Ligand Binding to Cytochrome *c* and Other Related Haem Proteins and Peptides. Part I. Equilibrium Studies

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Abstract

Investigation of ligand binding to native cytochrome *c*, carboxymethyl-Met 80-cytochrome *c*, myoglobin and haemhexapeptide revealed that the binding of exogenous ligands is modulated by the following factors:

(1) Hydrophobicity of the haem environment.

(2) Haem accessibility to exogenous ligands, termed the haem crevice 'open–closed' parameter.

(3) Steric interactions between the protein and the bound ligand.

Introduction

The binding of simple exogenous ligands, such as carbon monoxide, azide, imidazole and cyanide, to haem proteins has been extensively studied as a method for monitoring haem accessibility [1], electronic structure [2] and general haem protein conformation [3]. Reaction rates range from the very rapid, such as carbon monoxide binding to separated haemoglobin chains, to the very slow, such as the same reaction with cytochrome *c*.

One approach to understanding the functional and structural design of haem proteins is provided by a comparison of the affinity of various ligands for the model systems and for haem proteins; the effect of the protein moiety on reactivity can thus be analysed.

We have carried out a ligand binding study to broaden our understanding of the interactions between ligands and haem proteins. Equilibrium studies, as a function of pH and temperature, are reported for the binding of a number of ligands for the model systems. The haem proteins and peptides used were: N-cytochrome *c**, cm-cytochrome *c**, H-6-P*, H-11-P* and myoglobin. The structural differences among these systems are discussed in the text when appropriate. These studies are to probe the effects of steric constraints (simple coulombic interactions, the opening of the haem pocket and the metal–ligand bond strength) on the stability of ligand complexes and the dynamics of ligand binding to haem proteins.

*Abbreviations: N-cytochrome *c* = native cytochrome *c*; cm-cytochrome *c* = carboxymethyl-Met 80 cytochrome *c*; H-6-P = haemhexapeptide, residues 14–19 of native cytochrome *c*; H-11-P = haemundecapeptide, residues 11–21 of native cytochrome *c*.

Experimental

Materials

Horse heart cytochrome *c* (type III) was purchased from the Sigma Chemical Company. Sodium azide, sodium fluoride, sodium cyanide and imidazole were purchased from Fisons (Laboratory Reagents, U.K.). All other reagents used were of the purest grade available. Cytochrome *c* haemhexapeptide (H-6-P) was prepared as described by Peterson *et al.* [4]. Carboxymethylated cytochrome *c* (cm-cytochrome *c*) was prepared according to the method reported by Brunori *et al.* [5].

Ligand Binding to H-6-P

An aliquot (2.5 ml) of a solution of *ca.* 20 μM H-6-P, buffered at the desired pH, was pipetted into a 3-ml cuvette stoppered by a Subaseal vaccine cap. Aliquots of concentrated ligand solution at the same pH were added via a hypodermic needle connected to an Agla syringe. This latter was driven by a micrometer-screw gauge such that one revolution of the gauge delivered 10 μl from the syringe into the cuvette. Differential spectra were recorded using a Perkin-Elmer 575 spectrometer from 380 to 440 nm, as shown in Fig. 1, and the absorbance was corrected for dilution. The temperature of the solutions was monitored by an electronic thermometer (Comark type 1605) throughout the titration. All spectra were recorded after having allowed at least 10 min for thermal equilibration.

