Ligand Binding to Cytochrome *c* and Other Related Haem Proteins and Peptides. Part III. Temperature Dependence Studies

M. M. M. SALEEM

Chemistry Department, Kuwait Institute of Technology, Kuwait

and M. T. WILSON

Department of Chemistry, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, Essex, U.K.

(Received February 13, 1988)

Abstract

The temperature dependency of ligand binding processes lend support to the proposed mechanisms and the factors affecting ligand binding reported earlier in this series. The free energy contribution from each factor affecting ligand binding was estimated for a number of haem proteins. The structures of the haem proteins used, as conveyed from ligand binding data, are in agreement with the structures of these haem proteins as determined by other methods (e.g. X-ray crystallography, NMR, etc.). Therefore, ligand binding could be used as a facile probe to investigate some of the structural and functional properties of haem proteins. In this respect, it was concluded that the structure of native cytochrome *c* at pH 10 is similar to the structure of carboxymethyl-Met 80 cytochrome *c* between pH 7 and 10.

Introduction

The structure and function of haem proteins are affected by temperature, as for any other protein [1, 2]. The kinetics and affinity constants for their reactions with exogenous ligands are also affected by temperature [3].

In this study we explore the effect of temperature on the binding process of ligands such as cyanide, azide and imidazole to cytochrome *c* and other related haem proteins and peptides.* Such studies allow one to analyse the energetics of the factors affecting the binding of ligands to the haem moiety. In addition, they may yield a better understanding of the mechanism and parameters influencing the binding process outlined in our previous studies (see preceding two papers).

Experimental

The materials used and the methods employed were the same as reported in the earlier accompanying papers of this series.

Results

Equilibrium Studies

The dependences of the affinity constants on temperature for cyanide binding to H-6-P, cm-cytochrome *c* and N-cytochrome *c* at pH 7.5 and 10.0 are shown in Fig. 1A and B, respectively. The results were analysed according to the van't Hoff equation.

The thermodynamic parameters for binding to H-6-P, cm-cytochrome *c* and N-cytochrome *c* are reported in Tables I and II. The linear dependence on temperature of the affinity constant for cyanide binding to H-6-P suggests a single equilibrium is involved (Fig. 1A and B). The N-cytochrome–cyanide formation constant shows a linear dependence on temperature at pH 7.5 (Fig. 1A), also indicating a single equilibrium is involved. At pH 10 the affinity constant for cyanide binding to N-cytochrome *c* *versus* temperature (Fig. 1B) gave two limbs. This suggests the existence of two temperature-dependent equilibria. The affinity constant characterized in limb I seems to increase with temperature, while that in limb II decreases with increasing temperature. The reaction of cm-cytochrome *c* with cyanide, at both pH 7.5 and pH 10 as a function of temperature, gave similar results to the temperature-dependent binding of cyanide to N-cytochrome *c* at pH 10. It is well known that N-cytochrome *c* at pH 10 or greater lacks the 695 nm band characteristic of Met 80 binding to the central iron [4]. The loss of the 695 nm band results from the disruption of the native conformation of the protein which exists at pH 7.5. This disruption

*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*.

0020-1693/88/$3.50

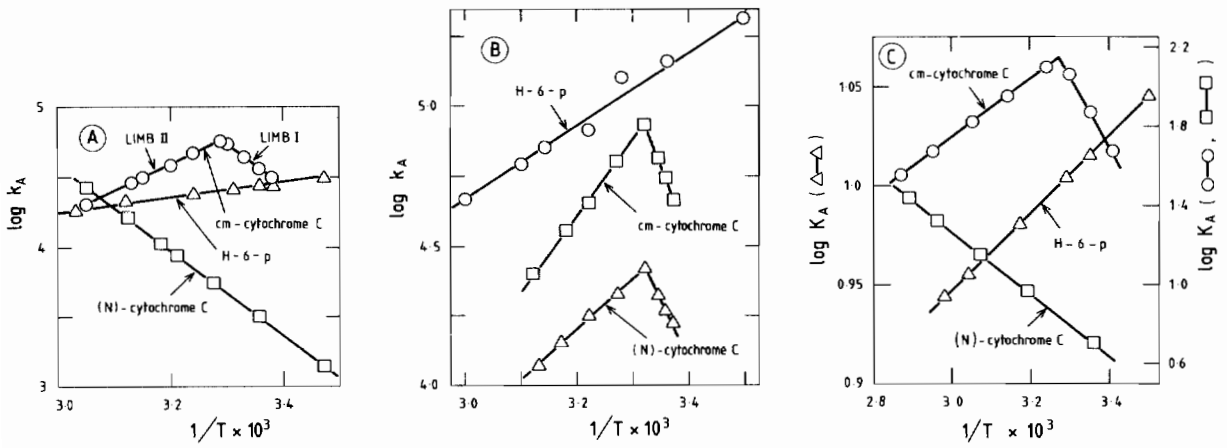

Fig. 1. Effect of temperature on the affinity constants for: (A) the binding of cyanide to H-6-P, cm-cytochrome *c* and N-cytochrome *c* at pH 7.5 in 0.15 M phosphate buffer; (B) the binding of cyanide to H-6-P, cm-cytochrome *c* and N-cytochrome *c* at pH 10 in 0.15 M borate buffer; (C) the binding of azide to H-6-P, cm-cytochrome *c* at pH 7.5 in 0.15 M phosphate buffer. In all sections limb I refers to the lower temperature region while limb II refers to the higher temperature region, as shown in (A).

