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Iron release from transferrin induced by mixed ligand complexes of copper(II)

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Summary. Copper(II) complexes CuL_1L_2 with the ligand pairs 3-phosphoglycerate (PG)/ethylenediamine (en), phosphoserine (PS)/ethylenediamine, phosphoserine/malonate (mal) are shown to be effective in inducing the release of both iron atoms from di-ferric transferrin (Fe_2Tf; human serum transferrin) at pH 7.3 in 1 M NaCl at 25° C. Half-times of the reaction with $Cu(PG)(en)^-$ were less than 1 min at 0.02 M concentration. The iron(III) products are polynuclear hydroxo complexes. There is weaker interaction with $Cu(PS)_2^{4-}$ and virtually none with Cu(serine)(en) nor Cu(PS)(2,2′-bipyridyl)$^-$, revealing crucial effects of the combined ligand sphere including the phosphomonoester group. The results suggest that the release of iron from Fe_2Tf, or from either monoferric transferrins, occurred due to the breakdown of the stability of iron binding in conjunction with the expulsion of the synergistic anion carbonate (or oxalate). The active copper(II) complexes are postulated to be models of membrane components that could liberate iron from transferrin succeeding its uptake at the receptor sites of cells.

Key words: Transferrin — Iron release — Mixed ligand copper(II) complexes

Introduction

The characteristic properties of transferrin have been thoroughly described by various authors (Aisen and Listowsky 1980; Brock 1985). The most outstanding feature is the binding of two ferric ions coupled compulsorily to simultaneous uptake of two so-called synergistic anions. Under physiological conditions carbonate is the anion preferred by the protein. It has been shown that a series of carboxylate anions such as oxalate, glycolate, and malonate are able to carry out the same function (Schlabach and Bates 1975). The stability of iron binding is limited to pH 6.5–8, otherwise polynuclear species and iron hydroxides are the thermodynamically more stable.

According to recent X-ray results on serum transferrin, in each of the two binding sites, the ferric ion is bound to four protein residues: two tyrosines, one histidine and one aspartate (Bailey et al. 1988). Since the two lobes of the polypeptide chain exhibit small structural differences, there are four chemically distinguishable forms of transferrin (leaving out forms differing in their carbohydrate content): diferric transferrin Fe_2Tf, two monoferric transferrins Fe_CTf and Fe_NTf and apotransferrin (apoTf).

There is a wealth of data on intracellular iron metabolism, most of it obtained from studies on iron consumption by reticulocytes and hepatocytes (Crichton and Chaloteaux-Wauters 1987). Although the transfer of iron from transferrin to the cytoplasm has been a subject of research for many years, it is not yet understood mechanistically. There is common consent that the receptor-mediated uptake at the cell surface is a prerequisite for the release of iron. Whether this step occurs directly at the membrane level or rather after the internalisation of coated pits, whereby the iron would be liberated in a vacuole with acidic pH, will probably continue to be the subject of controversy. Actually, a variety of experiments in this field suggest that the mechanisms used may differ depending on the cell type (Nunez and Glass 1983; Morley and Bezkorovainy 1985).

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Abbreviations: PG, phosphoglycerate; PS, phosphoserine; en, ethylenediamine; Fe_2Tf, diferric transferrin; Fe_CTf and Fe_NTf, transferrin with iron bound to the lobe containing the C- or N-terminus, respectively; apoTf, apotransferrin; K-3, all-*cis*-1,3,5-tris(trimethylammonio)-2,4,6-cyclo-hexanetriol; NTA, nitrilotriacetic acid; bipy, 2,2′-bipyridine; mal, malonate

In some studies it was shown that the iron was released from both binding sites at similar rates following the uptake by the reticulocyte membrane. The relevant half-time was of the order of minutes at most, i.e. much shorter than the time required for the internalization of the Fe_2Tf-receptor system including recycling of apoTf from the cell for re-utilization (Morgan and Baker 1969; Crichton and Chaloteaux-Wauters 1987). This prompted us to search for molecular interactions under in vitro conditions which involved iron release at physiological pH within the range of minutes or less. The internalization hypothesis is less attractive from the point of view of iron uptake by ferritin. It was shown (Schneider 1988) that ferritin is extremely selective with regard to the transient iron species admitted for iron deposition. The most favourable species involving the highest chemical potential is mononuclear $Fe(OH)_3 \cdot aq$, or mononuclear complexes derived from it, such as $FeOH(HPO_4)$. Endosomic release at pH around 5 rather increases the residence time of ferric species in the cytosolic space (Bakkeren et al. 1988). Consequently, for chemical reasons, membrane-induced release would be a superior mechanism with regard to iron storage in ferritin.

