iron from diferric human transferrin by chelator L_1 (1,2-dimethyl-3-hydroxypyrid-4-one). Results obtained from (△) change in absorbance at 460 nm and (○) quantitation of urea-polyacrylamide gels.

that the removal of iron is not a random process and the 2 chelators remove iron preferentially from different sites on the protein. In particular L_1, which was shown previously to react in a biphasic process with diferric transferrin [5], removes iron preferentially from the C-terminal site (fig.1), the thermodynamically more stable one [1,2]. Mimosine, however, shows a slight preference for iron in the N-terminal site (fig.2). This ability of mimosine to remove iron from transferrin may be partly associated with the toxic effects of this plant product [12].

When iron is transferred from diferric transferrin to L_1 there is an increase in absorbance at 460 nm which corresponds to the formation of the chelator-iron complex [5]. A similar increase has now been observed using mimosine. This increase, when normalised, is a measure of the percentage loss of iron from the protein. The time-dependent changes in the iron saturation of the transferrin, as determined from the densitometrically scanned urea-polyacrylamide gels, and the formation of the iron-chelator complexes, as determined from the absorption spectra, upon incubation with L_1 and mimosine are shown in figs 3 and 4. There is good agreement between the values obtained by the 2 procedures and although the 2 chelators remove iron preferentially from a different site on
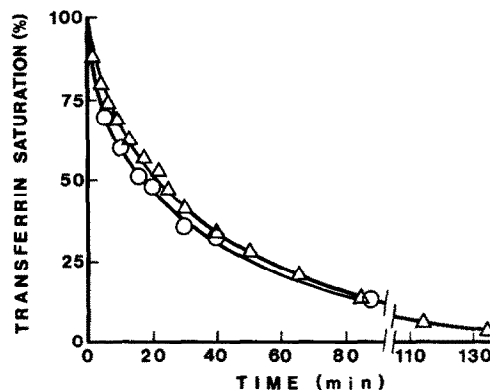
transferrin the overall reaction rates seem to be similar. Furthermore, the results of the dialysis experiments using [^{59}Fe]transferrin (table 1) confirm that both L_1 and mimosine can remove iron efficiently from the iron-saturated protein without the assistance of a mediator or reducing agent.

It is interesting to observe that although the chelating sites of L_1 and mimosine are the same they display different preferences for iron in the 2 sites on human transferrin. These differences may be related to the charge and size of the ring substituents in the two chelators. At physiological pH the 2 methyl substituents cause L_1 to be neutral whereas mimosine has a zwitterionic side chain. Subtle differences between the nature and arrangement of the ligands in the 2 iron-binding sites of transferrin would then be reflected in the way the 2 chelators remove iron from the protein. In a study of the iron-donating properties of NTA and 2 of its analogues [3] differences were observed in the order of occupancy of the N- and C-terminal sites.

The results of this study show that the 2 α-ketohydroxypyridine chelators, L_1 and mimosine, behave differently towards the 2 thermodynamically non-equivalent sites on transferrin although the overall rates of iron removal are very similar. From further studies on the removal of iron from transferrin by other analogues of these chelators it should be possible to correlate chelator structure with site specificity. This information
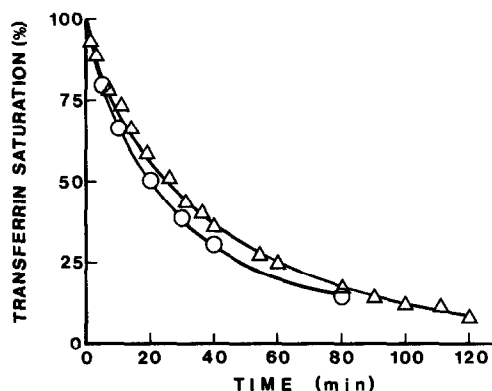
Fig.4. Time dependence of removal of iron from diferric human transferrin by mimosine. Results obtained from (△) change in absorbance at 460 nm and (○) quantitation of urea-polyacrylamide gels.

Table 1

^{59}Fe mobilisation from [^{59}Fe]transferrin

Conditions of experiment	% ^{59}Fe mobilised		
	L_1 (4 mM)	Mimosine (4 mM)	Buffer blank
10 μM [^{59}Fe]transferrin, 4 h, 26°C	89.5	80.0	0.5
5 μM [^{59}Fe]transferrin, 6 h, 26°C	88.0	89.0	1.0

will assist in the design and development of chelators which can be used as probes for studying iron metabolism and for the treatment of iron imbalance.

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