Biol Metals (1989) 2:65–68

BIOLOGY OF METALS

© Springer-Verlag 1989

Further evaluation of the biphasic kinetics of iron removal from transferrin by 3,4-LICAMS

Suzanne A. Kretchmar and Kenneth N. Raymond

Department of Chemistry, University of California, Berkeley, CA 94720, USA

Summary. Further evaluation of the kinetic data for Fe^{3+} removal from isolated differic and monoferric transferrins by the tricatechol ligand 3,4-LICAMS has allowed full characterization of the four microscopic rate constants. A very small cooperativity exists between the two iron-binding sites with respect to their rates of iron release. The activation free energy profile for the system is presented.

Key words: Transferrin — Biphasic kinetics — Microscopic rate constants — 3,4-LICAMS — Catechol ligand — Cooperativity

Introduction and discussion

In a previous paper (Kretchmar and Raymond 1986) describing the biphasic kinetics and temperature dependence of iron removal from transferrin (Tf) by the tetracatechol ligand 3,4-LICAMS [1,5,10-*N*,*N*′,*N*″-tris(5-sulfanato-2,3-dihydroxybenzoyl)-1,5,10-triazadecane], we presented a kinetic analysis of the rate of iron removal from this iron-transport protein (Aisen 1989; Chasteen 1983) which recognized that, in principle, the iron could be removed at different rates from the two sites (**a** and **b**) of the protein and that there might be some interaction between the two sites in the diferric form of the protein (Chasteen 1983; Kretchmar et al. 1988). Shown in Fig. 1 (upper right-hand corner) is the scheme for irreversible iron release, originally proposed by Baldwin (1980) that was used earlier to describe this system and which will be used here. Additional analysis of our data, prompted in part by the work of Bertini et al. (1988) and Harris et al. (1987) has shown that more information could be extracted from it and that there are some erroneous calculations in the original paper which we seek here to correct. We refer the reader to the earlier paper (Kretchmar and Raymond (1986) for experimental details and derivation of the kinetic expressions.

We assume that the rates of iron removal from Fe_aTf and Fe_bTf are the same within experimental error, and use Eq. (1) (Eq. 7 in the earlier paper (Kretchmar and Raymond (1986)) to determine

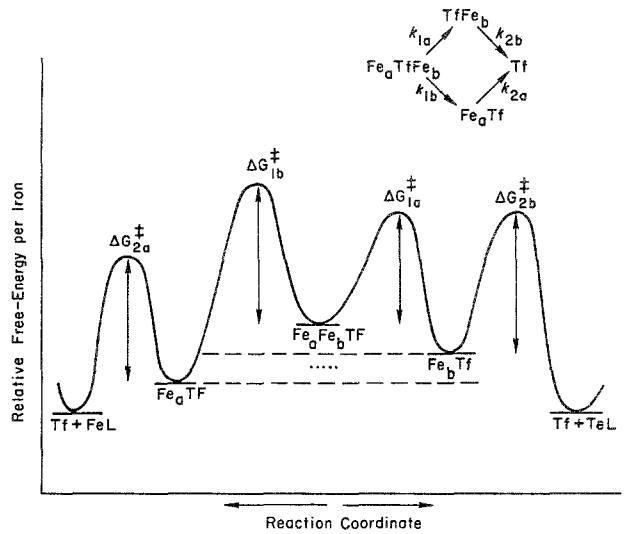

Fig. 1. Reaction coordinate diagram for the removal of iron from diferric transferrin (Fe_aFe_bTf) to form apotransferrin (Tf) and ferric ligand (FeL). The free energy of activation values ΔG^{+}, were calculated from: $\Delta G^{+} = -RT\ln[kh/\mathbf{k}T]$ (where k is the microscopic rate constant, h is Planck's constant, $\mathbf{k}$ is the Boltzmann constant, and T is the Kelvin temperature). The free energies are per iron bound and the average value of the free energy of the monoferric iron a and b sites are shown to illustrate cooperativity of binding between the two sites

Offprint requests to: K. N. Raymond

Table 1. Macroscopic rate constants for iron removal from 0.100 mM diferric transferrin by 3,4-LICAMS