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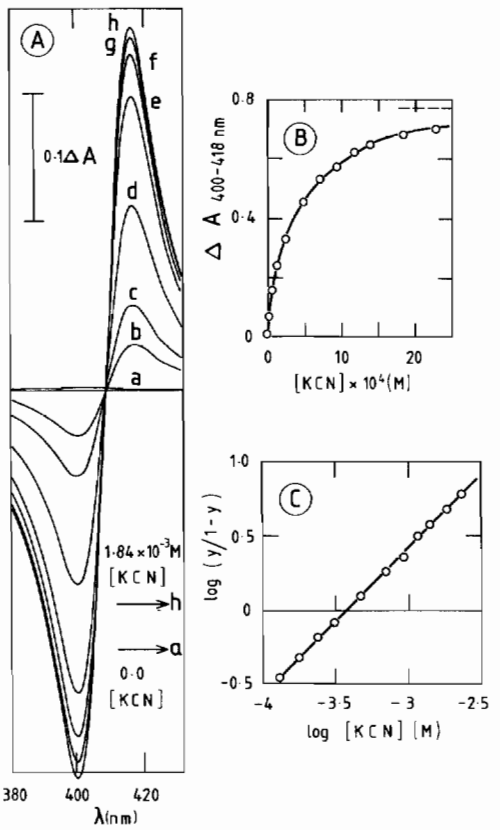

Fig. 1. The binding of cyanide ion to H-6-P at pH 6.25 in 0.15 M phosphate buffer at 25 °C. (A) Static difference spectrum [Fe(III)–CN minus Fe(III)] associated with successive additions of cyanide (a → h; 0.0; 4×10^{-6}; 6×10^{-6}; 2.4×10^{-4}; 7.3×10^{-4}; 1.14×10^{-3}; 1.4×10^{-3} and 1.84×10^{-3} M potassium cyanide respectively). (B) Saturation curve for the binding of cyanide ion to H-6-P (obtained from the spectra given in (A)). (C) Hill plot for the binding of cyanide ion to H-6-P, obtained from the data of (B).

Ligand Binding to N-Cytochrome c and cm-Cytochrome c

To each of ten spectrophotometer cells containing 3 ml of solution of *ca.* 30 μM protein, buffered at the desired pH and stoppered by a Subaseal vaccine cap, aliquots of concentrated ligand solution at the same pH were added such that each cell contained a different ligand concentration (e.g. to cell number 1 was added 10 μl increasing to 100 μl in cell number 10). The cells were incubated in the water which circulated around the spectrophotometer for about 2 h, then the differential spectra were taken from 380 to 440 nm. In order to ensure that the temperature in the reference cell in the spectrophotometer was the same as the cells in the water bath, two electronic thermometers were used, one to monitor the temperature in the refence cell and the other to monitor the temperature of the other cells in the water bath.

Results

Equilibrium Binding Studies

In common with other ferric haemoproteins with available coordination sites [6] the proteins considered here bind exogenous ligands giving rise to spectral changes. Figure 1 shows the result of titrating H-6-P with potassium cyanide at pH 6.25. Figure 1A indicates the presence of two spectral species with a well-defined isosbestic point at 408 nm, thus indicating that the binding conforms to a simple process. The titration curve in Fig. 1B gives a linear Hill plot with a slope of unity (Fig. 1C). This indicates that the haem sites are equivalent and independent. Similar binding curves were obtained from all ligand binding equilibrium experiments using the simple, single site, proteins and peptides under study. Table I reports the affinity constants (K_A) obtained from the Hill plot derived from such titrations.

TABLE I. Affinity Constants (K_A) for Ligand Binding to Ferric Haem Proteins (at 25 °C).

