The Influence of Charge on Reactions of Metalloproteins with Small Molecules

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A procedure for estimating the local effective charge on a protein surface from association constants for surface reactions of metalloproteins with inorganic complexes is outlined; results obtained are consistent with structural information.

An understanding of the reactions of enzymes with small molecule substrates is often impeded by lack of knowledge concerning the nature of the interaction of the surface of a protein with small charged reactants. A particularly important case is the binding of small inorganic ions, *e.g.* $[Fe(CN)_6]^{3-}$, to electron-transfer proteins, since such complexes are used extensively as an aid to the understanding of electron-transfer reactions in biological systems. In all cases the exact site of binding is rarely known and even where it is known the strength of binding has not been related to structure. Electrostatistics are often believed to be involved, but no clearly defined approach to the problem has emerged. This whole question is under investigation in our two laboratories in cases where the protein structure is known. In one case the kinetic method is being used to determine association constants for inorganic complexes of charge 5+ through to 5−. In the other the n.m.r. method is being used to detect binding sites with paramagnetic complexes as specific probes. We start by considering the basic theoretical and experimental approach to such interactions with reference to ion-pair formation between small anions and cations, where the energies involved are electrostatic in origin.

Figure 1 is a plot of experimental association constants K against charge product for interactions between small anions and cations of a purely electrostatic character. Only outer-sphere interactions between substitution-inert complexes and interactions of Group 1A and 2A ions are included.[1] The data avoid complications due to covalency effects although hydrogen-bonding could contribute to some secondary degree in some cases. Values given (all corrected to $I \rightarrow 0$) are compared with predictions made for ion-pair formation using Bjerrum's equation (1).[2] It can be seen that the experimental

$$K = \frac{4\pi N}{1000}\left(\frac{|z_1z_2|e^2}{DkT}\right)^3 \int_2^b y^{-4}\ \exp(y)dy \qquad (1)$$

$$y = |z_1z_2|e^2/DrkT; \quad b = |z_1z_2|e^2/DakT$$

values of log K follow the expected dependence on charge product z_1z_2, and are in good agreement with calculated values assuming an interionic distance in the range 5—7 Å. Thus this simple electrostatic theory provides a good method of relating association constants to the charge product.

Given these data, we can now turn to the experimental constants K (I = 0.10 M) for the outer-sphere association of inorganic complexes with metalloproteins; see Table 1.[3-6] Five metalloproteins have been selected which are representative of the three known structure types having (i) a single metal (the Cu containing protein plastocyanin[7]), (ii) a metal cluster (two Fe/S proteins[8,9]), and (iii) a heme Fe (cytochrome b_5 and cytochrome c[10,11]) active site. Expected ion-

Table 1. Formation constants for association of inorganic complexes with proteins (in reduced state) at 25 °C, I = 0.10 M (NaCl), pH 7.5.

		K/M^{-1}	Local effective charge	Charge at binding site from structure
PCu^{I} (−9)[a] (M.wt. 10 500)	*$[Co^{III}_2]^{5+}$	16 000	3.0−	4−
	*$[Pt(NH_3)_6]^{4+}$	22 000	4.0−	
	*$[Co(NH_3)_6]^{3+}$	580	3.8−	
	$[Co(phen)_3]^{3+}$	167	2.8−	
	*$[Cr(phen)_3]^{3+}$	176	2.8−	
[2Fe–2S](−17)[b] (M.wt. 10 500)	$[Co^{III}_2]^{5+}$	26 400	3.0−	3−
	$[Pt(NH_3)_6]^{4+}$	21 000	4.0−	
	$[Co(NH_3)_6]^{3+}$	998	4.0−	
	*$[Cr(NH_3)_6]^{3+}$	464	3.5−	
	*$[Cr(en)_3]^{3+}$	590	3.8−	
	$[Co(NH_3)_5Cl]^{2+}$	194	4.5−	
2[4Fe–4S](−12)[c] (M.wt. 6 500)	$[Pt(NH_3)_6]^{4+}$ [f]	2 500	3.4−	*ca.* 3−
	$[Co(NH_3)_6]^{3+}$	446	3.6−	
	*$[Cr(NH_3)_6]^{3+}$	212	3.0−	
Cytochrome b_5 (−9)[d] (M.wt. *ca.* 9 400)	$[Co^{III}_2]^{5+}$	16 600	3.0−	3−/4−
	$[Pt(NH_3)_6]^{4+}$	14 800	4.0−	
	$[Co(NH_3)_6]^{3+}$	600	3.8−	
	*$[Cr(en)_3]^{3+}$	309	3.3−	
Cytochrome c (+9)[e] (M.wt. 12 400)	$[Fe(CN)_6]^{3-}$	450 (I 0.18 M)	*ca.* 4+ [g]	3+/4+

[a] Parsley (ref. 3). [b] Parsley (ref. 4). [c] *Clostridium pasteurianum* (ref. 5). [d] Calves liver (ref. 6). [e] Horse heart in oxidised form (ref. 15). [f] pH 6.8. [g] Allowance made for higher I. * Indicates redox inactive complex; $[Co^{III}_2]^{5+}$ = $(NH_3)_5Co{\cdot}NH_2{\cdot}Co(NH_3)_5^{5+}$; phen = 1,10-phenanthroline; en = ethylenediamine.