is detected in a loosening of the haem crevice [4, 5]. As cm-cytochrome *c* does not have the 695 nm band, because Met 80 is modified, it may be reasonable to assume that cm-cytochrome *c* at both pH 7.5 and 10 has a very similar structure to N-cytochrome *c* at pH 10. Hence, it shows a very similar pattern in its reaction with cyanide at both pH 7.5 and 10 as a function of temperature to the alkaline form of N-cytochrome *c*. Figure 1C shows the affinity constant for azide binding to H-6-P, cm-cytochrome *c* and N-cytochrome *c* at pH 7.5 as a function of temperature, and it is consistent with the above argument. From Figs. 1A, B and C and Tables I and II, it is clear that the biphasic van't Hoff plots are a property of the protein (the alkaline form of N-cytochrome *c* and cm-cytochrome *c* and are independent of the ligand used.

TABLE I. Thermodynamic Parameters for Cyanide, Azide and Pyridine Binding to Haem Proteins (at 25 °C)

Protein	Ligand	pH	ΔH° (kcal mol^{-1})	ΔG° (kcal mol^{-1})	ΔS° (eu)	Remarks
N-Cytochrome *c*	cyanide	7.5	14.5	−4.8	+64.7	
cm-Cytochrome *c*	cyanide	7.5	14.3	−6.2	+68.85	Limb I
cm-Cytochrome *c*	cyanide	7.5	−8.9	−6.7	−7.6	Limb II
H-6-P	cyanide	7.5	−2.4	−6.0	+12.4	
Metmyoglobin[a]	cyanide	7.5	−18.6	−11.45	−24	
Maleylated cytochrome *c*[b]1 (no. 695 nm band)	cyanide	7.5	−9.4	−7.3	−7.0	
N-Cytochrome *c*	cyanide	10.0	17.4	−5.8	+77.9	Limb I
N-Cytochrome *c*	cyanide	10.0	−8.4	−6.1	−7.7	Limb II
cm-Cytochrome *c*	cyanide	10.0	23.5	−6.5	+100.8	Limb I
cm-Cytochrome *c*	cyanide	10.0	−12.0	−6.8	−17.4	Limb II
H-6-P	cyanide	10.0	−6.5	−7.0	+1.7	
N-Cytochrome *c*	azide	7.5	7.4	−0.95	+28	
cm-Cytochrome *c*	azide	7.5	16.3	−2.5	+63.4	Limb I
cm-Cytochrome *c*	azide	7.5	−7.0	−3.1	−12.9	Limb II
H-6-P	azide	7.5	−0.9	−1.4	+1.7	
Metmyoglobin[c]	azide	7.5	−11.6	−0.62	−18.4	
N-Cytochrome *c*	pyridine	10.0	4.6	−2.0	+22.2	Limb I
N-Cytochrome *c*	pyridine	10.0	−8.2	−2.6	−19	Limb II
cm-Cytochrome *c*	pyridine	7.5	9.2	−2.3	+38.5	Limb I
cm-Cytochrome *c*	pyridine	7.5	−9.2	−2.4	−19.8	Limb II

[a]From ref. 16. [b]From ref. 17 or data from ref. 18. [c]From ref. 19.

TABLE II. Thermodynamic Parameters of Cytochrome *c* Thermal Transitions (determined from the dependence of the 695 nm band on temperature)

Protein	pH	ΔH° (kcal mol^{-1})	ΔG° (kcal mol^{-1})	ΔS° (eu)
N-Cytochrome *c*[a] Stage 1	7.0	19.8	2.2	+67
N-Cytochrome *c*[b]	7.0	22	2.2	+66
N-Cytochrome *c*[c] Stage 2	7.0	45.1	4.3	+133
Maleylated cytochrome *c*[d]	7.0	41.5	−1.3	+144

[a] Stage 1 involves the displacement of Met 80 from iron coordination and small changes in the tertiary structure [6]. [b] Results from ref. 18 comparable to Stage 1 in ref. 6. [c] Stage 2 refers to a helix–coil transition, *i.e.* complete denaturation [6]. [d] From ref. 6. [e] From ref. 20.

TABLE III. Activation Parameters for Cyanide Binding to Haem Proteins

Protein	Ligand	pH	$\Delta H^\ddagger$ (kcal mol^{-1})	$\Delta G^\ddagger$ (kcal mol^{-1})	$\Delta S^\ddagger$ (eu)
N-Cytochrome *c*	cyanide	7.5	19.3	17.5	6.2
cm-Cytochrome *c*[a]	cyanide	7.5	14.9	16.45	−5.3
cm-Cytochrome *c*[b]	cyanide	7.5	24.1	17.7	21.4
Myoglobin[c]	cyanide	7.0	23.3	14.4	30
H-6-P[d]	cyanide	7.5	17.3	11.1	21
N-Cytochrome *c*[e]	imidazole	7.4	20.4	10.6	+33

[a] For the second-order rate of cyanide binding to cm-cytochrome *c*. [b] For the first-order rate-limiting step in cyanide binding to cm-cytochrome *c*. [c] From ref. 3. [d] For the fast phase (see Results section for explanation of phases). [e] Calculated from ref. 21.

