use. Most studies oxidised the commercial cytochrome c with a slight excess of potassium ferricyanide ($K_3Fe(CN)_6$) before use, separating the cytochrome from the oxidising agent by gel chromatography. For a number of reasons: (a) that ferric cytochrome c has been used on occasion without purification and sometimes without oxidation, (b) that Osheroff et al. (1980) state that the use or otherwise of ferricyanide did not alter results when titrating the 695 nm band, and (c) that chromatographic procedures are time consuming, it was decided to establish whether unpurified, unoxidised ferric cytochrome c could be used in binding studies without affecting the results.

The binding of perchlorate to unpurified cytochrome c in the absence of an added oxidising agent was compared with its binding to purified cytochrome c, and to purified cytochrome c in the presence of an oxidising agent. Cytochrome c was purified by eluting a sample dissolved in deionized water through a Sephadex G-50 Superfine column (1.5 x 30 cm) equilibrated with 0.5 M NH_4HCO_3, at a flow rate of 4 mℓ $cm^{-2}hr^{-1}$. The main band was collected and lyophilised. When $K_3Fe(CN)_6$ was used to oxidise cytochrome c it was separated from cytochrome c on the same column. $Fe(CN)_6^{3-}$ does in fact bind to cytochrome c at two or more sites (Ragg and Moore, 1984), but at the concentration required to oxidise the cytochrome c (<1 x 10^{-2} M $K_3Fe(CN)_6$) binding is minimal. The binding was confirmed qualitatively in this work: at 0.1 M $K_3Fe(CN)_6$, the A_{695} (corr.) of cytochrome c decreased by 20 per cent (pH 9.0).

A plot of $\log|A_o-A|/A$ against $\log[NaClO_4]$ for the three samples of cytochrome c (Figure 5.3) shows that all the points coincide and give a least squares line of slope. ca. 0.8 [cf. results in Section 5.5]. Thus there is no discernible change in binding due to purification or oxidation of ferricytochrome c. The remainder of the work in this study was carried out using unpurified cytochrome c (Sigma, Type VII) in the absence of an oxidising agent.

5.4 The pK_a of the alkaline isomerisation and estimation of ϵ_{695}

In the classic paper of Theorell and Åkesson (1941) five discrete conformational forms of cytochrome c were reported, the pK_a's of

which were reversible. The 'alkaline isomerisation' from Type III (present between ca. pH 4 and 8) to Type IV (present at alkaline pH values below pH 12) has received much attention in the literature. The pK_a of the alkaline isomerisation is known to vary from 8.9 to 9.3, depending on the ionic strength. Greenwood and Wilson (1971) reported a fall of 10 per cent in A_{695} at an ionic strength of $\mu = 1.0$; the pK_a was decreased by 0.3 on increasing the ionic strength from $\mu = 0.02$ to $\mu = 0.3$.

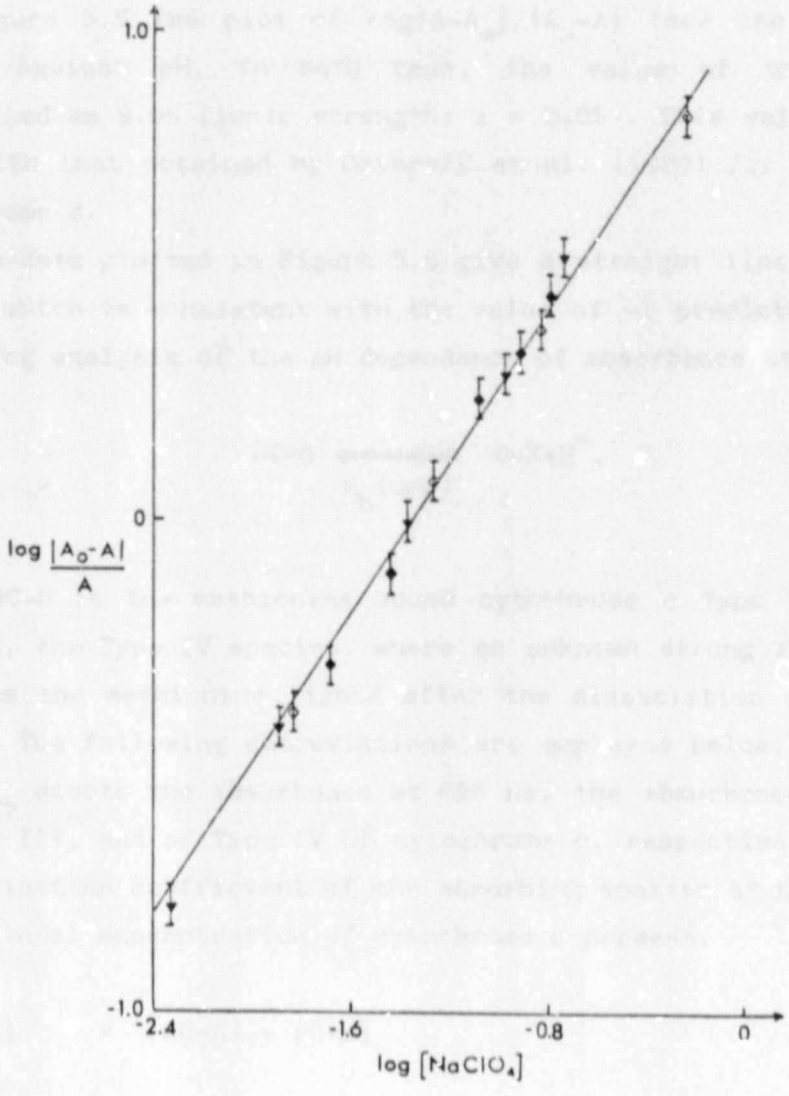


Fig. 5.3 The effect of purification and oxidisation (with $K_3Fe(CN)_6$) of cytochrome c on the binding of sodium perchlorate: ▼ purified cytochrome c; ◆ unpurified cytochrome c; ◇ purified and oxidised cytochrome c.

The value of the pK_a was determined in this study for comparison with literature values and to estimate the extinction coefficient, ϵ_{695}, both for the uncorrected A_{695} and A_{695} (corr.). This was deemed necessary as turbidity introduced during binding studies with sparingly soluble ligands [see Section 5.2], rendered A_{695} values meaningless, without affecting A_{695} (corr.). Recent determinations of the pK_a for the alkaline isomerisation of horse-heart cytochrome c and ϵ_{695} values for Types III and IV of cytochrome c (see below), are given in Table 5.1.

Figure 5.4 shows the plots of A_{695} and A_{695} (corr.) against pH, and Figure 5.5 the plot of $\log|A-A_\infty|/|A_o-A|$ (see the derivation below) against pH. In each case, the value of the pK_a was determined as 9.05 (ionic strength: μ = 0.05). This value compares well with that obtained by Osheroff et al. (1980) for horse-heart cytochrome c.

The data plotted in Figure 5.5 give a straight line with slope -0.96, which is consistent with the value of -1 predicted from the following analysis of the pH dependence of absorbance at 695 nm:

$$HC\text{-}S \underset{K_H(app)}{\rightleftharpoons} C\text{-}X + H^+,$$

where HC-S is the methionine bound cytochrome c Type III species and C-X, the Type IV species, where an unknown strong field ligand replaces the methionine ligand after the dissociation of a single proton. The following abbreviations are employed below: A_{695}, A^{o}_{695} and A^{∞}_{695} denote the absorbance at 695 nm, the absorbance at 695 nm of Type III, and of Type IV of cytochrome c, respectively; ϵ_{695} is the extinction coefficient of the absorbing species at 695 nm; $[C_T]$ is the total concentration of cytochrome c present.

$$[C_T] = [HC\text{-}S] + [C\text{-}X]$$

$$K_H(app) = \frac{[C\text{-}X][H^+]}{[HC\text{-}S]} \qquad (1)$$

$$[C\text{-}X] = [C_T]\,\frac{K_H(app)}{(K_H(app)+H^+)} \qquad (2)$$

$$A_{695} = [HC\text{-}S]\varepsilon^{HC\text{-}S}_{695} + [C\text{-}X]\varepsilon^{C\text{-}X}_{695}$$

$$= [C\text{-}X][\varepsilon^{HC\text{-}S}_{695} - \varepsilon^{C\text{-}X}_{695}] + [C_T]\varepsilon^{C\text{-}X}_{695} \quad (3)$$

$$= \frac{A_{695} - [C_T]\varepsilon^{C\text{-}X}_{695}}{\varepsilon^{HC\text{-}S}_{695} - \varepsilon^{C\text{-}X}_{695}} \quad (4)$$

$$[C\text{-}X] = [C_T] - [HC\text{-}S]$$

$$= \frac{[C_T]\varepsilon_{695} - A_{695}}{\varepsilon^{HC\text{-}S}_{695} - \varepsilon^{C\text{-}X}_{695}} \quad (5)$$