Protein	Ligand	pH	K_A (M^{-1})
N-Cytochrome *c*	cyanide	7.5	3.16×10^3
cm-Cytochrome *c*	cyanide	7.5	3.63×10^4
Myoglobin[a]	cyanide	7.5	1.6×10^5
H-6-P	cyanide	7.5	2.82×10^4
N-Cytochrome *c*	cyanide	10.0	1.82×10^4
cm-Cytochrome *c*	cyanide	10.0	5.5×10^4
Myoglobin[a]	cyanide	10.0	6.3×10^5
H-6-P	cyanide	10.0	1.45×10^5
N-Cytochrome *c*	azide	8.4	4.3
N-Cytochrome *c*	azide	10.4	2.2
cm-Cytochrome *c*	azide	8.4	7.5
cm-Cytochrome *c*	azide	10.4	2.1
Myoglobin[a]	azide	8.4	7.24×10^3
H-6-P	azide	6.2	17
H-6-P	azide	6.8	9.7
H-6-P	azide	8.4	2.8
H-6-P	azide	10.4	2.2
N-Cytochrome *c*	imidazole	7.5	32
N-Cytochrome *c*	imidazole	8.5	28.8
N-Cytochrome *c*	imidazole	10.4	28.2
cm-Cytochrome *c*	imidazole	7.5	4.9×10^2
cm-Cytochrome *c*	imidazole	8.5	66.7
cm-Cytochrome *c*	imidazole	10.4	29.2
Myoglobin	imidazole	8.5	1.12×10^2
H-6-P	imidazole	8.5	1.26×10^4
H-6-P	imidazole	10.4	2.5×10^4
N-Cytochrome *c*	pyridine	10.0	30.2
cm-Cytochrome *c*	pyridine	7.5	48
H-6-P	thiocyanate	6.8	2.2
H-6-P	thiocyanate	10.4	2.3
H-6-P	fluoride	6.8	2.9
H-6-P	fluoride	7.5	0.71
Myoglobin[a]	fluoride	7.5	1.53

[a]Calculated from data in ref. 6.

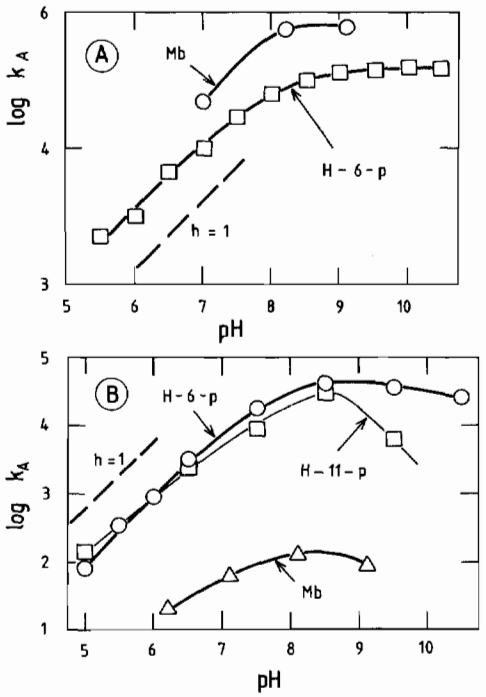

Fig. 2. Effect of pH on the affinity constant (K_A) of: (A) cyanide binding to H-6-P and metmyoglobin; (B) imidazole binding to H-6-P; H-11-P and metmyoglobin. Conditions: temperature = 25 °C; 0.15 M phosphate buffer for the pH region 5.5–8.5; 0.15 M borate buffer for the pH region 8.5–10.5. The dotted line represents a line of slope equal to unity. The solid line represents a theoretical line for the binding of cyanide ion to H-6-P (A) and imidazole to H-6-P (up to pH 9) (B) according to eqn. (1).

The affinity constants of cyanide and imidazole binding to the haem proteins used here were found to be pH dependent. Figure 2A shows the affinity constants for cyanide binding to H-6-P and metmyoglobin. From pH 5.5 to pH 7.5, the affinity constant increases rapidly with a slope of unity. Above pH 8.5, the affinity constant is almost pH independent. As the pK_a of hydrocyanic acid is 9.2 [7], this indicates that CN^- is the binding species. This type of binding may be described by eqn. (1)

$$\log K_A = \log K_1 + \log\left\{\frac{K_2}{K_2 + [H^+]}\right\} \quad (1)$$

where K_A is the measured affinity constant of the ligand binding to the haem protein, K_1 is the pH-independent affinity constant, K_2 is the ionization constant of the ligand and $[H^+]$ is the measured hydrogen ion concentration.

The pH dependence of the value of K_A for cyanide binding to H-6-P are consistent with eqn. (1) (see Fig. 2A).