Orthophosphate has a weakening effect on iron binding by transferrin. This applies particularly to exchange reactions with potent iron chelators, such as desferal (Morgan 1979). Unpublished experiments of our group with the ligand all-*cis*-1,3,5-tris(trimethylammonio)-2,4,6-cyclohexanetriol (K-3) showed that phosphoric acid monoesters also exhibit this impairing effect. Yet the rate of iron exchange is always slower than in the system K-3/orthophosphate.

The present paper shows that the effect of phosphoric acid monoester can be drastically enhanced by complexation with copper(II) and zinc(II) while nickel(II) is far less effective.

Materials and methods

Deionized water was used throughout. Glassware were washed by 6 M hydrochloric acid prior to use. Buffers and solutions for photometrical measurement were separated from insoluble matter by 0.22-μm membrane filtration (Millipore GS). Analytical polyacrylamide electrophoresis was performed by using a modified Makey-Seal procedure (Zak and Aisen 1986). Reagents of highest available purity were from Merck or Fluka with the exception of apotransferrin and 3-phosphoglycerate (Sigma).

Diferric transferrin. Human serum apotransferrin 0.1 mM in 0.2 M Tris, 0.02 M $NaHCO_3$ pH 7.5 was treated with an excess of 1.5 equivalents of 1.2 mM FeNTA (Fe:NTA = 1:1.05; pH 2). After 12 h the protein was separated from unbound iron by gel filtration in a similar way as described by Smit et al. (1981). A 1 mM stock solution was obtained by microconcentration of the eluate.

Mono-Fe_CTf. An established procedure (Baldwin and de Sousa 1981) was followed with some but important modifications: iron release by EDTA was stopped after 35 min by addition of 0.98 equivalent of $ZnSO_4$ (referring to the remaining free EDTA), and continuous diafiltration (see below) was used instead of the ten subsequent concentration/dilution steps.

Mono-Fe_NTf. The standard prescription (Kretchmar and Raymond 1986) was followed except for applying continuous diafiltration instead of dialysis. The analytical polyacrylamide electrophoresis revealed that the monoferric preparations contained traces of diferric transferrin.

Diferric transferrin with oxalate as synergistic anion. This was obtained by mixing apotransferrin with FeNTA under CO_2-free atmosphere in the presence of a fourfold excess of oxalate (Schlabach and Bates 1975). Separation from unbound chelates was carried out under N_2 by continuous diafiltration.

Solutions of copper complexes. To acid solutions of ligand (L) and $CuCl_2$, NaOH was added very slowly through a capillary to adjust the pH. In some cases tiny amounts of precipitate appeared which were removed by filtration. The total concentrations were L, 0.025 M; Cu^{2+}, 0.023 M; Cl^-, 1 M; Mops, 0.1 M; pH 7.3 unless otherwise indicated.

Ultrafiltration. A 50-ml cell from Berghof with BM-100 or BM-10 membranes (exclusion size 10 and 1 kDa, respectively) was used as well as Centricon 30 or Centricon 10 microconcentrators from Amicon (exclusion size 30 or 10 kDa, respectively).

Continuous diafiltration. The 50-ml Berghof cell or a Centricon micro-concentrator was supplied with appropriate fittings through which the eluant was added (only for the Berghof cell is this equipment commercially available). In diafiltration, the eluant can be continuously added to the protein during ultrafiltration, which provides a very simple way to change the buffer or rinse out low-molecular-mass components without changing the volume of the protein solution.

Measurement of iron release. This was carried out spectrophotometrically in 1-cm quartz cuvettes at 460 nm at 25° C (Uvikon 810 photometer from Kontron, equipped with a thermostatted holder for six cuvettes). As a rule, a 1 mM solution of transferrin was mixed with the iron-releasing medium directly in the cuvette giving a protein concentration of about 0.09 mM. The reference solution was obtained by diluting the releasing medium with buffer to the same concentration as in the test solution. In order to reach the equilibrium from the apoTf side, a 1 mM solution of the latter was mixed with the releasing medium, and an equivalent of hydrated Fe^{2+} was added in an N_2 atmosphere. The very slow oxidation of iron was achieved by circulating this solution in a closed system where oxygen was admitted by gas-permeable silicon tubing. The fast oxygenation occurs in the trivial opening of the cuvette.