At pH 7.0, $[H^+] \gg K_H(app)$ so $[HC\text{-}S] \sim [C_T]$

and $A^{\circ}_{695} = [C_T]\varepsilon^{HC\text{-}S}_{695}$ (6)

At pH 11.0, $[H^+] \ll K_H(app)$ so $[C\text{-}X] \sim [C_T]$

and $A^{\infty}_{695} = [C_T]\varepsilon^{C\text{-}X}_{695}$ (7)

Taking the ratio of (5) and (4) and substituting (6) and (7) gives:

$$\frac{[HC\text{-}S]}{[C\text{-}X]} = \frac{A_{695} - A^{\infty}_{695}}{A^{\circ}_{695} - A_{695}} \quad (8)$$

Rewriting (1) as

$$\log[H^+] = \log K_H(app) + \log \frac{[HC\text{-}S]}{[C\text{-}X]} \quad (9)$$

and substituting (8) into (9) gives:

$$pH = pK_H(app) - \log \frac{|A_{695} - A^{\infty}_{695}|}{|A^{\circ}_{695} - A_{695}|} \quad (10)$$

Equation (10) is plotted in Figure 5.5.

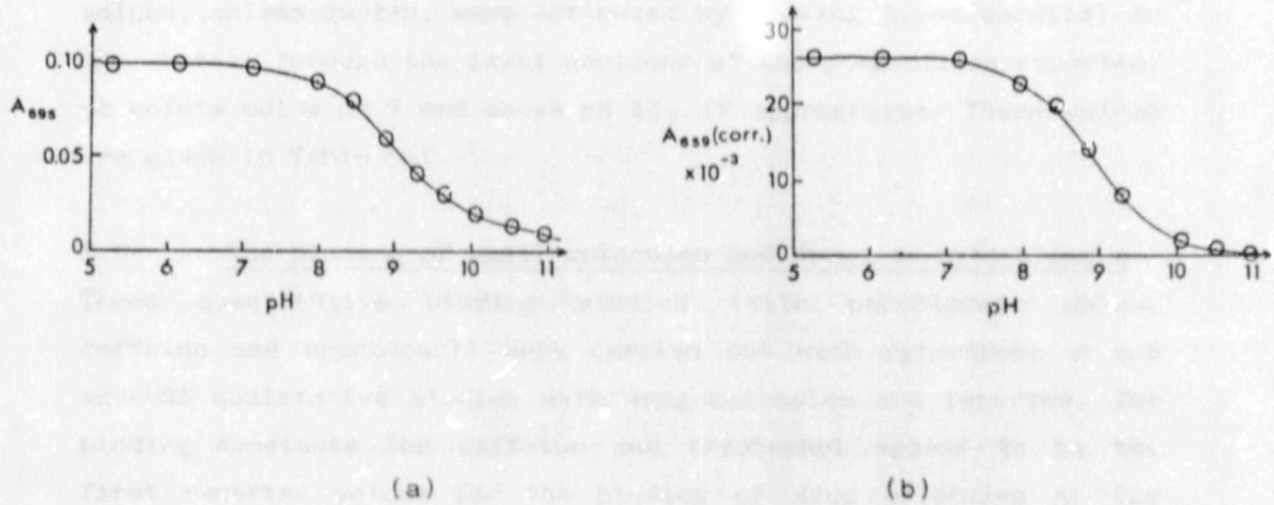
TABLE 5.1: Recent determinations of the pK_a for the alkaline isomerisation, and ϵ_{695} for Types III and IV of cytochrome c.

Source	pK_a	Ionic strength	$^*\epsilon^{III}_{695}$/$mM^{-1}cm^{-1}$	$^*\epsilon^{IV}_{695}$/$mM^{-1}cm^{-1}$
Smith and Millett (1980)	8.9	0.05	867	256
Andersson et al. (1980)	9.3	-	1000	55
Osheroff et al. (1980)	9.05	-	1000	-
Davis et al. (1974)	9.0	0.1	600	90
Greenwood and Wilson (1971	9.2 8.9	0.02 0.3	780 -	200 -
Lemberg and Barrett (1973)	-	-	810	-
This work	9.05	0.05	867	83

* These values were estimated as described in Section 5.4

The cytochrome c concentration used in the pH-titration was estimated using the extinction coefficient at 550 nm for ferri cytochrome c (ϵ_{550} = 8.50 x 10^3 $M^{-1}cm^{-1}$ (Kaminsky et al., 1973)). The cytochrome c concentration was thus 1.13 x 10^{-4} M. This value may represent a slight overestimation due to the fact that a small proportion of commercial cytochrome c is expected to be in the reduced form ($\epsilon_{red} > \epsilon_{oxid}$ at 550 nm).

From the A_{695} values and the value of the concentration of cytochrome c, ϵ_{695} for Types III and IV of cytochrome c were estimated as 867 $mM^{-1}cm^{-1}$ and 83 $mM^{-1}cm^{-1}$, respectively. These values may represent slight underestimations due to the possible



(a) (b)

Fig. 5.4 Acid-base titration of the 695 nm absorption band of 1.13×10^{-4} M cytochrome c at 25 °C: (a) A_{695} against pH; (b) A_{695} (corr.) against pH (μ = 0.05).

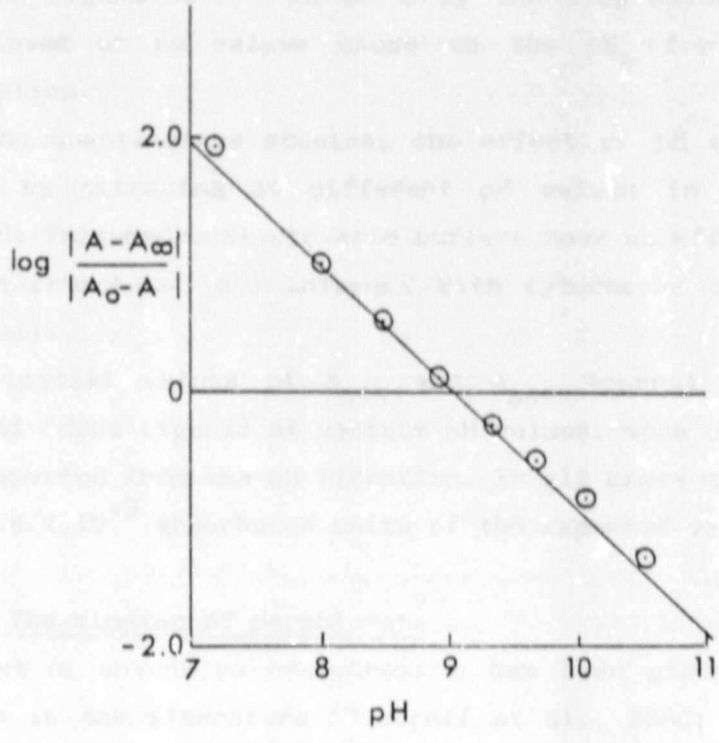


Fig. 5.5 The data in Fig. 5.4 (a) are plotted according to equation 10 [Section 5.4].

overestimation of the cytochrome c concentration.

The value of ε_{695} (corr.) at pH 6.5 (Type III) was 230 $mM^{-1}cm^{-1}$. No value of ε_{695} (corr.) has been published, so no basis for comparison exists. The ε_{695} values for Types III and IV of cytochrome c compare well with the literature values. Literature values, unless quoted, were estimated by drawing lines parallel to the abcissa through the level portions of the pH-profiles reported, at points below pH 7 and above pH 11, if appropriate. These values are given in Table 5.1.

5.5 The binding of small molecules and drugs to cytochrome c

Three quantitative binding studies (with perchlorate anion, caffeine and tryptophol) were carried out with cytochrome c and several qualitative studies with drug molecules are reported. The binding constants for caffeine and tryptophol appear to be the first reported values for the binding of drug molecules to the cytochrome c haem centre.

The method of titration and analysis of results was discussed in Section 5.2. The binding constants of these molecules were expected to be low. To facilitate the displacement of the methionine ligand to cytochrome c by the drug molecules, binding was followed at pH values close to the pK_a for the alkaline isomerisation.