Figure 2B shows the affinity constant of imidazole binding to H-6-P, H-11-P and metmyoglobin *versus* pH. As with the binding of cyanide (Fig. 2A), the K_A for the formation of the imidazole adduct was found to be pH-dependent, increasing up to pH 7.5, thus indicating the binding species was deprotonated imidazole. Above pH 8, the affinity constant (K_A) of imidazole binding to H-11-P and metmyoglobin decreases as the pH increases. This may be explained by assuming that an ionizable group with a pK_a higher than 8 is competing effectively with imidazole. Such a group could be an ϵ-amino group of a lysine residue ($pK_a = 10.53$). H-11-P contains a number of amino groups which could play the above role [8]. Metmyoglobin also contains a number of lysine residues in its structure, but the distal histidine would be more likely. H-6-P contains only the amino terminal of Cys 14. From the molecular models of H-6-P it appears that binding of this nitrogen to the haem is sterically unfavourable, and this is reflected in the smaller decrease in K_A above pH 8. Intermolecular binding of the terminal amino group of one peptide to the iron atom of another is possible but this requires two molecules to come into a very close proximity to each other. Table I shows the binding of other ligands to H-6-P (N_3^-, SCN^- and F^-). These ligands do not show such marked pH dependence in their respective K_A values with H-6-P.

Discussion

Factors Affecting Ligand Binding

The process of exogenous ligand binding to the haem in haem proteins is complex and depends on a number of chemical and steric factors. It is, however, possible to go some way in rationalizing the effect of the protein or peptide moiety on the binding constant by considering the following factors.

1. Hydrophobicity of the haem environment

A hydrophobic environment is equivalent to a medium of lower dielectric constant [9, 10]. As all the ligands under consideration are either negatively charged or possess a lone-pair of electrons, proteins should provide an environment that stabilizes the adduct with the positively charged iron, and thus leads to an enhanced binding constant.

2. The haem crevice 'open–closed' equilibrium

The accessibility of exogenous ligands to haem varies between the proteins used in this study, e.g. depending on whether the protein provides a sixth ligand to the central iron atom and on the nature of this ligand: (i) native cytochrome *c* presents a closed haem crevice to ligands [11]; (ii) cm-cytochrome *c* presents a partially open haem crevice; (iii) myoglobin represents an open haem crevice; (iv) H-6-P represents a direct binding site for attacking ligands.

We will refer to the accessibility of ligands to the haem of the various proteins and peptides used as the haem crevice 'open–closed' parameter.

3. Steric interactions

This factor depends on the configuration of the attacking ligand. Cyanide ion binds linearly and perpendicularly to the haem plane [10]. Azide, however, binds non-linearly and therefore forms an angle with the haem [10]. Imidazole is a bulky ligand and consequently should cause a steric effect upon ligation. The influence of these factors combine to effect the binding of exogenous ligands to the haem in the various haemoproteins.

Relative Binding Stabilities of Ligand–Protein Complexes

In the following section we attempt to rationalize the data reported in terms of the three factors mentioned above. These factors are seen to combine in various ways to determine the relative stabilities of the ligand–protein complexes.