Analytical methods. pH was measured with a combined glass electrode (Ingold) and a digital potentiometer (Metrohm). Calibration was carried out by titration of a standard solution of HCl with a standard solution of NaOH (Merck Titrisol). Total

protein was determined by the Bio-Rad method. In the determination of total iron in the presence of Cu(II), the latter was masked with triethylenetetramine (Wang and Cheng 1982). For the determination of total iron in the presence of EDTA, concentrated HCl was added to the sample (1 ml) up to 3 M and 120 mg ascorbic acid was added also. The solution was heated for 10 min to 60° C and mixed with 10 ml phenanthroline reagent (1 M acetate/acetic acid, pH 4.5, 0.25 mg/ml phenanthroline and 4 mg/ml hydroxylamine hydrochloride). Absorbances were measured in 5-cm cuvettes.

Results and discussion

Figure 1 combines data on the effect of a variety of copper complexes containing either phosphoserine (PS) or 3-phosphoglycerate (PG). The mixed ligand complex $Cu^{II}(PG)(en)$ is the most powerful species in the acceleration of iron removal with respect to free phosphoserine as a reference. On the other hand, $Cu^{II}(PS)(bipy)$ is virtually noninteracting in contrast to $Cu^{II}(PS)(en)$, $Cu^{II}(PS)(mal)$, and $Cu(PS)_2$. The absorbance at 460 nm, used to follow iron removal, may contain some contributions from products in the later stage of the reaction, e.g. polynuclear hydroxo-complexes of iron(III), or Cu(II) complexes with protein residues, or deprotonated peptide groups. Hence, the asymptotic value of A_{460} is not as well defined as in the initial part (see section on *Approaching the equilibrium from the apotransferrin side*).

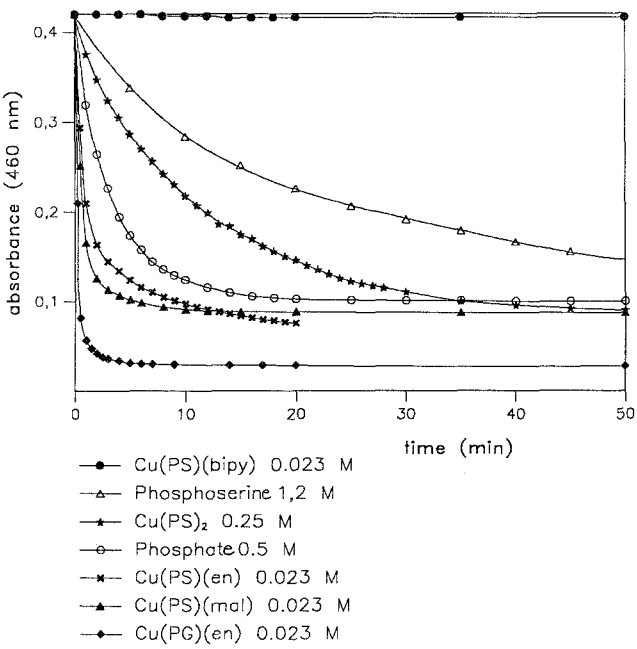

Fig. 1. Iron removal from diferric transferrin induced by organic phosphates and their copper(II) complexes. Conditions: total Tf 0.09 mM; NaCl 1 M; Mops 0.1 M; pH 7.3 (25° C)

For comparison, the iron release in 0.5 M phosphate is shown in Fig. 1. Apart from facilitating iron removal, it is a scavenger agent, since Fe_2Tf is thermodynamically unstable with respect to apoTf and $Fe^{III}PO_4 \cdot aq$ (s) at pH 7.3.

The stability of the copper complexes used

Theoretical consideration and visible absorption spectra of the complexes indicate that in a ternary system of Cu^{2+}, phosphoric acid monoester and diamine (or dicarboxylic acid), mixed ligand complexes are favoured. The ligands used in this study were phosphoserine (PS), 3-phosphoglycerate (PG), ethylenediamine (en), malonate (mal) and 2,2′-bipyridine (bipy). Sigel clearly demonstrated that the equilibrium

$$MY_2 + ML_2 \rightleftarrows 2\,MYL$$

is shifted to the right when M is Cu^{2+}, Y is bipy and L an oxygen ligand (Sigel 1967). Apart from this argument, the striking effectiveness of the mixture of Cu^{2+}, PS and en provides an independent proof for the mixed-ligand complex as the prevailing species because neither $Cu(en)_2$ nor $Cu(PS)_2$ are able to liberate iron in corresponding concentrations.