In the quantitative studies, the effect of pH on binding was examined by titrating at different pH values in the range pH 8.6 - 9.5. Tris-hydrochloric acid buffers have no effect on binding (although phosphate ions interact with cytochrome c (Osheroff et al., 1980)).

The initial values of A_{695} and A_{695} (corr.) (i.e. in the absence of added ligand) at various pH values, were compared to the values expected from the pH titration. In all cases the values fell within 0.6×10^{-3} absorbance units of the expected value.

5.5.1 The binding of perchlorate

The effect of anions on cytochrome c has been given considerable attention in the literature (Osheroff et al., 1980; Greenwood and Wilson, 1971; Nicholls, 1974). Anions such as phosphate, citrate, malate and nucleotide triphosphates and diphosphates appear to

share a common binding site on cytochrome c, located not far from the haem crevice (Osheroff et al., 1980; Borden et al., 1978). Phosphate and citrate at low concentrations stabilise the closed form of the haem crevice. The ability of phosphate to stabilise the haem crevice has been confirmed by thermal (Kaminsky et al., 1973) and pulse radiolysis (Land and Swallow, 1976) studies, in the presence of alcohols. Anions like chloride, acetate, borate and cacodylate do not interact with this site, and their effect at low concentrations on the haem crevice is minimal. Osheroff et al. (1980) found an increase in the pK_a for the alkaline isomerisation with high concentrations of chloride or acetate, rather than the decrease reported by Greenwood and Wilson (1971). They attribute the increase to a general ionic strength effect, rather than one of specific binding. However, a destabilising effect on the haem crevice due to sodium bromide was also reported by Kaminsky et al. (1973): an increase in the ionic strength from $\mu = 0.15$ to $\mu = 0.34$ facilitated a thermally induced crevice opening in cytochrome c in the presence of alcohols.

The effect of perchlorate on cytochrome c was first mentioned by Ilan et al. (1976); its effect on the oxidation of ferrous cytochrome c by $Fe(CN)_6^{3-}$ was seen to be similar to that of alcohols i.e. the oxidation was slowed. The effect was interpreted as being due to perchlorate's water structure breaking ability, water probably being involved in some stage of the redox reaction (Margalit and Schejter, 1973).

A more comprehensive study of the effect of perchlorate on cytochrome c was conducted using ^{1}H and $^{35}C\ell$ NMR, ESR and optical spectroscopy (Andersson et al., 1980). The pK_a for the formation of Type IV, and for its disappearance, were found to be displaced from 9.3 to 8.3 and 10.4 to 10.9, respectively, an effect dependent upon perchlorate concentration below 0.1 M. The effects were attributed to specific binding of perchlorate to cytochrome c at a 'new site', in or near the haem crevice. Perchlorate was seen to have a higher affinity for Type IV than III, and to stabilise it. Complex formation with cyanide caused a loss of the 'new binding site', thought to be present in the alkaline form in the presence of perchlorate.

It is clear that direct binding between perchlorate and

cytochrome c occurs which induces a conformational change in the cytochrome c. First, increasing perchlorate concentrations cleave the iron-methionine bond, as evidenced by the disappearance of A_{695}. Secondly, ESR measurements show that cyanide, known to displace methionine 80 as the sixth haem ligand resulting in a small conformational change (Wütrich, 1969), causes a loss of the binding site for perchlorate. Thirdly, the greater affinity of perchlorate for the open, unstrained conformation of the alkaline form and its stabilisation of this form, are consistent with the binding of this anion. Finally, the decreased pK_a for the alkaline isomerisation in the presence of perchlorate, is also consistent with the binding effects of the anion. However, the nature of the interaction between perchlorate and cytochrome c is not well understood, and a binding constant has not been reported.

Accordingly, it was decided to investigate the binding of perchlorate. The decrease of the optical charge-transfer band at 695 nm (representative of the integrity of the iron-methionine bond) was followed with increasing perchlorate concentrations, for four pH values near the pK_a of the alkaline isomerisation. In contrast to the effect of chloride, where a 10 per cent decrease in A_{695} was found at $\mu = 1.0$, a 60 per cent decrease in A_{695} was found with perchlorate (pH 8.8) at a tenth of this concentration. Approximately one anion was found to bind to the site at each pH; the affinity of perchlorate for the alkaline form was clearly demonstrated. The near infra-red spectrum for a titration of cytochrome c by perchlorate at pH 8.8, is shown in Figure 5.6; and Figure 5.7 shows plots of A_{695} (corr.) against perchlorate concentration for four different pH values. The binding was evaluated by plotting $\ell og|A_o-A|/A$ against $\ell og[NaC\ell O_4]$ [see Section 5.2], where the slope = n, the number of anions bound, and the intercept = $\ell og\ K_{app}$. These plots, for four pH values, are shown in Figure 5.8. The transition from Type III to Type IV of cytochrome c is accompanied by a single ionization with the number of protons lost per molecule ranging from 0.8 to 1.1 (Osheroff et al., 1980). The values of K_{app} from the ordinate-intercepts of the plots in Figure 5.8 were used to estimate K_{eq} [see Section 5.2]: $\ell og\ K_{app}$ was plotted against pH giving $\ell og\ K_{eq}$ as the ordinate-intercept (see Figure 5.9). The results are summarised in Table 5.2.

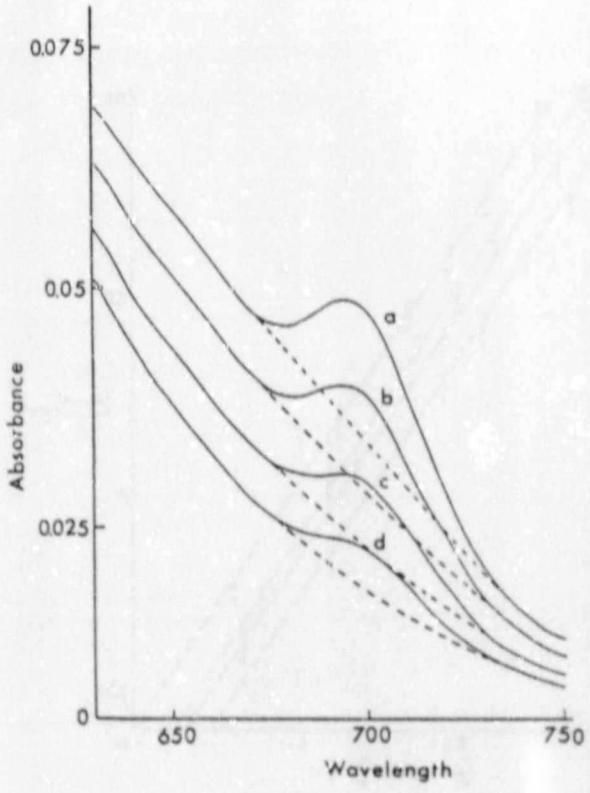


Fig. 5.6 Spectra of a titration of the 695 nm absorption band of ca. 1 x 10^{-4} M cytochrome c by sodium perchlorate, pH 9.0: A = 0 M $NaC\ell O_4$; B = 0.014 M $NaC\ell O_4$; C = 0.053 M $NaC\ell O_4$; D = 0.146 M $NaC\ell O_4$.

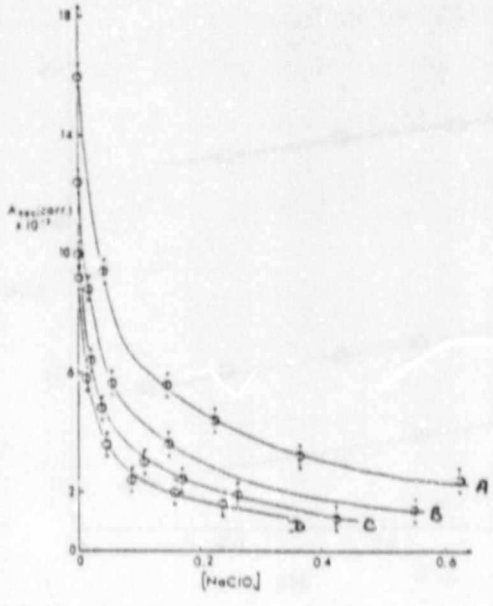


Fig. 5.7 The corrected absorbance values at 695 nm as a function of sodium perchlorate concentration: A = pH 8.8, B = pH 9.0, C = pH 9.3 and D = pH 9.5; cytochrome c concentration ca. 1 x 10^{-4} M (tris-hydrochloric acid buffers, μ = 0.1).