It is interesting to note that the point where limb I and limb II intersect is almost invariant and corresponds to a temperature of 30–35 °C.

Kinetic Studies

The apparent second-order rates at pH 7.5 for cyanide binding to N-cytochrome *c* and cm-cytochrome *c* are temperature dependent; Figure 2A shows this dependency. The results were analysed according to the Arrhenius equation. Table III contains the activation parameters derived from Figs. 2A and B. The rate-limiting step was also found to be sensitive to temperature and to have a direct relationship to temperature, as shown in Fig. 2B.

Discussion

From Fig. 1 it is clear that the affinity of ligands for cytochrome *c* exhibiting the 695 nm band increases with temperature in a single equilibrium process, while cytochrome *c* lacking the 695 nm band shows a biphasic temperature dependence.

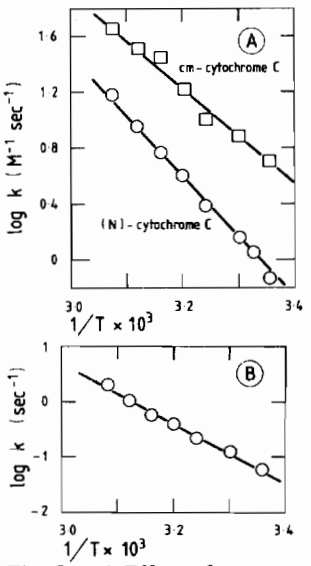

Fig. 2. (A) Effect of temperature on the apparent second-order rate constants for the binding of cyanide to N-cytochrome *c* and cm-cytochrome *c* at pH 7.5 in 0.15 M phosphate buffer. (B) Effect of temperature on the limiting rate kinetics for the binding of cyanide to cm-cytochrome *c* at pH 7.5 in 0.15 M phosphate buffer.

Temperature effects the conformation of native cytochrome *c* in two stages [6]. The first stage starts at about 35 °C; it involves the displacement of Met 80 from the iron coordination and small changes in the tertiary structure. This stage proceeds to about 60 °C [1] and the second stage begins at about 70 °C in a helix–coil transition. For cytochrome *c* lacking the 695 nm band, the native-type conformation (stage 1) is lost at much lower temperatures [6].

The temperature dependence of the affinity of cyanide for N-cytochrome *c* and cm-cytochrome *c* at pH 7.5 (Fig. 1A) reflects these structural changes. The binding of cyanide to N-cytochrome *c* is an endothermic process. Increasing the temperature results in a more open crevice, thus enhancing cyanide binding. For cm-cytochrome *c*, limb I (Fig. 1A) represents an endothermic process with thermodynamic parameters similar to those of the native molecule (Table IA). Therefore, limb I represents a native-type conformation of the haem crevice, as reported by Schejter *et al.* [6]. At about 30–35 °C there is an inflection point where limb I meets limb II. Limb II represents an exothermic process. This indicates that at temperatures higher than 35 °C the native-type conformation of the haem crevice in cm-cytochrome *c* is disrupted. At pH 10 the reaction of N-cytochrome *c* with cyanide exhibits biphasic behaviour similar to cm-cytochrome *c*, with limb I (an endothermic process) having thermodynamic parameters similar to those of native cytochrome *c* at pH 7.5 (Table Ia). The thermodynamic parameters of limb II are similar to cm-cytochrome *c* limb II at pH 7.5 (see also myoglobin, Table I). The differences in thermodynamic parameters at pH 10 between N-cytochrome *c* and cm-cytochrome *c* may be attributed to perturbations in the cm-cytochrome *c* structure because of chemical modifications. The ligand binding equilibrium studies (temperature and pH dependencies) seem to suggest that the cm-cytochrome *c* haem crevice is somewhat more open than the N-cytochrome *c* haem crevice at pH 10. Similar results are obtained with pyridine binding to N-cytochrome *c* at pH 10 and cm-cytochrome *c* at pH 7.5 (Table I).

The ligand binding data (see also Part II of this series) suggests that the structure of ferricytochrome *c* at pH 10 resembles that of cm-cytochrome *c* between pH 7 and 10. This is consistent with the ^{1}H NMR studies of both proteins [7] and with X-ray studies [8], where similar conclusions were reached.

Thermodynamic Drive in Cyanide Binding to Haem Proteins

At neutral pH values the reactions of myoglobin with cyanide are driven by a favourable enthalpy change (*i.e.* exothermic), while the reaction of N-cytochrome *c* with cyanide is driven by a favourable entropy change [9]. This can be rationalized in structural terms. In myoglobin, the cyanide displaces a water molecule; this is an exothermic process, so the only contribution to the overall entropy change arises from the loss of the cyanide molecule from solution and is partly compensated for by the release of a water molecule. In N-cytochrome *c*, binding of the cyanide necessarily displaces Met 80, so that the heat liberated by the formation of the cyanide–iron bond is compensated by the heat required to break the Met 80–iron bond and by the movement of the side-chains of the protein residues. This entropy change contributed by N-cytochrome *c* amounts to about 65 eu. This implies some reordering of the protein conformation. Part of these changes may be those required to accommodate the Met 80 side-chain in its new position. Other movements in the protein side-chains may occur depending on the exogenous ligand bound to the iron. For example, cyanide binds linearly and perpendicularly to the haem plane, causing a steric effect on the protein which results in movement of some side-chains [7]. Imidazole, a bulky ligand, almost certainly will cause movements similar to those caused by cyanide binding.