[3,4-LICAMS] (mM)	$m_1 \times 10^2$ (min^{-1})	$m_2 \times 10^2$ (min^{-1})	% m_1	$k_{obsd} \times 10^2$ (min^{-1})
6.00	7.0 (1)	1.95 (2)	31 (1)	2.1
4.00	5.2 (1)	1.48 (8)	30 (2)	1.8
3.00	4.7 (1)	1.38 (2)	29 (1)	1.3
2.00	3.36 (8)	1.02 (4)	28 (2)	1.1
1.00	1.91 (6)	0.52 (4)	31 (2)	0.63
0.40	0.9 (1)	0.19 (8)	37 (3)	0.30

Data are taken from Kretchmar and Raymond (1986), measured in 0.050 M Hepes pH 7.4, 25° C, $I=0.082$ M. $m_1=k_{1a}+k_{1b}$; $m_2=k_{2b}$; $\%m_1=50[2-m_1/(m_1-m_2)]$. Values of k_{obsd} are from Baldwin 1980 (0.1 M Tris pH 7.4, 25° C, $I=0.082$ M). Here, and in other tables, the estimated standard deviation (in the least significant digits) is presented in parentheses following the corresponding parameter, i.e. the first value for $m_1=7.0\pm0.1\times10^{-2}$ min^{-1}

m_1 and m_2 for iron removal from diferric transferrin. $m_1 > m_2 \approx m_3$

$$A-A_\infty = B_0\varepsilon l\left[\left(2-\frac{m_1}{(m_1-m_2)}\right)e^{-m_1t} + \left(\frac{m_1}{(m_1-m_2)}\right)e^{-m_2t}\right] \quad (1)$$

This assumption was subsequently independently verified by directly measuring k_{2a} and k_{2b} for iron removal from the monoferric transferrins at 25° C; it means that $\Delta G^{\ddagger}_{2a}$ and $\Delta G^{\ddagger}_{2b}$ (Fig. 1) are equal. Here A is the absorbance at 520 nm as a function of time, A_∞ the equilibrium absorbance, B_0 the initial Fe_2Tf concentration, ε the molar extinction coefficient per iron atom, l the cell path length, $m_1=k_{1a}+k_{1b}$, $m_2=k_{2b}$, and $m_3=k_{2a}$ (see Fig. 1).

The question of whether the two sites affect one another as a second iron atom is bound remains; the converse of this is whether the rates of iron release by the k_{1a} and k_{1b} paths differ significantly on average from the k_{2a} and k_{2b} paths. From Table 1 it can be seen that the values (Carrano and Raymond 1979) for the percentage of iron released during the first phase, $\%m_1$, range over 28%–37%. (The values of $\%m_1$, Tables 1 and 2 of the earlier paper (Kretchmar and Raymond 1986), are corrected in Tables 1 and 2 of this paper.)

In the following discussion it will be useful to use the free energy diagram (Fig. 1) to describe the relative rates of iron removal from the different sites of the iron-loaded protein. It will be of particular importance here to describe precisely what we mean by 'cooperativity' of iron binding in diferric transferrin. The free energy diagram is intended to describe the free energy per iron site. The iron is bound more strongly at the **a** site than at the **b** site. This relative strength of binding is also paralleled in the kinetics. If there were no interaction between the two sites when iron is bound at both of them, the free energy per iron for the diferric protein molecule should be just the average of the two monoferric proteins. The absence of interactions can be seen in Fig. 1 as the midway position of the two dashed lines. Any interaction between the two sites (cooperativity) will strengthen or weaken (as shown in Fig. 1) their average binding energy and correspondingly change the average rate of iron removal. As can

Table 2. Rate constants for iron removal from 0.100 mM labeled diferric transferrin by 6.00 mM 3,4-LICAMS