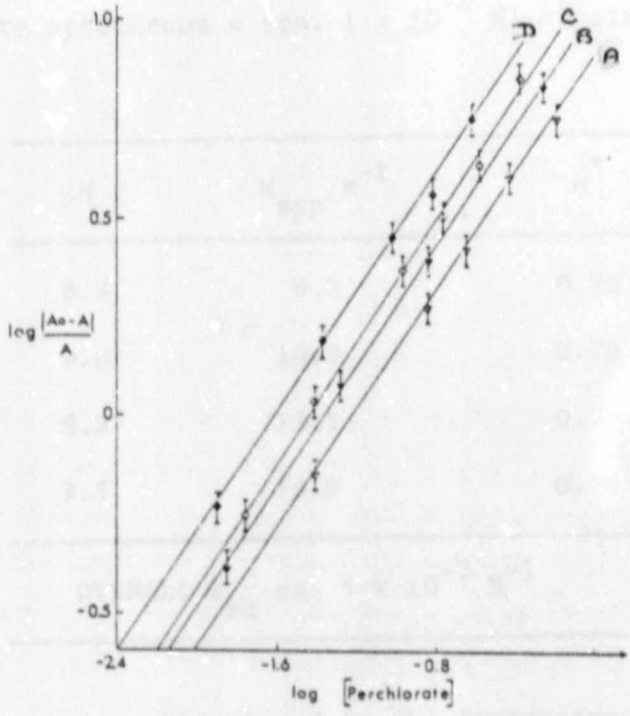


Fig. 5.8 The binding of sodium perchlorate to ca. 1 x 10^{-4} M cytochrome c: A = pH 8.8, B = pH 9.0, C = pH 9.3 and D = pH 9.5 (tris-hydrochloric acid buffers, μ = 0.1).

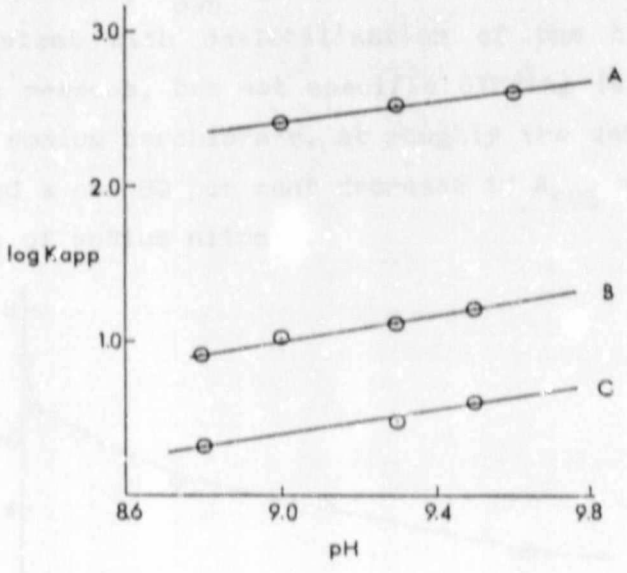


Fig. 5.9 The plots of ℓog K_{app} against pH for perchlorate, caffeine and tryptophol binding to ca. 1 x 10^{-4} M cytochrome c. The value of ℓog K_{eq} is given by the ordinate intercept: A = tryptophol; B = perchlorate and C = caffeine.

TABLE 5.2: Equilibrium constants for the binding of perchlorate to cytochrome c (ca. 1 x 10^{-4} M) in alkaline solution.

pH	K_{app}/M^{-1}	n*
8.8	8.2	0.75
9.0	10.8	0.76
9.3	13.1	0.78
9.5	16.6	0.76
OVERALL K_{eq} ca. 5 x 10^{-3} M^{-1}		

* These values are rationalised in the discussion.

The effect of sodium nitrate on the 695 nm band was examined in this study for comparison with perchlorate. At ionic strength μ = 3.0 (sodium nitrate), A_{695} was reduced in intensity by ca. 50%; an effect consistent with destabilisation of the haem crevice for electrostatic reasons, but not specific binding (see Figure 5.10). In contrast, sodium perchlorate, at roughly the same pH value (ca. 9.2), effected a ca. 80 per cent decrease in A_{695} at a tenth of the concentration of sodium nitrate.

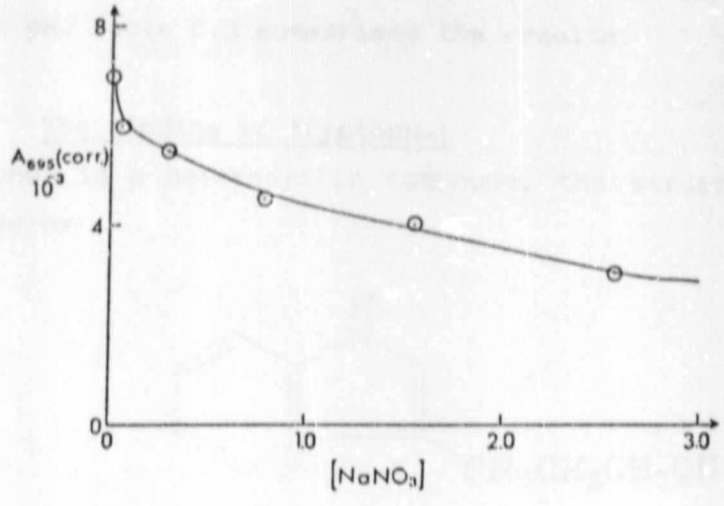
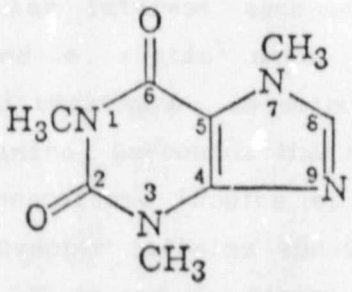


Fig. 5.10 The 695 nm absorbance of ca. 1 x 10^{-4} M cytochrome c as a function of the increased concentration of sodium nitrate.

5.5.2 The binding of caffeine

The structure of caffeine, a heterocyclic compound, is shown below:

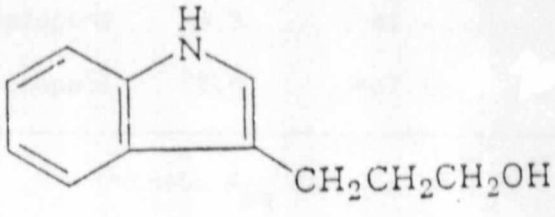


Caffeine has no pK_a's in the alkaline region, and while it is known to hydrolyse in solution, less than 1 per cent decomposition is present after 3 hours (Campbell, 1980). Studies with caffeine are of interest, because it has been shown to interact with haemin forming a monomeric complex in aqueous solution (Campbell, 1980). Gallagher and Elliot (1967) ascribed a low value for ΔG and a high ΔS found for the dissociation of the haemin/caffeine adduct to hydrophobic bonding between caffeine and haemin. Barry et al. (1973) showed that haemin and caffeine form a donor-acceptor complex in $CDC\ell_3$. Hence, the nature of the interactions in the monomeric haemin/caffeine complex (K_{caff} ca. $7.5 \times 10^4\ M^{-1}$) are likely to comprise both hydrophobic and π-π interactions (caffeine has an extended π system) (Campbell, 1980).

The binding of caffeine to cytochrome c with an accompanying conformational change to a Type IV complex (where A_{695}(corr.) tends to zero), was examined at three pH values, by similar methods to those described in the preceding section. The plots yielding K_{app} are shown in Figure 5.11, and Figure 5.9 shows the plot of $\log K_{app}$ against pH. Table 5.3 summarises the results.

5.5.3 The binding of tryptophol

Tryptophol is a heterocyclic compound, the structure of which is shown below:



It has no pK_a's in the pH region under consideration and has no effect on the ionic strength of buffered solutions, pH 8.5 - 9.5. It is of particular interest because of its close structural similarity to indole acetic acid (of importance in plant physiology) and to tryptophan, an amino acid which, together with histidine and arginine, surrounds the sixth coordination position in cytochrome c peroxidase (Poulos et al., 1980). The effect of forming a donor-acceptor caffeine adduct with haemin is to shift the Soret band to 402 nm and the visible band to 606 nm (Campbell, 1980). A similar effect was obtained when tryptophol was added to 1×10^{-5} M haemin in aqueous alkaline solution (pH 8) (Aron (1982)). Thus tryptophol probably engages in donor-acceptor interactions with haemin. The binding of tryptophol to cytochrome c at three pH values was investigated by similar methods to those described in the previous section. Figure 5.12 shows the plots yielding K_{app} and Figure 5.9 the plot of $\log K_{app}$ against pH. The results are summarised in Table 5.3.