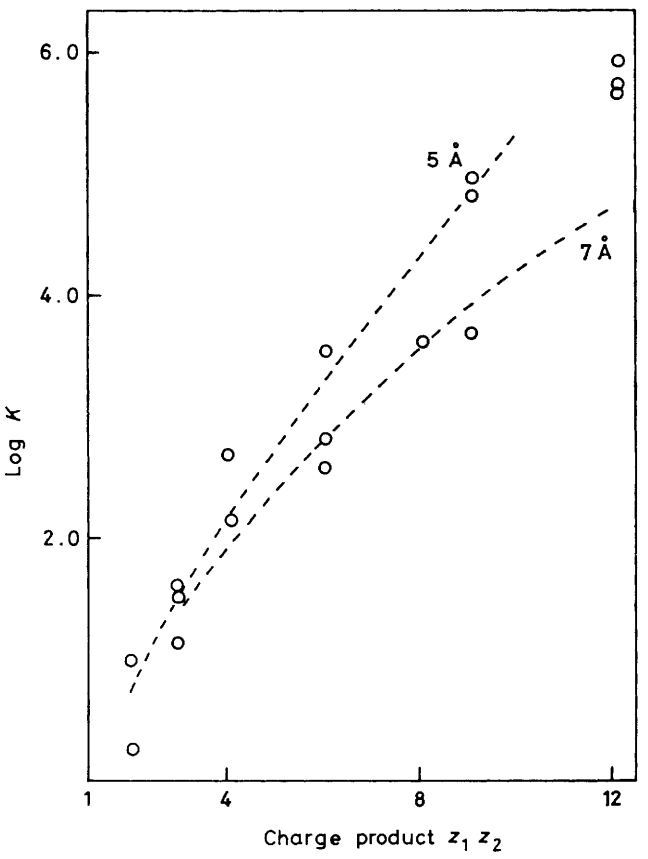

Figure 1. The variation of experimental association constants (K) with charge product at $I \rightarrow 0$. The correspondence of experimental points (ref. 1) with curves generated from equation (1) for 5 and 7 Å separation of reactants is noted.

pair equilibrium constants from equation (1), at an estimated separation of 6 Å, can be corrected to I = 0.10 M using equation (2).[12] This has been done for a range of different

$$\log K_{(I)} = \log K_{(I\rightarrow 0)} - 1.018 z_1 z_2 \, [I^{\frac{1}{2}}/(1 + I^{\frac{1}{2}}) - 0.3I] \quad (2)$$

charge interactions, and similar curves to those in Figure 1 have been calculated for I = 0.10 M. Knowing the charge on the small molecule and experimental association constants (as in Table 1), we can then read off the local effective charge at the protein binding site. It has been demonstrated in the case of plastocyanin from n.m.r. line broadening effects using paramagnetic complex ion probes,[13] as well as from competitive inhibition, Cr^{III} protein modification, and pH effects on rate constants, that a highly conserved negative patch, incorporating consecutive acid residues 42—45, is a reaction site of high specificity for cationic reactants. This binding site has a charge of 4− as expected. With the [2Fe–2S] ferredoxin there are two patches of negative (3−) charge, 65—67 or 92—94, which are possible binding sites,[14] and cytochrome b_5 is known to have clusters of negative charge $\geqslant$3− around the exposed heme edge from the peptide chains 37—48 and 56—60. With the smaller 2 [4Fe–4S] protein, acid residues 39 and 55 and the partly exposed negatively charged cluster constitute a likely reaction site. In the case of cytochrome c(III) the n.m.r. method has given a binding constant of 450 M^{-1} for $[Fe(CN)_6]^{3-}$ (I *ca.* 0.18 M)[15] consistent with a local effective charge of 3+/4+. There are several positive residues (4—6) in the sequence 72—88 which are implicated in these studies.†

† Note that from kinetic studies on the reaction of $[Fe(CN)_6]^{4-}$ with cytochrome c(III) K < 200 M^{-1} at 25 °C, pH 7.2, I = 0.10 M (NaCl).[16] This value relates to the more specific association at or close to Lys 72 which is the dominant site for electron-transfer.[17]

In each of the above five cases therefore it would appear that the binding constant is adequately explained by electrostatic interaction based on the local effective charge.‡ For comparison, from n.m.r. studies, neurotoxins and lysozymes have no sites of high affinity (log $K \leqslant 2$ and $I = 0.10$ M) for cations or anions of charge ± 3, *e.g.* for $[Cr(CN)_6]^{3-}$ or $[Cr(NH_3)_6]^{3+}$. Therefore it can be concluded that there are no sites of local effective charge $\geqslant 3$ on the protein surfaces. Inspection of their structures shows this to be true. In all cases, the effect of the net charge on the protein (see Table 1) appears to be of no consequence in binding, nor would there appear to be any reason to involve dipolar field effects.

We conclude that local electrostatic effects are of considerable importance in determining the magnitude of the binding of ions at zero ionic strength, but net protein charges and dipolar effects are not. The latter are important however in the consideration of ionic strength effects. It is important to note that the theoretical prediction given is for spherical ions. Consideration of non-spherical charge distribution and the effects of matching spatially disposed opposed charges will be given elsewhere. Further terms will have to be added if hydrophobic ions are considered.‡ It should also be noted that the prediction of charges from Figure 1 is based on the binding in a continuous dielectric, that of water. The protein environment may offer a different dielectric medium. For these reasons we cannot expect exact correspondence between theory and experiment.

Received, 7th June 1983; Com. 744

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‡ It should be noted that other studies on the oxidation of negatively charged proteins ACu^I, PCu^I and the high-potential Fe/S protein with $[Co(4,7\text{-DPSphen})_3]^{3-}$ as oxidant[18] (4,7-DPSphen = 4,7-diphenylsulphonato-1,10-phenanthroline) give high K values ($\geqslant 2750\ M^{-1}$) which are not consistent with a simple electrostatic approach. These may be the result of the high aromaticity of the 4,7-DPSphen ligand.