Parameter	Value for N-labeled site		Value for C-labeled site	
	^{55}Fe(N)-Fe(C)Tf ($\times10^2$ min^{-1})	^{59}Fe(N)-Fe(C)Tf ($\times10^2$ min^{-1})	^{55}Fe(C)-Fe(N)Tf ($\times10^2$ min^{-1})	^{59}Fe(C)-Fe(N)Tf ($\times10^2$ min^{-1})
m_1	6.9 (2)	7.0 (2)	6.3 (1)	6.3 (1)
m_2	2.35 (6)	1.94 (6)	1.79 (4)	1.93 (2)
% m_1	24 (2)	31 (2)	30 (1)	28 (1)
k_{1a}[Fe(N)]	4.5 (3)	4.8 (2)	5.5 (3)	5.1 (4)
k_{1b}[Fe(C)]	3.0 (2)	2.2 (4)	0.9 (1)	1.2 (4)
$m_1[k_{1a}+k_{1b}]$	7.5 (4)	7.0 (4)	6.4 (3)	6.3 (6)
m_1[Fe(T)]/$m_1[k_{1a}+k_{1b}]$	0.92 (6)	1.0 (1)	0.98 (5)	1.0 (1)
$[k_{1a}\cdot k_{1b}]/[k_{2a}\cdot k_{2b}]$	3 (1)	3 (1)	1.2 (3)	1.5 (7)

Here k_{1a} and k_{1b} are determined from Eqs. (4) and (5). The degree to which cooperativity exists between the **a** and **b** sites is defined as the ratio $[k_{1a}\cdot k_{1b}]/[k_{2a}\cdot k_{2b}]$. Measurements were made in 0.050 M Hepes pH 7.4, 25° C, $I=0.082$ M. $m_1=k_{1a}+k_{1b}$; $m_2=k_{2b}$. $\%m_1$ is defined in Table 1. [Fe(T)] refers to the total amount of iron in diferric transferrin; [Fe(N)] and [Fe(C)] refer to the iron located in the N- and C-terminal sites of diferric transferrin, respectively. From the kinetic data, it is concluded that [Fe(N)] corresponds to site **a** and [Fe(C)] to site **b**

be seen from the free energy diagram, a change in any one of the activation barriers can occur independently, so that all four are linearly independent. If, however, it is assumed that the average energy per iron site for the monoferric transferrins is the same as that for the corresponding site in diferric transferrin, then $\Delta G_{1a}^{\ddagger}+\Delta G_{1b}^{\ddagger}=\Delta G_{2a}^{\ddagger}+\Delta G_{2b}^{\ddagger}$. Therefore the ratio $(k_{1a}\cdot k_{1b})/(k_{2a}\cdot k_{2b})$ can be used as a measure of the cooperativity of the binding between the two sites of transferrin, with a ratio >1 corresponding to a negative interaction.

A stronger assumption is that the rate of iron release from the **a** or **b** sites is independent of whether it is the monoferric or diferric transferrin. In previous studies (cited in Kretchmar and Raymond 1986) this has been the tacit definition of 'cooperativity'. We earlier made no attempt quantitatively to fit the rate of iron removal from the radioisotopically labeled monotransferrins using the model that we described (Kretchmar and Raymond 1986). However, from that mathematical model one can define the rate of iron removal form the monoferric iron sites (described as Fe_aTf and Fe_bTf) versus Fe_2Tf for the differic transferrin).

Amount of radioactive iron in the C-terminal site
$$=[Fe_2Tf]+[Fe_bTf] \tag{2}$$

Amount of radioactive iron in the N-terminal site
$$=[Fe_2Tf]+[Fe_aTf]. \tag{3}$$

Substitution of the time-dependent expressions for the concentrations of the diferric and monoferric transferrins (Kretchmar and Raymond 1986) gives:

Fraction of radioactive iron in the C-terminal site
$$=e^{-m_1t}+\frac{k_{1a}}{m_2-m_1}[e^{-m_1t}-e^{-m_2t}] \tag{4}$$

Fraction of radioactive iron in the N-terminal site
$$=e^{-m_1t}+\frac{k_{1b}}{m_3-m_1}[e^{-m_1t}-e^{-m_3t}] \tag{5}$$

where the **a** site has been equated to the N-terminal site and the **b** site to the C-terminal site. Upon substitution of the macroscopic rate constants m_1 and m_2 (obtained from the visible spectroscopic data) into Eqs. (4) and (5), the microscopic rate constants k_{1a} and k_{1b} were determined using non-