TABLE 5.3: Equilibrium constants for the binding of caffeine and tryptophol to cytochrome c (ca. 1×10^{-4} M) in alkaline solution.

Ligand	pH	K_{app}/M^{-1}	n
caffeine	8.8	2.1	0.69
caffeine	9.3	3.0	0.76
caffeine	9.5	4.1	0.78
OVERALL K_{eq} ca. 6×10^{-4} M^{-1}			
tryptophol	9.0	262	1.59
tryptophol	9.3	341	1.50
tryptophol	9.5	407	1.48
OVERALL K_{eq} ca. 9×10^{-2} M^{-1}			

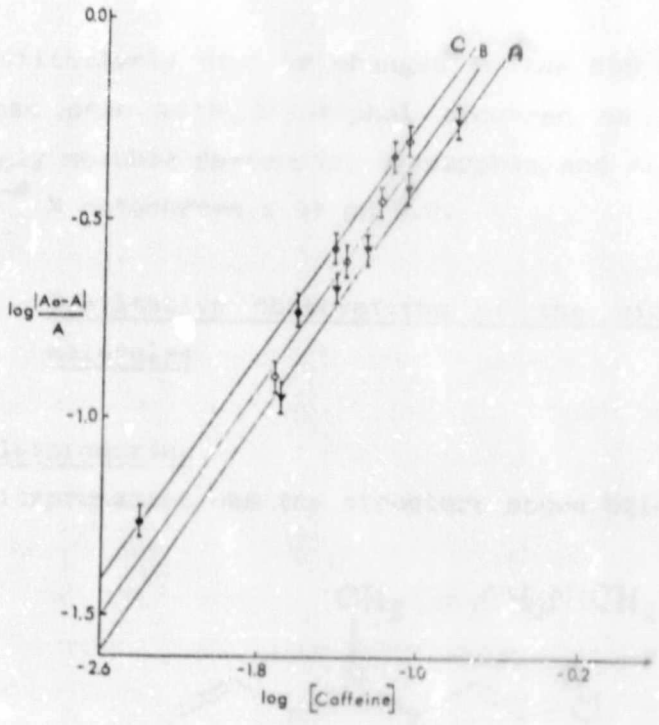


Fig. 5.11 The binding of caffeine to ca. 1 x 10^{-4} M cytochrome c: A = pH 8.8, B = pH 9.3 and C = pH 9.5 (tris-hydrochloric acid buffers, μ = 0.1).

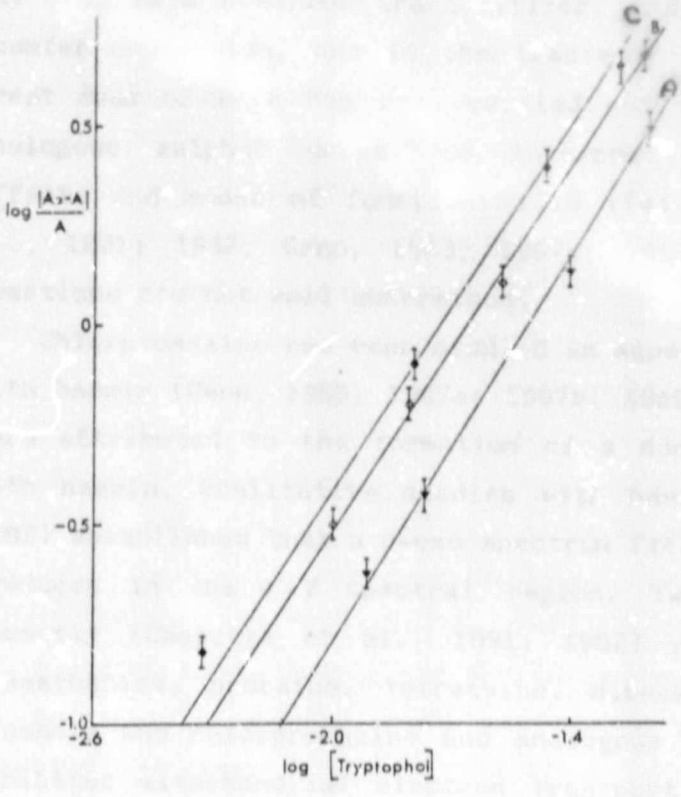


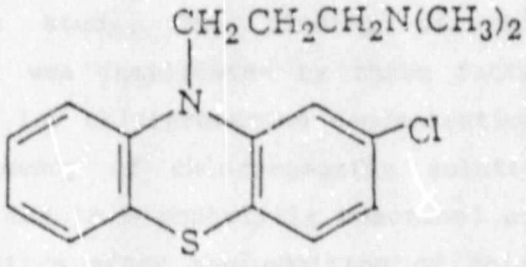
Fig. 5.12 The binding of tryptophol to ca 1 x 10^{-4} M cytochrome c: A = pH 9.0, B = pH 9.3 and C = pH 9.5 (tris-hydrochloric acid buffers, μ = 0.1).

Qualitatively similar changes in the 695 nm absorbance region to those seen with tryptophol occurred on the addition of the sparingly soluble compounds, tryptophan and indole acetic acid, to 1×10^{-4} M cytochrome c at pH 9.0.

5.5.4 Qualitative observations of the binding of other drug molecules

(a) Chlorpromazine

Chlorpromazine has the structure shown below:



The drug is a powerful tranquillizer, and is administered to counter depression, and in the treatment of schizophrenia. A great deal of work has been carried out using this drug and analogous sulphur drugs, to interpret its anti-psychotic effects and modes of functioning in vivo (e.g. Chazotte et al., 1981; 1982; Cann, 1965; 1967a; 1967b; 1969). Yet its reactions are not well understood.

Chlorpromazine has been studied in aqueous solution at pH 6 with haemin (Cann, 1965; 1967a; 1967b; 1969). Spectral effects were attributed to the formation of a donor-acceptor complex with haemin. Qualitative studies with haemin at pH 8 (Aron, 1982) established that a μ-oxo spectrum (see Campbell, 1980) is produced in the α-β spectral region. Two papers published recently (Chazotte et al., 1981; 1982) reported that local anaesthetics, procaine, tetracaine, dibucaine and lidocaine, alcohols and chlorpromazine and analogous drug, procainamide, inhibited mitochondrial electron transport at several points along the chain. One of the sites was concerned with cytochrome c oxidase. Chlorpromazine was regarded as the most potent inhibitor (412 times more effective than n-butanol), with a 50

per cent inhibitory concentration of 1 x 10^{-3} M for cytochrome c oxidase electron transfer. All disruptions to electron transport were found to be reversible. The order of increasing inhibitory ability in alcohols was directly proportional to the chain length of the molecules: a linear relationship between log[octanol/water partition coefficients] and log[concentration for 50 per cent inhibition] was found. For the anaesthetics and chlorpromazine, it was concluded on the basis of sedimentation and electrophoretic studies that direct interactions with the protein occurred.

In this study, the binding of chlorpromazine with cytochrome c was complicated by three factors: the onset of turbidity at low chlorpromazine concentrations (ca. 1.5 x 10^{-4} M); the tendency of chlorpromazine solutions to acquire a purple hue (due to a photolytic reaction) within an hour; and by slow kinetics after the addition of chlorpromazine to the cytochrome c solution. Nevertheless, the same qualitative binding pattern to cytochrome c was shown by chlorpromazine at very low concentrations. Thus, A_{695}, presumably due to a π-π interaction with the porphyrin ring, diminished by 40 per cent on the addition of 1 x 10^{-4} M chlorpromazine to the cytochrome c solution (in contrast to ca. 0.1 M sodium perchlorate, for the same decrease). Qualitative results with the model complex MP-8 are discussed in Chapter 7: again evidence for binding was obtained.

Future studies with this drug could be facilitated in water/methanol or water/ethylene glycol mixtures: its hydrophobicity (critical micellar concentration is ca. 5 x 10^{-3} M at pH 6) precludes a study in pure aqueous solution. Indeed, the environment of chlorpromazine in the mitochondria (as studied by Chazotte et al., 1981; 1982) is lipid based.