The entropy change in cyanide binding to myoglobin is −24 eu (Table I). This implies that when cyanide binds to myoglobin the system becomes more ordered. Cyanide ligation will cause a restricted conformation of the haem crevice, which results in a more ordered structure and hence negative entropy [10].

The endothermic equilibria (limb I) for cyanide binding to cm-cytochrome *c* and to the alkaline form of N-cytochrome *c* are similar to those for N-cytochrome *c* and have been discussed earlier (Table I).

The exothermic equilibria (limb II) for cyanide binding to cm-cytochrome *c* at pH 7.5 and N-cytochrome *c* at pH 10 yield entropy changes of −7.6 and −7.7 eu, respectively. This indicates that cyanide ligation results in a more ordered system, but possibly the protein side-chain movements upon cyanide ligation are more pronounced than with myoglobin, although solvent effects should occur and cannot be ignored or quantitized here. The general picture that emerges from the exothermic equilibrium is that the protein cm-cytochrome *c* and alkaline cytochrome *c* lose their native-type conformation. The degree of haem exposure, while not identical to that in myoglobin, is nevertheless similar enough to be comparable.

Cyanide binding to H-6-P is driven by a favourable enthalpy change. The entropy change of +12.4 eu implies disordering upon cyanide ligation. This is understood in terms of two oppositely charged ions forming a neutral product. The release of tightly solvated water molecules from the ions forming the complex is generally considered to be the source of the increased entropy and corresponds to a decrease in electrostriction.

The thermodynamic parameters for azide and pyridine binding to haem proteins (Table I) confirm the conclusions reached from cyanide binding.

How Do the Factors Affecting Ligand Binding to Haem Proteins Influence the Thermodynamic Parameters?

Haem proteins provide a hydrophobic environment for the haem. This is in contrast to the polar environment in H-6-P in aqueous solutions. The haem environment in the proteins would then be equivalent to a medium of lower dielectric constant, which results in a more negative ΔH° for the electrostatic interaction of the positively charged haem and the negatively charged ligands (cyanide and azide). This is illustrated by comparing ΔH° for cyanide binding to H-6-P at pH 7.5 with ΔH° for cyanide binding to myoglobin, cm-cytochrome *c* (limb II) and maleylated cytochrome *c* at the same pH (Table I). This result is supported by cyanide binding to haem proteins at pH 10 and azide binding to haem proteins at pH 7.5. Thus the hydrophobicity of the haem crevice will stabilize the complex to a greater extent than the aqueous environment of H-6-P.

Conformational changes due to steric interaction of the bound ligand (e.g. cyanide) with the protein will contribute to a more positive ΔH° in haem proteins compared to H-6-P. This factor is dependent on the 'open-closed' haem crevice parameter. In a closed crevice as in cytochrome *c*, a more positive ΔH° is expected when compared to an open crevice as in myoglobin. Steric interactions would be more significant in closed haem structures than in those with an open haem crevice. ΔH° values for cyanide binding to N-cytochrome *c* and cm-cytochrome *c* (limb I) at pH 7.5 are significantly more positive than ΔH° values for cyanide binding to myoglobin, cm-cytochrome *c* (limb II), maleylated cytochrome *c* and H-6-P (Table I), which serves to illustrate this point.

The 'open–closed' haem crevice parameter makes a significant contribution to the ΔH° of ligand binding, the sign of which depends on the state of the haem crevice. An open haem crevice as in myoglobin would result in a more negative ΔH°, as such a site presents only partial hindrance to exogenous ligands and at the same time preserves the hydrophobicity of the haem environment. A closed haem crevice as in N-cytochrome *c* results in a more positive ΔH°, as the closed structure represents a considerable barrier to the attacking ligand and ligation may result in a considerable steric effect (e.g. cyanide binding), as mentioned earlier. If, for example, ΔH° for cyanide and azide binding to N-cytochrome *c* and myoglobin at pH 7.5 are compared, then the importance of this factor becomes apparent. While N-cytochrome *c* has a positive ΔH° in its reaction with cyanide and azide, myoglobin has a negative ΔH° value. The other data presented in Table I are consistent with this argument.

In conclusion, the hydrophobicity of the haem crevice results in a negative ΔH° value, while steric effects result in a positive ΔH° value. A relatively more open haem crevice results in a more negative ΔH° and a closed crevice in a more positive ΔH°. These factors interplay and the apparent ΔH° measured is a reflection of their net effect in proteins.

Evaluation of the Relative Importance of the Factors Affecting Ligand Binding to Haem Proteins

The simplest approach is to express the free energy obtained from the binding of an exogenous ligand to the haem protein as the sum of the following individual energy contributions:

(i) ΔG_i which is the intrinsic free energy which depends primarily on the metal–ligand (M–L) bond strength.

(ii) ΔG_s is the free energy of the steric interactions between the ligand and the haem protein.