At pH 7.5 the affinity constant for cyanide binding to H-6-P is an order of magnitude higher than the affinity constant for cyanide binding to N-cytochrome *c* (Table I). H-6-P has a direct binding site for cyanide ligation [8], while N-cytochrome *c* has a closed haem crevice. The exogenous ligand has to enter the crevice and replace the endogenous ligand (met 80) [12]. Also the binding of CN^- to H-6-P is linear and normal to the haem plane and thus causes no steric effect on ligation [9, 10], while it results in a steric effect (a structural rearrangement involving side-chain movements of protein residues) when bound to N-cytochrome *c* [13]. However, the hydrophobicity of the haem environment in N-cytochrome *c* enhances the electrostatic interactions of the anionic cyanide and the cationic haem, as the hydrophobic environment is equivalent to a medium of lower dielectric constant and thus stabilizes the adduct formed [9, 10, 14]. The importance of the latter factor is further revealed in the affinity constant of cyanide binding to cm-cytochrome *c*. The affinity constants for cyanide binding to H-6-P and to cm-cytochrome *c* are comparable (Table I), even though the haem of the latter is not completely accessible to cyanide ligation [1] and in spite of the steric effect of cyanide ligation [9, 13] to the haem iron. This seems to confirm that cm-cytochrome *c* still has, to a large extent, an integral haem crevice [1, 13, 15]. The affinity of cyanide binding to myoglobin (Table I and Fig. 2A) supports the above argument, where the affinity constant of cyanide binding to metmyoglobin is an order of magnitude greater than that of H-6-P, reflecting the structural factors affecting cyanide binding. Metmyoglobin has a relatively open crevice and a relatively hydrophobic environment [9, 10] around the haem. In spite of the steric effect (involving a structural rearrangement which includes movement of a number of the protein side-chains [16]), the open site of myoglobin allows the cyanide ion to approach the central iron and to form an adduct, the stability of which is enhanced by the hydrophobicity of the haem environment.

The reported affinity constants for azide binding at pH 8.4 to H-6-P, N-cytochrome *c*, cm-cytochrome *c* and myoglobin (Table I) are also in accordance with the foregoing argument. Azide, unlike cyanide, is known to form bent or angular bonds with metals and therefore need not have a significant steric effect on the haem pocket [9, 10]. The 'open–closed' parameter of the haem pocket and the hydrophobicity of the haem crevice are, therefore, the dominant factors affecting azide binding to haemoproteins. The metmyoglobin affinity constant is about three orders of magnitude greater than the azide affinity constant for H-6-P, reflecting the open haem pocket of myoglobin and the hydrophobicity of the haem pocket. The affinity constant of cm-cytochrome *c* for azide is three times greater than the azide affinity constant to H-6-P, which is in accordance with the partially open haem pocket of the protein and the preserved hydrophobicity of the haem pocket. The azide affinity constant of N-cytochrome *c* is comparable to that of H-6-P, thus reflecting the importance of the hydrophobicity of the haem crevice in spite of the closed crevice of N-cytochrome *c*.

The reported affinity constants for imidazole binding at pH 8.5 to H-6-P, N-cytochrome *c*, cm-cytochrome *c* and myoglobin (Table I) reflect the importance of the haem pocket 'open–closed' equilibrium and steric factors, as imidazole is considerably more bulky than cyanide or azide. Thus the affinity constant of imidazole for H-6-P, a direct binding site, is an order of magnitude greater than that of metmyoglobin, an open site, and about three orders of magnitude higher than that of cm-cytochrome *c*, a partially open site. In turn, the imidazole affinity constant for cm-cytochrome *c* is double that of N-cytochrome *c*, thus reflecting the more open site of cm-cytochrome *c* compared to the native protein, but it is obviously not open enough for the imidazole affinity constant to be as high as with the open haem pocket of myoglobin. This is supported by the affinity constants of alkyl isocyanide to metmyoglobin (at pH 9.1, [6]), where the results demonstrate clearly that the affinity constant decreases with an increase in the size of the ligand, *i.e.* methyl ($52\ M^{-1}$) > ethyl ($28\ M^{-1}$) > n-propyl ($14\ M^{-1}$) > isopropyl ($2\ M^{-1}$) > isobutyl ($0.5\ M^{-1}$) > tert-butyl ($0.2\ M^{-1}$).

N-cytochrome *c* is known to have an alkaline isomerization [17] with a p*K* value of about 9.2. Thus, the closed structure of the N-cytochrome *c* haem crevice is preserved up to pH 8.5 [11]. At pH 10 or greater, N-cytochrome *c* has a haem crevice similar, if not identical, to cm-cytochrome *c* [17, 18].