In estimating the overall stability of Cu(PS)(en) according to Sigel (1967), we add an increment of 0.5 to the sum of $\log K_1$ values of $Cu(PS)^-$ and $Cu(en)^{2+}$, i.e. $\beta = [Cu(PS)(en)]/[Cu][PS][en] = 10^{20 \cdot 3}$.

Thermodynamic factors favouring iron release

It is recommended that one check whether Cu(II) ions are able to displace the transferring iron due to the stability of CuTf.

Supposing that the equilibria (1) to (4) account for the removal of iron from the weaker iron binding site (charges have been omitted):

$$Fe + apoTf \rightleftarrows FeTf$$

$$K'_{FeTf} = \frac{[FeTf]}{[Fe] \cdot [apoTf]} = 10^{19.3} \qquad (1)$$

$$Fe + 3\,OH \rightleftarrows Cu(PS)(en)$$

$$K_{so} = [Fe] \cdot [OH]^3 = 10^{-39} \qquad (2)$$

$$Cu + PS + en \rightleftarrows Cu(PS)(en)$$

$$\beta = \frac{[Cu(PS)(en)]}{[Cu]\cdot[PS]\cdot[en]} = 10^{20.3} \quad (3)$$

$$Cu + apoTf \rightleftarrows CuTf$$

$$K'_{CuTf} = \frac{[CuTf]}{[Cu]\cdot[apoTf]} \quad (4)$$

In which the value of K'_{FeTf} was obtained by Aisen et al. (1978) while that for β is only an estimated value (see text above), then we deduce expression (5) for further consideration.

$$Q = \frac{[FeTf]}{[CuTf]} = K'_{FeTf}\cdot K_{so}\cdot K'^{(-1)}_{CuTf}\cdot[Cu]^{(-1)}\cdot[OH]^{(-3)} \quad (5)$$

(The concentration of Cu^{2+} is constant since under the experimental conditions Cu(PS)(en) acts as a buffer for the free metal ion.)

In the range $100 \geq Q \geq 0.01$, Eq. (5) is compatible with $4.8 \leq \log K'_{CuTf} \leq 8.8$ for our experimental conditions including the total concentrations of $[Cu^{2+}(PS)(en)] = 0.023$ M, $[FeTf] = 0.09$ mM and $[HCO_3^-] = 0.1$ mM (corresponding to the partial presence of CO_2 in ambient air).

Hence, iron release (Eq. 6) would be conceivable if $Q \approx 1$ and consequently $\log K'_{CuTf} \approx 7$.

$$Cu(PS)(en) + FeTf \rightleftarrows CuTf + PS + en + Fe(OH)_{3(s)} \quad (6)$$

However, it is demonstrated by the set of three experiments (A), (B) and (C) as described below that the conditional stability constant of CuTf must be even lower than 10^5 and that chelation of copper by the protein residues of the iron binding site is not a condition for iron release.

Experiment A: Iron release from $Fe_2Tf(oxalate)_2$ induced by Cu(PS)(en). Figure 2 shows that the iron exchange reactions of $Fe_2Tf(CO_3)_2$ and $Fe_2Tf(oxalate)_2$ with Cu(PS)(en) only differ in their reaction rates but not in the equilibrium situation. Taking into account that both Fe_N and Fe_C are liberated, it is concluded that the formation of Cu_2Tf cannot be a condition for iron release, since this species is not accessible with oxalate as a synergistic anion (Zweier and Aisen 1977).

Experiment B: Fe_2Tf and Cu(serine)(en) at equilibrium. Upon addition of an equivalent of $FeCl_3$ to a mixture of apotransferrin and 0.023 M Cu(serine)(en), the protein was saturated with iron very slowly (within several days) as monitored by the increase in A_{460nm}. Although the