(iii) ΔG_{o-c} is the free energy of the 'open–closed' parameter of the haem crevice.

(iv) ΔG_{es} is the free energy of the electrostatic interaction.

Thus,

$$\Delta G_{Pr-L} = \Delta G_i + \Delta G_s + \Delta G_{es} + \Delta G_{o-c}$$

where Pr–L is the haem protein–ligand complex.

A similar analysis can be performed for H-6-P, but $\Delta G_s = 0$ and $\Delta G_{o-c} = 0$ as both these terms are protein (haem crevice) specific terms which vanish in H-6-P. So the total change in free energy is given by

$$\Delta G_{H6P-L} = \Delta G_i + \Delta G_{es}$$

By taking the difference between the free energies of ligation in the haem protein and H-6-P, *i.e.*

$$\Delta G_{Pr-L} - \Delta G_{H6P-L} = \Delta\Delta G_L = \Delta\Delta G_i + \Delta G_s + \Delta\Delta G_{es} + \Delta G_{o-c}$$

estimates of these free-energy components can be obtained. Huang and Kassner [11] have reported a similar treatment and we have used their mathematical procedure to calculate these free-energy components. The values of the free energies (in kcal mol^{-1}) of these components are listed in Table IV. These values were calculated making the following additional assumptions:

(i) $\Delta\Delta G_i = 0$ for proteins possessing the same prosthetic group, e.g. N-cytochrome *c* and cm-cytochrome *c*.

(ii) ΔG_s values are comparable for cyanide and imidazole and are close to zero for small ligands such as fluoride.

It is clear from the relationship of $\Delta G^\circ = -RT \ln K_A$ that a more positive free-energy change results in a lower affinity constant, while a more negative free-energy change results in a larger affinity constant. The steric interaction free-energy component, therefore, will cause a decrease in the affinity constant in N-cytochrome *c* and cm-cytochrome *c*, while in

TABLE IV. Free Energy[a] Contributions for Ligation to Haem Proteins Compared to H-6-P

Protein	$\Delta\Delta G_i$	ΔG_s	$\Delta\Delta G_{es}$	ΔG_{o-c}
N-Cytochrome *c*	0	+0.83	−2.48	+2.92
cm-Cytochrome *c*	0	+0.97	−2.31	+1.16
Metmyoglobin	+8.16	−0.65	−8.61	−4.3

[a]The free energy calculated is in kcal/mol at pH 7.5 and 25 °C.

myoglobin this factor promotes ligand binding (possibly by steric interactions leading to hydrogen bonding between the distal histidine and the bound ligand [11]. The electrostatic free-energy component enhances the affinity constant in all proteins. The haem crevice 'open–closed' parameter free-energy component in both N-cytochrome *c* and cm-cytochrome *c* is disadvantageous, while in myoglobin it enhances the affinity constant at least relative to H-6-P, possibly due to the fact that H_2O or OH^- ligands are more readily displaced from an 'out-of-plane' iron atom.

The values of the free-energy components are clearly related for a given protein, e.g. the value of ΔG_{o-c} is reflected in ΔG_s. In myoglobin ΔG_{o-c} is −4.3 kcal mol^{-1}, which results in ligands causing minimum steric effect on the protein and stabilization of the bound ligand by hydrogen bonding, which is reflected in the negative value of ΔG_s. This in turn is reflected in the large negative value of $\Delta\Delta G_{es}$, as in the absence of serious steric interactions the electrostatic interactions between the positive haem and anionic ligands will be the dominant factor influencing ligand binding. In N-cytochrome *c* the ΔG_{o-c} is positive which is reflected in a positive ΔG_s, thus the disadvantage of the ΔG_{o-c} will offset the advantageous ΔG_{es}. This is in accordance with the structure of both proteins. These free-energy components and their inter-relationships support the arguments employed in analysing the ligand binding data and support the conclusions reached in the earlier papers of this series.

The intrinsic free energy ($\Delta\Delta G_i$) taken for N-cytochrome *c* and cm-cytochrome *c* is zero or vanishingly small as both these proteins have the same haem group as H-6-P and therefore it is expected that these terms will cancel. In myoglobin the haem moiety is type *b*, while in H-6-P the haem is type *c*. This is reflected in $\Delta\Delta G_i$ = +8.155 kcal mol^{-1}, which suggests some difference in the metal–ligand bond strengths. The positive value indicates that the bond formed in Mb–L is weaker than the bond formed in (H-6-P)–L. This is consistent with the electron-pair donation from the distal histidine into ligands with low-energy empty orbitals, e.g. cyanide, azide, imidazole and CO [11, 12]. This will cause tilting in the bound ligand, thus resulting in a weaker bond than the untilted ligand bonds in H-6-P [13]. This effect should not be taken as a steric interaction, as steric interactions result in protein conformational changes which is clearly not the case here.