The reported values for cyanide affinity constants to N-cytochrome *c* and cm-cytochrome *c* at pH 10 (Table I) are of the same order, thus affirming that the haem pockets of the two proteins are similar. This is supported by imidazole and azide binding to both proteins at pH 10.4 (Table I). The affinity constant for cyanide binding to H-6-P at pH 10 is about an order of magnitude higher than the affinity constants for both N-cytochrome *c* and cm-cytochrome *c*. This is in keeping with the foregoing argument as far as the 'open–closed' crevice equilibrium is concerned, as H-6-P has a direct site for cyanide ligation, while both proteins have a partially open haem crevice. Also this is consistent with the steric effect on both proteins resulting from cyanide binding; such steric restraints are absent in H-6-P. The protein–cyano complex would be stabilized by the hydrophobic environment of the haem pocket in both native and cm-cytochrome *c*, as mentioned earlier, since the cyanide affinity constant at pH 7.5 for cm-cytochrome *c* exceeds that of H-6-P.

One may expect, intuitively, the same to apply at pH 10 for both proteins, as their haem pockets resemble that of cm-cytochrome *c* at pH 7.5. Clearly this is not the case, as both proteins have lower cyanide affinity constants than H-6-P at pH 10 (Table I). This apparent contradiction is explained in terms of the type of endogenous ligand replaced by cyanide at pH 7.5 and pH 10 in cm-cytochrome *c*. At pH 7.5 approximately half the protein molecules have a weak-field ligand, *i.e.* a water molecule, at the sixth position of the haem iron [18], while at pH 10 the sixth coordination position is occupied either by a lysine residue [17, 18], or a hydroxyl anion [19], which are both intermediate-to-strong-field ligands. Thus, cyanide (being a strong-field ligand) will replace the water molecule at pH 7.5 with little competition, while at pH 10 the endogenous ligand (lysine or a hydroxyl anion) competes more effectively with cyanide for the sixth coordination position. Thus the lower cyanide affinity for cm-cytochrome *c* and N-cytochrome *c* than that of H-6-P is in keeping with the structure of both proteins. The cyanide affinity of myoglobin at pH 10 is greater than the H-6-P cyanide affinity constant. This is in keeping with the open site of myoglobin and the enhancement of cyanide binding by the hydrophobicity of the haem pocket, which outweighs the steric effect resulting from cyanide ligation.

The azide affinity to H-6-P at pH 10.4 is the same as the azide affinity for N-cytochrome *c* and cm-cytochrome *c* at the same pH (Table I). This enforces the argument for the presence of a stronger endogenous ligand in both proteins at pH 10 than that bound to the haem of cm-cytochrome *c* at pH 7.5. Therefore, in spite of the lack of a steric effect of azide ligation and the enhancement of the affinity constant by the partially open haem pocket and the hydrophobicity of the haem pocket, it is apparent that azide does not compete effectively to replace the endogenous ligand.

Imidazole (an intermediate-to-strong-field ligand) affinity constants to H-6-P, cm-cytochrome *c* and N-cytochrome *c* at pH 10.4 are reported in Table I. The imidazole affinity constant for cm-cytochrome *c* at pH 7.5 is greater than the affinity constant of imidazole binding to cm-cytochrome *c* at pH 10.4. This is in agreement with the concept of a stronger endogenous ligand at pH 10.4. As with cyanide and azide binding at pH $>$ 10 (Table I) the affinities for both native and cm-cytochrome *c* are comparable. This supports the argument of a similar haem pocket and a partially open haem crevice in both proteins. The affinity constant of imidazole binding to H-6-P is three orders of magnitudes greater than the imidazole affinity constants for both native and cm-cytochrome *c*. This enforces the argument of the haem pocket 'open–closed' parameter, as the affinity of imidazole to both N-cytochrome *c* and cm-cytochrome *c* is greatly reduced by the size of the imidazole molecule, in spite of the partially open site in both proteins at pH 10.4.

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