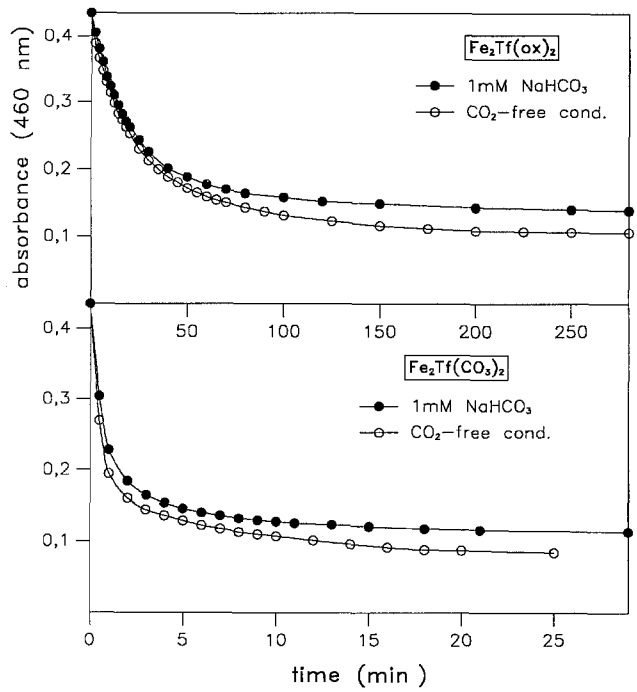

Fig. 2. Reaction of two transferrins differing in their synergistic anion with Cu(PS)(en). The bicarbonate-containing solution serves as a control showing that the absence of this synergistic anion has no effect on the rate of iron release. Conditions: total Tf 0.09 mM; Cu(PS)(en) 0.023 M; NaCl 1 M; Mops 0.1 M; pH 7.3 (25° C); test solution flushed with N_2 gas

overall stability of Cu(serine)(en) is 1.5 (log value) lower than those of Cu(PS)(en), equilibrium (6) is distinctly shifted to the left side. It is obvious that this statement is independent of the quality of the estimates about K'_{CuTf}. The result of this experiment is extremely important in drawing attention to the indispensable role of the phosphate group in Cu(PS)(en), Cu(PS)(mal), and Cu(PG)(en).

Experiment C: determination of filterable iron. Further information about the thermodynamics of iron release is delivered by the analysis of filterable iron in the solution containing Fe_2Tf and Cu(PS)(mal). When using a membrane with an exclusion limit of 10 kDa, 50% of total iron had permeated the pores after an incubation time of 3 h. The corresponding portion was lower after three days (27%). Using a 1-kDa membrane, the filterable iron amounted to 14% after three days. These results indicate that polynuclear iron hydroxide species emerge from iron release. Obviously, their size is shifted to larger values upon progressive aging (Schneider and Schwyn 1987). Clearly, the conditional stability of Fe_2Tf is lowered in the presence of Cu(PS)(mal).

Approaching the equilibrium from the apotransferrin side

Neither the equilibration time nor the amount of iron removed can be reliably inferred from Fig. 1. It was verified that, in the range 450–700 nm, spectral absorption due to polynuclear hydroxo complexes of iron(III), as well as Cu_xTf and the mixed ligand copper(II) complexes, cannot be analyzed in terms of independent variables. That is why no true reference solution can be prepared which filters out all the background absorption to provide the spectrum of unreacted Fe_xTf in the later state of iron removal.

In view of these inherent complications, it is all the more pleasing that the spectra 1 and 2 in Fig. 3 are nearly equivalent. They refer to the equilibrium system (7) as approached by different routes: (a) the educt solution containing apoTf, Cu(PS)(mal) and Fe^{2+} is oxygenated; (b) the mixture of Fe_2Tf and Cu(PS)(mal) is equilibrated.

$$Fe_2Tf \xrightleftharpoons{Cu(PS)(mal)} Tf + 2\,Fe(OH)_{3(PN)} \qquad (7)$$

At equilibrium there is some small fraction of Fe_xTf in both systems where polynuclear $Fe(OH)_3$ is the major component. However, different properties of $Fe(OH)_3$ emerge from oxygenation of Fe^{2+} (route a) on the one hand, and from release of Fe^{3+} from Fe_xTf on the other hand. It was abundantly shown that iron hydrolysis products depend on the specific pathway of formation (Schneider and Schwyn 1987). Consequently, the spectra 1 and 2 cannot be expected to be truly identical. There is no contradiction to this in the finding that spectrum 2 virtually does not depend on the rate of oxygen admission. In view of these results, it can be concluded that the asymptotic values of A_{460} in Fig. 1 correspond to almost complete loss of iron from Fe_2Tf.