Kinetic Studies

The enthalpies of activation for cyanide binding (Table III) to N-cytochrome *c*, cm-cytochrome *c*, metmyoglobin and H-6-P at neutral pH reflect the energy barrier for the binding process, where $\Delta H^{\ddagger}$ for myoglobin > N-cytochrome *c* > H-6-P > cm-cytochrome *c*. N-Cytochrome *c* has a closed haem crevice and an endogenous intermediate-to-strong-field ligand, cm-cytochrome *c* has a partially open haem crevice and thus requires less energy than N-cytochrome *c* to reach the transition state. H-6-P has a direct binding site, but the polar environment of the haem is responsible for the higher $\Delta H^{\ddagger}$ compared to cm-cytochrome *c*, which has a hydrophobic haem environment and a partially open site for ligation, resulting in a low energy barrier for cyanide binding. The high $\Delta H^{\ddagger}$ for myoglobin reflects the displacement of a water molecule by a cyanide molecule and the subsequent release of this water molecule [13].

$\Delta H^{\ddagger}$ for the rate-limiting step in cm-cytochrome *c* is higher than $\Delta H^{\ddagger}$ for N-cytochrome *c* and myoglobin, supporting the argument presented earlier about the nature of this barrier.

If the entropy of activation, $\Delta S^{\ddagger}$, of cyanide binding is negative, this implies that the reacting molecules must order themselves in the transition state before they may form a product [14]. Cyanide binding to cm-cytochrome *c* has a $\Delta S^{\ddagger}$ of −5.3 eu which implies that cyanide and cm-cytochrome *c* must orient themselves for cyanide to bind, and upon binding a highly ordered structure results. The $\Delta S^{\ddagger}$ for the first-order rate limit is +21.4 eu which is a property of the protein alone. This is another piece of evidence supporting the proposed mechanism by which cm-cytochrome *c* binds exogenous ligands. As the polypeptide containing Lys 79 must be swung away to allow the cyanide to approach the haem iron, movement of the lysine residue must lead to a greater flexibility of the protein backbone. The entropy of activation for cyanide binding to N-cytochrome *c* has a value of +6.2 eu, while myoglobin has a $\Delta S^{\ddagger}$ value of +30 eu. This indicates that upon cyanide binding the protein in the transition state is in a relatively more rigid structure [13] than myoglobin. This also reflects the nature of the endogenous ligand replaced by cyanide in both proteins. In N-cytochrome *c* at pH 7.5 cyanide replaces Met 80 which is a protein ligand and thus the entropy change is mainly contributed by the protein residues readjusting themselves upon cyanide binding, while in myoglobin at pH 7 cyanide is replacing a water molecule, which requires the haem crevice opening to be enlarged to release the metal-

bound water and allow a cyanide molecule to enter [13], and by doing so increases $\Delta S^{\ddagger}$. This is also supported by the imidazole $\Delta S^{\ddagger} = 30$ eu for cytochrome *c* (Table III).

A positive $\Delta S^{\ddagger}$ for cyanide binding to H-6-P (Table III) is expected, since the reaction entails two oppositely charged ions forming a neutral product. The release of tightly solvated water molecules from the ions upon forming the activated complex is the source of the increased entropy and corresponds to a decrease in electrostriction [2].

The Overall Energetics of Cyanide Binding to Haem Proteins and H-6-P

In Figs. 3A and B the overall picture of ligand binding to haem proteins is depicted. Figure 3A shows the different barriers involved and their relative magnitudes for cyanide binding to haem proteins and H-6-P. Figure 3A also shows the energy content of the different systems upon cyanide ligation. Figure 3B shows the free energy each system requires to lift the reactants to the energy level of the transition state and also indicates the overall affinity. Both these Figures summarize the energetics of cyanide binding to the haem proteins and H-6-P. N-Cytochrome *c*, for example, upon cyanide binding has a large positive enthalpy, while the binding is characterized by a small negative free-energy change. According to the relationship $\Delta H^{\circ} = \Delta G^{\circ} + T\Delta S^{\circ}$, therefore, a large positive entropy change is involved. This is manifested also in the large activation barrier for cyanide binding and, therefore, the large free energy of activation required to lift a cyanide molecule and N-cytochrome *c* from their ground states to the transition state. Therefore, cyanide ligation to N-cytochrome *c* is not an energetically favourable process. This is in accordance with the physiological role of cytochrome *c* being an electron-carrier protein. On the other hand, myoglobin has a large negative free energy and a negative ΔH°. In spite of the large $\Delta H^{\ddagger}$ for cyanide binding to myoglobin, the overall process is energetically favourable. This is reflected in $\Delta S^{\circ} = -24$ eu which indicates a highly ordered protein structure upon cyanide ligation. This may be in keeping with the physiological function of myoglobin being a ligand-carrier protein. In cyanide binding to cm-cytochrome *c* the binding is biphasic, the first phase of which is to a native-type conformation which has almost the same energetics as that of the native protein. However, the second phase, which is the disrupted native-type conformation, is more comparable to the energetics of myoglobin.

The haem groups in both cytochrome *c* and myoglobin are very similar, although structurally different [15]. Disrupting the conserved structure of cytochrome *c* (as in cm-cytochrome *c*) induces the protein to go some way towards a myoglobin-like molecule.