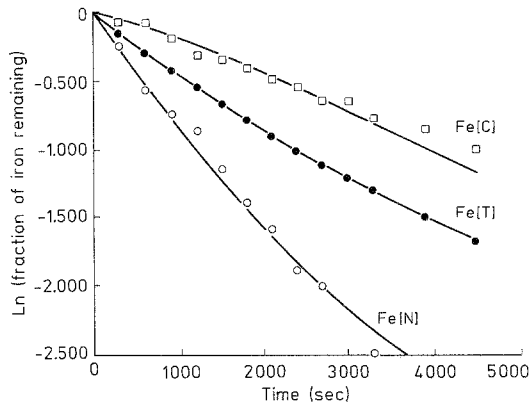

Fig. 2. Iron removal from 0.100 mM ^{55}Fe(C)-Fe(N) transferrin by 6.00 mM 3,4-LICAMS (0.050 M Hepes pH 7.4, 25° C, $I=0.082$ M). (●) The total iron removed [Fe(T)]; (□) iron removed from the C-terminal site [Fe(C)]; (○) iron removed from the N-terminal site [Fe(N)]. All data have been normalized in order to fit on the same plot. Data for iron removal from the individual sites have been fitted to Eqs. (4) and (5)

linear-least-squares regression (Table 2). Plots of these fits are shown in Fig. 2. A summary of the combined results of the four experiments is given in Table 3.

It is apparent that iron is removed from the N-terminal site at higher rates than from the C-terminal site, as noted previously. However, there is also a small cooperativity between the two sites that leads to a factor of 2 between the products of the rate constants for the first versus second iron-removal steps. More important, these independent refinements of the monoferric sites allow the independent measurement of all four of the microscopic rates constants (k_{1a}, k_{1b}, k_{2a}, k_{2b}). In the Baldwin kinetic model (Baldwin 1980) the sum $k_{1a}+k_{1b}$ should equal m_1. As can be seen from the ratios of the values of m_1 determined from the individual monoferric versus diferric kinetic data, these are essentially the same (Table 2).

Table 3. Microscopic rate constants for iron removal from ferric transferrin by 3,4-LICAMS (see Fig. 1)

Microscopic rate constant	Value ($\times 10^{-2}$ min^{-1})
k_{1a}	5.0 (4)
k_{1b}	1.8 (9)
k_{2a}	2.3 (5)
k_{2b}	1.8 (2)
$(k_{1a}\cdot k_{1b})/(k_{2a}\cdot k_{2b})$	2 (1)

Values were determined in 0.050 M Hepes pH 7.4, 25° C, $I=0.082$ M. k_{1a} and k_{1b} were determined from the average of the results of the four experiments presented in Table 2 while k_{2a} and k_{2b} were determined from the average of five experiments

Acknowledgement. This research is supported by National Institutes of Health grant AM 32999.

References

Kretchmar SA, Raymond KN (1986) Biphasic kinetics and temperature dependence of iron removal from transferrin by 3,4-LICAM. J Am Chem Soc 108:6212–6218

Aisen P (1989) Physical properties of the transferrins: Update 1984–1988. In: Loehr TM (ed) Iron carriers and iron proteins. VCH Press, Weinheim

Chasteen ND (1983) Transferrin: a perspective. In: Theil EC, Eichorn GL, Marzilli LG (eds) Advances in inorganic biochemistry, vol 5. Elsevier, New York, pp 201–233

Kretchmar SA, Teixeira M, Huynh B-H, Raymond KN (1988) Mössbauer studies of electrophoretically purified monoferric and diferric human transferrin. Biol Metals 1:26–32

Baldwin D (1980) The kinetics of iron release from human transferrin by EDTA. Effects of salts and detergents. Biochim Biophys Acta 623:183–198

Bertini I, Hirose J, Lichinat C, Messori L, Piccioli M, Scozzafava A (1988) Kinetic studies on metal removal from transferrins by pyrophosphate. Inorg Chem 27:2405–2409

Harris WR, Rezvani AB, Bali PK (1987) Removal of iron from transferrin by pyrophosphate and tripodal phosphonate ligands. Inorg Chem 26:2711–2716

Carrano CJ, Raymond KN (1979) Ferric ion sequestering agents. 2. Kinetics and mechanism of iron removal from transferrin by enterobactin and synthetic tricatechols. J Am Chem Soc 101:5401–5404

Received March 18, 1989