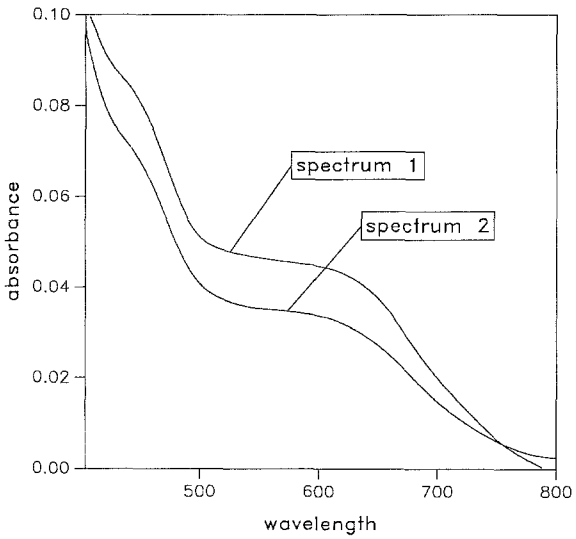

Fig. 3. Spectra of solution prepared in a different way but approaching nearly equivalent final states. Spectrum 1, the mixture of apoTf (0.09 mM), Cu(PS)(mal), and $Fe^{II}SO_4$ was prepared anaerobically; oxygen was admitted slowly as described in the text. Spectrum 2, the mixture of Fe_2Tf and Cu(PS)(mal) after 20-h incubation. Conditions: total Tf 0.09 mM; Fe 0.18 mM; Cu 23 mM; NaCl 1 M; pH 7.3; 25° C. The reference cuvette contained the electrolyte (NaCl) as well as Cu(PS)(mal)

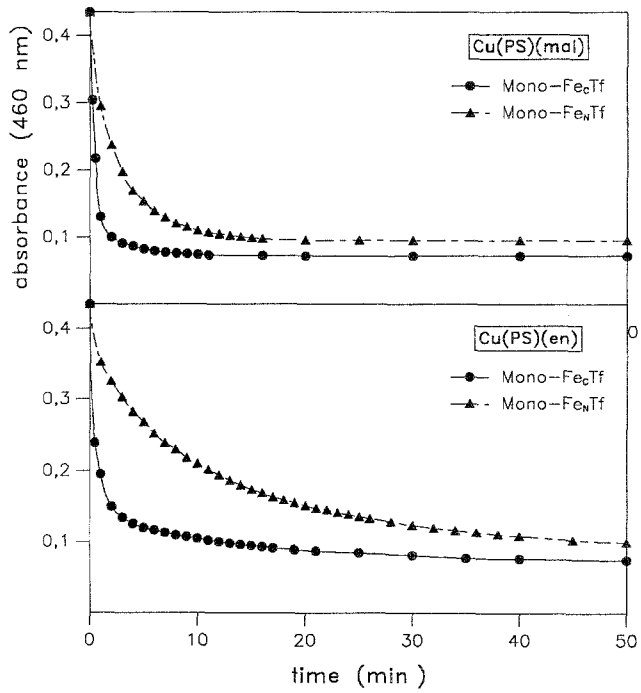

Fig. 4. Iron release from monoferric transferrins induced by the copper complexes of phosphoserine indicated in the figure. Conditions: total Tf 0.09 mM; Cu 23 mM; NaCl 1 M; Mops 0.1 M; pH 7.3; 25° C

Nonequivalence of the two iron-binding sites

In the presence of Cu(PS)(en), as well as in the presence of Cu(PS)(mal), the monoferric transferrins Fe_CTf and Fe_NTf showed different reactivity (Fig. 4). Neither of these reaction profiles fitts a first-order rate law. There is a particularly fast release within the initial period of some 15 s when 50% of iron is removed from Fe_CTf by Cu(PS)(mal).

Concluding remarks

The experiments in this study clearly demonstrate that iron is liberated because the stability of Fe_2Tf breaks down due to the interactions with the copper complexes. We postulate that this interaction is regiospecific because the first coordination sphere of copper(II) is absolutely essential for the effectiveness. This is born out by the key role of the phosphate group in Cu(PS)(en), which is very active in contrast to the inactive Cu(serine)(en). Furthermore, $Fe_2Tf(ox)_2$, containing two oxalates as synergistic anions, reacts about ten times more slowly compared to $Fe_2Tf(CO_3)_2$. Hence, the regiospecific interaction most likely involves the competition between the carbonate and the phosphate groups in Cu(PS)L (where L = PS; mal; en) as well as in Cu(PG)(en), the most effective species. Recent studies on the kinetics of iron removal by pyrophosphate (PP_i) show that PS is less efective than PP_i (Bertini et al. 1988).

A more detailed mechanistic interpretation certainly requires molecular graphics display methods in conjunction with molecular mechanics considerations. However, with regard to biological relevance, it is fair to say that active mixed-ligand copper complexes represent molecular models for iron release by components of the plasma membrane phase in cells.

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