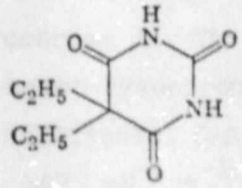
A definite decrease in the 695 nm absorbance band was seen in this study on the addition of the local anaesthetic procaine, to ca. 1 x 10^{-4} M cytochrome c at pH 9.0, but precipitation of the protein occurred as the concentration of procaine was raised.

(b) Other drug molecules

Semi-quantitative studies were carried out with barbitone, ephedrine, salicylic and benzoic acids.

(i) Barbital

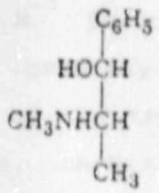
Barbitone (the sodium salt, barbital, is used to give buffered solutions at ca. pH 9.4) is a drug belonging to the barbiturate family. It is employed medically as an anti-depressant. The structure of barbitone is shown below:



Binding was examined at pH 9.0 by following the decrease in A_{695} with increasing barbital concentration. The binding constant for one molecule of barbital binding to cytochrome c was K_{app} ca. 10 M^{-1}.

(ii) Ephedrine

Ephedrine, shown below, has a pK of 9.54 and is also used medically in the treatment of depression:



The titration curve of A_{695} for increasing concentrations of ephedrine was sigmoidal: at concentrations greater than 0.4 M ephedrine, A_{695} was reduced by 50 per cent. In contrast, 0.05 M tryptophol completely removes the band. A plot of $\log|A_o - A|/A$ against log[Ephedrine] was linear with a slope of 1.6. The effect appears to be one of hydrophobicity of the added reagent destabilising the haem crevice and disrupting the iron-methionine bond in this manner, rather than by direct binding. The result is similar to that achieved by alcohols and denaturants.

(iii) Salicylic and Benzoic Acids

Both salicylic and benzoic acids removed the 695 nm absorption band, appearing to bind one molecule to cytochrome c at pH 9.0 with an apparent equilibrium constant for salicylic acid of ca. 80 M^{-1}.

5.6 Discussion

It was shown in this study that the use of commercial cytochrome c without further purification, or after oxidation by $K_3Fe(CN_6)$, gave identical results for the binding of the perchlorate anion, to purified, oxidised cytochrome c. The pK_a value for the alkaline isomerisation of horse-heart cytochrome c was confirmed to be 9.05, and the extinction coefficients for Type III and Type IV were estimated as ϵ_{695} = 867 $mM^{-1}cm^{-1}$ and ϵ_{695} = 83 $mM^{-1}cm^{-1}$, respectively. The initial absorbance values at different pH's of solutions of cytochrome c were found to correspond to the expected value from the pH titration. Titrations of A_{695} of cytochrome c at various pH values close to the pK_a of the alkaline isomerisation, with the perchlorate anion, caffeine and tryptophol, suggested binding to cytochrome c. An analysis of the binding gave slopes of ca. 0.8 (i.e. probably one ligand bound) for caffeine and perchlorate and ca. 1.5 (i.e. probably two ligands bound) for tryptophol, and binding constants: K_{eq}: ca. 6 x 10^{-4} M^{-1} (caffeine), ca. 5 x 10^{-3} M^{-1} (perchlorate) and ca. 9 x 10^{-2} M^{-1} (tryptophol). Qualitative studies, where the elimination of A_{695} was effected on addition of the tranquillizer chlorpromazine, the local anaesthetic procaine, barbital (K_{app} ca. 10 M^{-1}, at pH 9.0) and salicylic and benzoic acids, to cytochrome c, suggested similar binding interactions. Ephedrine did not appear to bind to the porphyrin: reduction of A_{695} with increasing ephedrine concentration gave a sigmoidal curve and 0.4 M ephedrine gave a 50 per cent loss of A_{695}.

The probable nature of the interactions between cytochrome c and anions, drugs and other molecules studied in this work, responsible for the cleavage of the Fe-S band of cytochrome c and formation of its Type IV open haem crevice structure, are discussed below.

5.6.1. Interaction causing a destabilisation of the haem crevice

It was shown in Section 5.1 that cleavage of the Fe-S bond of cytochrome c, which is probably coupled with a conformational change yielding a more open haem crevice structure, is induced under the following conditions:-

1. Alkaline pH: increasing pH causes an alkaline isomerisation due to the loss of a single proton from an unknown residue, and displacement of the methionine 80 by a strong field ligand (possibly lysine 79); the pK_a of the alkaline isomerisation is a measure of the stability of the crevice.
2. High temperature: increasing temperature induces the alkaline isomerisation; transition temperature ($t_{\frac{1}{2}}$) values are a measure of the crevice stability.
3. Denaturants: the efficacy of alcohols, and other denaturants, in cleaving the Fe-S bond and facilitating crevice opening, is a function of their hydrophobicity; the pK_a is decreased in their presence.
4. Anions: two classes of behaviour are seen: some anions (e.g. phosphate) bind to a site located near the haem crevice and stabilise the Type III structure at low concentrations; other anions (e.g. chloride) probably bind to cytochrome c, but at a different site, and slightly destabilise the Type III structure where a small reduction in A_{695} is the only spectroscopic effect observed.
5. Haem-ligands: cyanide, azide and imidazole bind to the haem iron, effecting Fe-S bond cleavage and isomerisation, with spectral changes at A_{695} and A_{530}.
6. Drugs and aromatic molecules: in this work, caffeine, tryptophol and chlorpromazine were shown to cleave the Fe-S bond and appeared to bind to cytochrome c, with spectral changes at A_{695} and A_{530}.

The diversity of conditions effective in inducing cleavage of the Fe-S bond suggests there are several different forces which influence the stability of the haem crevice, either directly or indirectly. Direct effects include:

(i) binding via coulombic interactions with the porphyrin or charged protein residues (e.g. by anions or charged aromatic molecules);

(ii) ligation to the haem iron (e.g. by cyanide);

(iii) donor-acceptor (π-π) interactions with the porphyrin (e.g. by aromatic molecules);

(iv) hydrogen bonding to the protein (e.g. by denaturants or drugs).

An indirect influence on the haem crevice stability could be exerted by an alteration of the nature of the ordered water structure within the hydrophobic crevice (e.g. by perchlorate, denaturants or drugs) or by steric effects due to the bulk of the added reagents.

5.6.2 The perchlorate anion

An analysis of the binding of perchlorate to cytochrome c gave $K_{eq} = 5 \times 10^{-3}\ M^{-1}$, with a slope of ca. 0.8 (one ligand bound). It is unlikely that this represents direct ligation to the metal site: the binding between perchlorate and metals is usually weak. Given the value of the slope ($n \sim 1$), a weak association between perchlorate and the protein (where $n \gg 1$) may be excluded. Binding to the protein therefore most likely occurs at a specific site, which may be the phosphate binding site.

Correlation of the pK_a value for the alkaline isomerisation and amino acid sequences for several mammalian species (Osheroff et al., 1980) identified as important in maintaining the closed crevice structure, surface residue interactions between lysine 13 and glutamic acid 90 (a salt bridge verified by X-ray crystallography) constituting the 'top haem crevice bond', and between lysine 79 and residue 47 (either serine or threonine) (a hydrogen bond verified by X-ray crystallography) constituting the 'bottom haem crevice bond' (see Figure 5.13). The phosphate binding site (and hence possibly the perchlorate binding site) is situated near the top haem crevice bond (Osheroff et al., 1980).