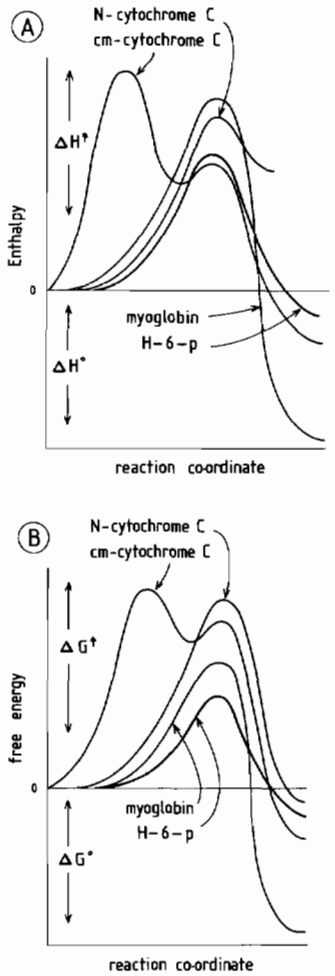

Fig. 3. (A) Enthalpy profile for the binding of cyanide to H-6-P, myoglobin, cm-cytochrome *c* and N-cytochrome *c* at pH 7.5. (B) Free energy profile for the binding of cyanide to H-6-P, myoglobin, cm-cytochrome *c* and N-cytochrome *c* at pH 7.5 (for experimental conditions, see text).

It is clear, therefore, that cyanide binding may be used as a probe in exploring structure, function and structure–function relationships in haem proteins.

References

1 L. Kaminsky, V. Miller and A. Davison, *Biochemistry, 12*, 2215 (1973).
2 A. Fersht, in 'Enzymes, Structure and Mechanism', W. H. Freeman, Reading, U.K., 1977, p. 44.
3 E. Antonini and M. Brunori, in 'Hemoglobin and Myoglobin in their Reactions with Ligands', North-Holland, Amsterdam, 1971.
4 W. Osheroff, D. Orden, W. Koppenol and E. Margoliash, in T. E. King (ed.), 'Cytochrome Oxidase', Elsevier/North-Holland Biochemical Press, Amsterdam, 1979, pp. 385–397.

5 R. Timkovich, in D. Dolphin (ed.), 'The Porphyrins', Vol. III, Academic Press, New York, 1979, p. 241.
6 A. Schejter, M. Zuckerman and I. Aviram, *J. Biol. Chem., 254,* 7042 (1979).
7 A. Boswell, *Ph.D. Thesis,* Oxford University, Oxford, 1981.
8 A. Colosimo, M. Brunori, F. Andreasi and S. Mobilio, *J. Inorg. Biochem., 15,* 179 (1981).
9 A. Schejter, I. Aviram, R. Marglit and T. Goldkorn, *Ann. N.Y. Acad. Sci., 244,* 51 (1975).
10 D. Blumenthal and R. Kassner, *J. Biol. Chem., 255,* 5859 (1980).
11 Y. Huang and R. Kassner, *J. Biol. Chem., 256,* 5327 (1981).
12 J. Maxwell and W. Caughey, *Biochemistry, 15,* 388 (1976).
13 H. Smith and G. McLendon, *J. Am. Chem. Soc., 102,* 5666 (1980).
14 P. Glansdorff and I. Prigogine, in 'Thermodynamic Theory of Structure, Stability and Fluctuations', Wiley–Interscience, New York, 1971.
15 R. Dickerson and I. Geis, in 'The Structure and Function of Proteins', W. A. Benjamin, Menlo Park, Calif., 1969, pp. 44–66.
16 P. George, in D. Green (ed.), 'Currents in Biochemical Research', Wiley–Interscience, New York, 1956, p. 358.
17 I. Aviram and A. Schejter, *J. Biol. Chem., 255,* 3020 (1980).
18 G. Pettigrew, I. Aviram and A. Schejter, *Biochem. J., 149,* 155 (1975).
19 J. Bailey, J. Beetlestone and D. Irvine, *J. Chem. Soc. A,* 241 (1969).
20 R. Izatt, J. Christenson, R. Pack and R: Bench, *Inorg. Chem., 1,* 828 (1962).
21 C. Greenwood and G. Palmer, *J. Biol. Chem., 240,* 3660 (1965).

Appendix

The following is the mathematical treatment used to obtain the free energy contribution of the factors affecting ligand binding to haem proteins. For definition of ΔG_i, ΔG_s, ΔG_{es} and ΔG_{o-c}, see text.

$$\Delta G_{Pr-L} = \Delta G_i + \Delta G_s + \Delta G_{es} + \Delta G_{o-c} \quad (1)$$

(where Pr–L is the protein–ligand complex).

$$\Delta G_{H6P-L} = \Delta G_i + \Delta G_{es} \quad (2)$$

(where H6P–L is the cytochrome *c* haemhexapeptide–ligand complex).

In order to examine the effect of the binding of a specific ligand to a given protein, subtract eqn. (2) from eqn. (1).

$$\Delta G_{Pr-L} - \Delta G_{H6P-L} = \Delta\Delta G_L =$$

$$\Delta\Delta G_i + \Delta G_s + \Delta\Delta G_{es} + \Delta G_{o-c} \quad (3)$$

$\Delta\Delta G_i$ could be omitted if the protein used has the same prosthetic group as H-6-P, which is true for N-cytochrome *c* and cm-cytochrome *c*. Therefore eqn. (3) simplifies to

$$\Delta\Delta G_L = \Delta G_s + \Delta\Delta G_{es} + \Delta G_{o-c} \quad (4)$$

Equation (4) will be used to obtain these free energy contributions (in kcal/mol) for native and cm-cytochrome *c* at pH 7.5 and 25 °C.

I. Native Cytochrome c

$$\Delta\Delta G_{N_3^-} = 0.44 \text{ kcal/mol} = \Delta\Delta G_{es} + \Delta G_{o-c} \quad (5)$$

(Here ΔG_s is taken to be of negligible value as azide binding causes little steric effect. See text for discussion.)

$$\Delta\Delta G_{imidazole} = 3.75 \text{ kcal/mol} = \Delta G_s + \Delta G_{o-c} \quad (6)$$

$$\Delta\Delta G_{CN^-} = 1.27 \text{ kcal/mol} = \Delta G_s + \Delta\Delta G_{es} + \Delta G_{o-c} \quad (7)$$

If the value of $\Delta\Delta G_{es} + \Delta G_{o-c}$ from eqn. (5) is used in eqn. (7)

$$\Delta\Delta G_{CN^-} = \Delta G_s + 0.44 \text{ kcal/mol} = 1.27 \text{ kcal/mol}$$

$$\Delta G_s = 0.83 \text{ kcal/mol}$$

If ΔG_s resulting from cyanide binding to haem protein is considered to be as small or comparable to ΔG_s resulting from imidazole binding to haem proteins, then

$$\Delta\Delta G_{CN^-} = 3.75 \text{ kcal/mol} + \Delta\Delta G_{es} = 1.27 \text{ kcal/mol}$$

$$\Delta\Delta G_{es} = -2.48 \text{ kcal/mol}$$

$$\Delta\Delta G_{CN^-} = 0.83 - 2.48 + \Delta G_{o-c} = 1.27 \text{ kcal/mol}$$

$$\Delta G_{o-c} = 2.92 \text{ kcal/mol}.$$

II. cm-Cytochrome c (for the Endothermic Phase)

$$\Delta\Delta G_{N_3^-} = -1.55 \text{ kcal/mol} = \Delta\Delta G_{es} + \Delta G_{o-c}$$

$$\Delta\Delta G_{imidazole} = 2.13 \text{ kcal/mol} = \Delta G_s + \Delta G_{o-c}$$

$$\Delta\Delta G_{CN^-} = -0.18 \text{ kcal/mol} = \Delta G_s + \Delta\Delta G_{es} + \Delta G_{o-c}$$

Following the same procedure used with native cytochrome *c*, the following results are obtained:

$$\Delta G_s = 0.97 \text{ kcal/mol}$$

$$\Delta\Delta G_{es} = -2.31 \text{ kcal/mol}$$

$$\Delta G_{o-c} = 1.16 \text{ kcal/mol}$$

III. Metmyoglobin

Here $\Delta\Delta G_i$ cannot be neglected, as myoglobin has a somewhat different prosthetic haem group to that of H-6-P; therefore eqn. (3) will be used, and calculation is on a similar footing to that used with native cytochrome *c*.

$$\Delta\Delta G_{N_3^-} = -4.76 \text{ kcal/mol} = \Delta\Delta G_i + \Delta\Delta G_{es} + \Delta G_{o-c} \quad (8)$$

$$\Delta\Delta G_{imidazole} = 3.21 \text{ kcal/mol} = \Delta\Delta G_i + \Delta G_s + \Delta G_{o-c} \quad (9)$$

$$\Delta\Delta G_{CN^-} = -5.41 \text{ kcal/mol} = \Delta\Delta G_i + \Delta G_s + \Delta\Delta G_{es} + \Delta G_{o-c} \quad (10)$$

$$\Delta\Delta G_{F^-} = -0.455 \text{ kcal/mol} = \Delta\Delta G_i + \Delta\Delta G_{es} \quad (11)$$

Fluoride is a very small ligand and thus the contribution of ΔG_{o-c} is considered to be negligible and ΔG_s is almost certainly negligible. This is a reasonable assumption (see ref. 11).

Using the value of eqn. (8) in eqn. (10), one obtains

$$\Delta\Delta G_{CN^-} = \Delta G_s - 4.76 \text{ kcal/mol} = -5.41 \text{ kcal/mol}$$

$$\Delta S_s = -0.65 \text{ kcal/mol}$$

Similarly, using the value of eqn. (9) in eqn. (10), one obtains

$$\Delta\Delta G_{CN^-} = \Delta\Delta G_{es} + 3.21 \text{ kcal/mol} = -5.41 \text{ kcal/mol}$$

$$\Delta\Delta G_{es} = -8.61 \text{ kcal/mol}$$

Using the value of the calculated $\Delta\Delta G_{es}$ in eqn. (11), one obtains

$$\Delta\Delta G_{F^-} = \Delta\Delta G_i = 8.155 \text{ kcal/mol}$$

Using all the calculated values in eqn. (10), ΔG_{o-c} is obtained

$$\Delta\Delta G_{CN^-} = \Delta G_{o-c} + \Delta G_s + \Delta\Delta G_{es} + \Delta\Delta G_i = -5.41 \text{ kcal/mol}$$

$$\Delta\Delta G_{CN^-} = \Delta G_{o-c} - 0.65 - 8.61 + 8.155 = -5.41 \text{ kcal/mol}$$

$$\Delta G_{o-c} = -4.305 \text{ kcal/mol}$$