Perchlorate may also influence the Fe-S bond, indirectly, via its ability to act as a water structure breaking agent. It is likely that protein function would be sensitive to an alteration of

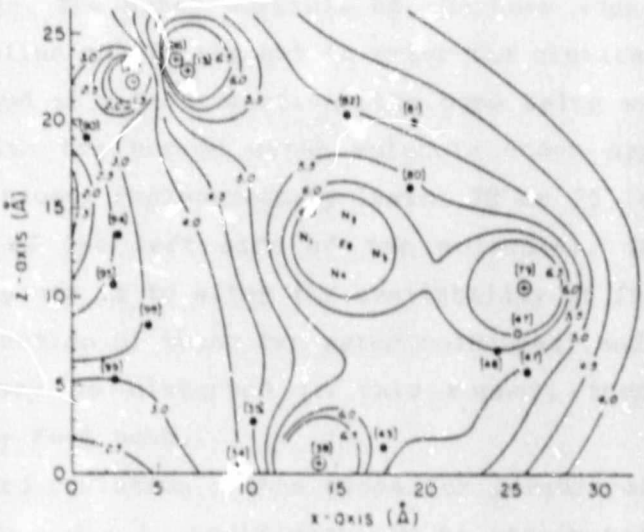


Fig. 5.13 Representation of the electric potential field generated within human ferricytochrome c (source: Osheroff et al. (1980)). The view is from the left of the protein looking at a cross-section which lies approximately along the dipole axis and contains both the top haem crevice salt bridge between the ε-amino group of lysine 13 and the α-carboxyl group of glutamic acid 90 and the bottom hydrogen bond between the ε-amino group of lysine 79 and the backbone carbonyl of residue 47. The italicised numbers denote the strength of the electric potential lines, in kTD_w/eD_p.

the highly ordered water structure found in the interior of the hydrophobic haem crevice. The X-ray studies of Takano and Dickerson (1981a; 1981b) have identified two water molecules, one invariant at the haem edge, and one buried below and bonded to asparagine 52

(and also bonded to tyrosine 67 and threonine 78), thought to be involved in the alkaline transition. The buried molecule is 4.7 Å from the methionine 80 sulphur in ferric cytochrome c, but 5.4 Å away in ferrous cytochrome c. This serves, together with the 0.16 Å outward shift of the ferric haem iron, to give the haem a more hydrophyllic environment in ferric cytochrome c, and stabilises the positive charge. The water molecule at the haem edge (which would be OH^- at alkaline pH) is thought to enter the crevice cleaving the ferric Fe-S bond in a fast reaction (the bond being weakened by an interaction with the buried water molecule under asparagine 52), followed by a slower replacement by lysine 72 or 79 (and a drastic rearrangement of the left side of the molecule). The effect of chaotropic reagents is to alter the availability of free water; the delicate interaction of these two water molecules (and others) with cytochrome c may be disturbed in this manner, thereby inducing cleavage of the Fe-S bond.

The downward deviation of the slope for perchlorate binding to cytochrome c from n = 1, could possibly be attributed to an onset of chaotropic effects at higher concentrations of perchlorate.

5.6.3 Drugs and other aromatic molecules

Quantitative studies with caffeine and tryptophol suggested binding to cytochrome c with K_{eq} values 6×10^{-4} M^{-1} and 9×10^{-2} M^{-1}, respectively, and slopes of ca. 0.8 and 1.5 (i.e. one and two bound ligands), respectively. A possible explanation for the deviation of the slope from n = 1 or n = 2 could be an alteration of the availability of water within the haem crevice with increasing concentration of the hydrophobic drug molecules. Qualitative studies with chlorpromazine, barbital, benzoic and salicylic acids showed the same elimination of A_{695}, a decrease in A_{530} and broadening of the peak at 530 nm with an isosbestic point at ca. 545 nm. Several forces probably combine to effect Fe-S bond cleavage and subsequent isomerisation, due to the addition of these reagents:

(i) hydrogen bonding to the protein or porphyrin;
(ii) coulombic interactions (e.g. with chlorpromazine);
(iii) an alteration of ordered water structure by these hydrophobic molecules;

(iv) donor-acceptor interactions between the aromatic molecules and the tetrapyrrole ring system of the porphyrin.

It has been suggested that planar π-π complexes can occur either directly over the metal or at the periphery with single pyrrole rings (La Mar et al., 1974). The quantitative binding results observed probably correspond to the formation of planar, 1:1 and 1:2, π-bonded porphyrin/aromatic molecule complexes. The literature results discussed below corroborate this view.

Purines are known to form donor-acceptor complexes. The X-ray structures of caffeine with pyrogallol (Arnone and Marchessault, 1968) and caffeine with 5-chlorosalicylic acid (Shefter, 1968) display plane to plane stacking, characteristic of π-π interaction. Barry et al. (1973) examined the caffeine interaction with several metallic porphyrins by NMR, and found plane to plane structures likely to be π-π molecular complexes. The nature of the metal was not found to affect the formation of complexes. Hill et al. (1973) found the same type of interaction with steroid/porphyrin complexes, and Mohr and Scheller (1969) with haemin/alkyl pyridinium complexes.

It is known that the dimerisation of porphyrins in solution is in part due to π-π interactions on planar stacking (Viscio and La Mar, 1978). These interactions also have a rôle in maintaining the position of the haem in the hydrophobic pocket created by the protein folding. For example, in myoglobin (Mb) the haem resides in hydrophobic crevice with two aromatic rings (from phenylalanine residues) parallel to the pyrrole rings or vinyl groups of the porphyrin. Donor-acceptor interactions have been implicated in the positioning of this haem (Kendrew, 1962). Cann (1965; 1967a; 1967b; 1969) found that several aromatics including benzene, indole butyrate, β-naphthoate, chlorpromazine, methyl viologen and toluene exert a strong and specific effect, enhancing the rate of reaction of Zn^{2+} with Mb, and its denaturation by urea. Binding via donor-acceptor interactions, at two sites on Mb, thus disrupting the phenylalanine/haem interactions, was held responsible for the rate enhancement. Alcohols had no effect on the reaction rate.

A spectrum of the chlorpromazine/haemin complex (Cann, 1967b) showed a similar spectral effect to that of caffeine, which was

interpreted as splitting the dimer (Campbell, 1980), although this was not suggested by Cann. The unusually strong electron donating ability of the phenothiazine ring system of chlorpromazine, probably outweighs coulombic repulsion due to a positively charged tertiary amine group. It has been suggested that the therapeutic activity of this anti-psychotic drug is due to charge-transfer interactions with receptor sites in the brain (Karreman et al., 1959). A charge-transfer complex between Mb and the anaesthetic, xenon, has also been reported (Schoenborn et al., 1965).

Theoretical studies of hypothetical complexes between cytochrome P-450 and quinoline molecules, suggested that donor-acceptor interactions on planar contact between cytochrome P-450 and its substrates are likely (Nagata et al., 1976). This view is supported by recent studies with a model for cytochrome P-450 (Adams et al., 1984). Both theoretical calculations and experimental evidence (examining the effect of aniline ring substituents and the effect of axial ligands on the haemin/aniline interaction) indicated that planar π-bonding was the probable mode of interaction between haemin and aniline. It was also suggested that as the haemin/aniline interaction necessarily precedes oxygen activation, donor-acceptor interactions are biochemically significant in the function of cytochrome P-450.

Thus, there is a growing body of evidence that π-π interactions are very important in vivo.

5.7 Summary

The iron-methionine 80 bond in ferric cytochrome c, which is essential to its in vivo function, can be monitored using a charge-transfer band in the near IR (695 nm), present only when the bond is intact. It was confirmed for horse-heart cytochrome c that the bond is broken by increasing pH in an alkaline isomerisation from Type III to Type IV of cytochrome c, with a pK_a of 9.05 (25 °C). Extinction coefficients for Type III and Type IV at 695 nm were calculated to be 867 and 83 $mM^{-1} cm^{-1}$, respectively. The binding of perchlorate to cytochrome c was examined at various pH values near the pK_a at one perchlorate anion appeared to be bound per molecule of cytochrome c with K_{eq} ca. 5 x 10^{-3} M^{-1} (25 °C). Caffeine and tryptophol formed 1:1 and 1:2 donor-

acceptor complexes with cytochrome c, respectively, with K_{eq} ca. 6×10^{-4} M^{-1}, for caffeine, and K_{eq} ca. 9×10^{-2} M^{-1}, for tryptophol (25 °C). The formation of donor-acceptor complexes between cytochrome c and chlorpromazine, procaine, barbitone, salicylic and benzoic acids was assessed qualitatively.

CHAPTER 6 - PREPARATION OF MICROPEROXIDASE-8 AND ITS ANALYSIS BY THIN-LAYER CHROMATOGRAPHY

6.1 Introduction

Cytochrome c is unique amongst haemoproteins in that its prosthetic group, the haemin c of Hill and Keilin (1930), is built into the protein of the cytochrome by thioether linkages between two cysteine residues (cysteine 14 and cysteine 17) and two vinyl side chains of the haem (Lemberg and Barrett, 1973). It is precisely this feature which permits the derivation of haem-peptides of varying size from cytochrome c by the digestion of its peptide chain. The thioether linkages, haem c and histidine 18, which is the fifth ligand to the iron, remain unaltered.

6.1.1 Haem-peptides of cytochrome c as models for haemoproteins

The products of proteolytic digestion of cytochrome c present a unique opportunity for a study of the regulatory rôle of the protein on the reactivity of the active site. A series of haem-peptides have been synthesized, differing only from one other and cytochrome c in the length of their peptide chains, and perhaps the identity of the sixth axial ligand. From horse-heart cytochrome c alone (104 residues), digestions utilising a variety of enzymes have yielded:

1. eighty residue (1-80), sixty-five residue (1-65) and thirty-eight residue (1-38) haem-peptides (Wilgus et al., 1978);
2. a twenty-six residue haem-peptide (Margoliash and Smith, 1961;1962);
3. a sixteen residue haem-peptide (11-26) (Margoliash, 1962a; 1962b);
4. nine residue (14 - 22) and ten residue (14 - 23) haem-peptides (Tuppy and Bodo, 1954);
5. eight residue (14-21) and eleven residue (11-21) haem-peptides (Tuppy and Paleus, 1955; Harbury and Loach, 1960a).

In addition, a six residue haem-peptide (14 - 19) (Titani and Nanta, 1964) has been isolated from yeast cytochrome c.

Comparing the reactivities and properties of these haem-peptides with one another and cytochrome c could illuminate the rôle of the protein in the parent enzyme.

As indicated in Chapter 1, haem-peptides of cytochrome c have several advantages over the conventional models for haemoproteins. The study of 'naked' iron porphyrins, such as haemin, is largely restricted to alkaline aqueous solution, in which they are soluble, but exist mainly as dimers (Shack and Clark, 1947). Synthetic analogues of haem-peptides are also subject to aggregation; synthetic complexity probably precludes the preparation of a diverse range of peptides. Both models present difficulties where it is desired to model a mono-imidazole haemoprotein (Walker et al., 1976).

The proteolytic digests, in contrast are:

1. relatively easily prepared in good yield;
2. water soluble over a wide range of pH and temperature;
3. developed from a parent enzyme, easily isolated and purified on a large scale;
4. able to participate in ligand binding as 'five-coordinate' mono-imidazole species (the sixth ligand (e.g. water) is easily removed);
5. monomeric in aqueous solution (below 2×10^{-7} M) or in the presence of small volumes of methanol (below ca. 2×10^{-6} M in 20 per cent methanol solution) (Aron et al., 1986);
6. able to maintain the haem in an aqueous environment (i.e. free of the constraints of the protein);
7. unmodified with respect to the haem c, thioether linkages, histidine 18 axial haem ligand, and in some cases (e.g. MP-11) in the α-helices of the peptide. Thus direct comparison with cytochrome c is feasible.

However, the development of methods of preparation and purification for MP-8 that give samples of consistently high purity, and a more complete investigation of the solution properties of haem-peptides, are essential in order fully to exploit these as enzyme models.

6.1.2 Microperoxidase-11 and microperoxidase-8

The amino acid sequences of haem-peptides MP-8 and MP-11 are shown below in Figure 6.1.

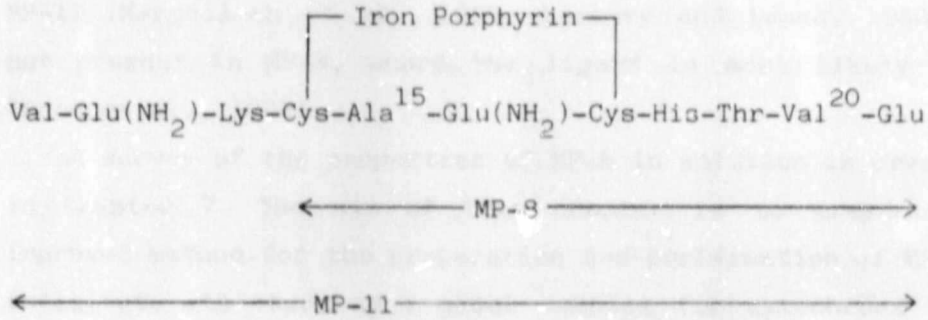


Figure 6.1 The amino acid sequences of the undecapeptide of cytochrome c (MP-11), and its tryptic product, the octapeptide (MP-8).

MP-11 has been particularly well characterised: its spectral (Harbury and Loach, 1960a), redox (Kimura et al., 1981; Harbury and Loach, 1959), pH (Wilson et al., 1977), binding (Harbury and Loach, 1960b; Baumgartner et al., 1974) and magnetic properties (Jehanli et al., 1976) have been studied by a variety of techniques including electronic spectroscopy, NMR, ESR (electron spin resonance spectroscopy), Raman spectroscopy and Mössbauer spectroscopy. By contrast, much less time has been invested in MP-8.

The choice of MP-8 rather than MP-11 as the preferred haemoprotein model for this work, is justified as follows:

1. Aggregation problems are more pronounced with MP-11 than MP-8 (Harbury and Loach, 1960a); it is feasible to study monomeric MP-8 below 2×10^{-7} M in aqueous solution or below 2×10^{-6} M in 20% MeOH:H_2O (v/v) (Aron et al., 1986) or in ethylene glycol (Blumenthal and Kassner, 1979).
2. MP-8 is less well characterized; a study of the conditions for preparation, purification, the existence of a monomeric species, as well as investigation of ligand binding and other properties, could lead to useful comparisons with the better studied MP-11, and the parent cytochrome c molecule.
3. A further advantage of MP-8 over MP-11 is that there is no

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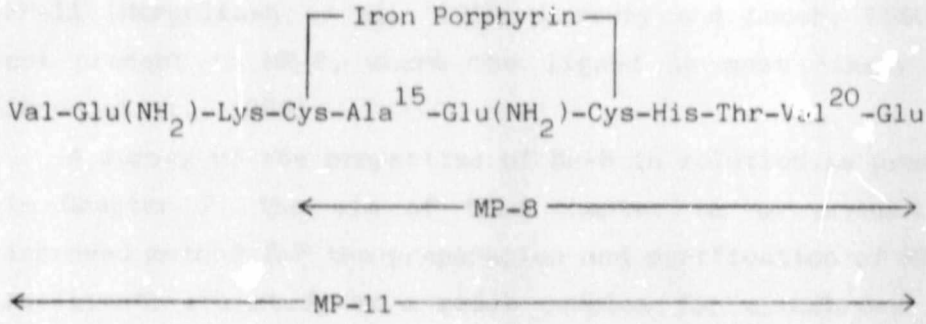

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3. A further advantage of MP-8 over MP-11 is that there is no

ambiguity concerning the identity of the sixth haem ligand. The ϵ-NH_2 group of lysine 13, a coordinating nitrogen group in MP-11 (Margoliash et al., 1959; Harbury and Loach, 1960a) is not present in MP-8, where the ligand is most likely water (Aron et al., 1986).

A survey of the properties of MP-8 in solution is presented in Chapter 7. The aim of this chapter is to present an improved method for the preparation and purification of MP-8 to facilitate its study as a model complex for cytochrome c and other haemoproteins. MP-8 is available commercially, but is expensive and of limited purity.

6.1.3 A survey of preparative methods for MP-11 and MP-8

A procedure for the isolation of MP-11 from beef-heart cytochrome c was first described by Tsou (1949; 1951a; 1951b), and Tuppy and Paleus (1955) reported its purification by partition chromatography, and the chemical structure of the purified product. In the method derived by Tsou, the peptic digestion of cytochrome c was carried out at pH 1.5 for 16-20 hours at 24°C. Subsequently, the pigment was recovered from the neutralised solution by repeated precipitation with ammonium sulphate and trichloroacetic acid, and dialysis against phthalate. Tuppy and Paleus considerably extended this time of digestion in a two stage peptic digestion: it was thought that a pepsin inhibitor present in the cytochrome c as a result of its purification on an ion-exchange resin was causing incomplete digestion. In the purification, partition chromatography with Hyflo-Supercel and the two phases of butanol/glacial acetic acid/water [4:1:5] was followed by dialysis against phthalate buffer, and finally against deionized water.

A detailed report of the preparation of MP-8 was presented by Harbury and Loach in 1960, based on the methods described above. The tryptic digestion was carried out in alkaline solution in dialysis tubing for a total of 24 hours at 38°C, with two additions of trypsin. Tryptic activity was subsequently destroyed at 100°C. Separation of MP-8 from undigested MP-11 and other impurities was accomplished with the same partition chromatographic method used to purify MP-11.

A novel method of purification of MP-11 by affinity

Author Aron Janine
Name of thesis The Coordination Chemistry Of Some Cobalt Corrinoid And Iron Porphyrin Complexes. 1986

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