Physicochemical Properties of Two Atypical Cytochromes *c*, *Crithidia* Cytochrome *c*-557 and *Euglena* Cytochrome *c*-558

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Cytochrome *c*-557 from *Crithidia oncopelti* and cytochrome *c*-558 from *Euglena gracilis* are mitochondrial cytochromes *c* that have an atypical haem-binding site. It was of interest to know whether the loss of one thioether bond affected the physicochemical properties of these cytochromes. The thermodynamic parameters of the redox potential were measured. The reaction with imidazole, the kinetics and thermodynamics of the alkaline isomerization and the effect of heating on the visible spectrum are described for the ferricytochromes. The kinetics of the loss of cyanide, the spectral changes occurring on reduction with dithionite at alkaline pH values and the reactivity with CO are described for the ferrocytochromes. In many respects the cytochromes of the two protozoans are very similar to the cytochromes of horse and yeast. The ferricytochromes do, however, undergo a reversible transition to high-spin species on heating, which may be due to the more flexible attachment of the prosthetic group. Similarly the alkaline isomers of cytochromes *c*-557 and *c*-558 give rise to high-spin proteins above pH 11. The alkaline isomerization of cytochrome *c*-558 involves a pK_{obs} of 10 and kinetics which do not obey the model of Davis *et al.* [(1974) *J. Biol. Chem.* **249**, 2624–2632] for horse cytochrome *c*. It is proposed that a model involving two ionizations, followed by a conformational change, may fit the data. Both cytochromes *c*-557 and *c*-558 combine slowly with CO at neutral pH values.

Over 60 amino acid sequences of mitochondrial cytochrome *c* are known (references compiled in Dayhoff, 1972). The protein can apparently tolerate large changes in primary structure without functional properties (Byers *et al.*, 1971) and physicochemical properties (Aviram & Schejter, 1969) being greatly affected. Certain residues are, however, unvaried and we can assume that some of these are essential for the structural and functional integrity of the cytochrome *c* molecule and that mutational changes at these positions could not have become fixed in the course of the evolution of eukaryotes.

Until recently, the two cysteine residues which form thioether linkages to the haem group (Theorell, 1939) were thought to be examples of such invariant residues. However, neither *Crithidia* cytochrome *c*-557 (Pettigrew, 1972) nor *Euglena* cytochrome *c*-558 (Lin *et al.*, 1973; Pettigrew, 1973) have cysteine at position 14 and the haem group is therefore bound covalently through only one thioether linkage.

It was decided to investigate whether the absence of the thioether bond is reflected in the physicochemical properties of the proteins.

Experimental

Cytochrome *c*-557 and *c*-558 were prepared from *Crithidia oncopelti* and *Euglena gracilis* respectively

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as described by Pettigrew *et al.* (1975). Imidazole was Sigma Grade III (Sigma Chemical Co., St. Louis, Mo., U.S.A.). Potassium ferrocyanide was from Riedel de Haën A.G. (Seelze-Hannover, Germany). Sodium dithionite was from E. Merck A.G. (Darmstadt, W. Germany) and for the calculation of concentration was assumed to constitute 90% by weight of the reagent.

Spectrophotometry

Spectra were recorded on a Cary 118 spectrophotometer, except in the heating experiments, where a Varian Techtron spectrophotometer fitted with a slave recorder was used.

Autoxidizability and CO binding

The cytochrome *c* was fully reduced with sodium ascorbate and the excess of reagent was removed by gel filtration on Sephadex G-25 in 50 mM-KH₂PO₄-K₂HPO₄, pH 7.0. The rate of change of absorbance of the α peak was recorded.

CO binding in 5 mM-KH₂PO₄-K₂HPO₄, pH 7.0, was measured as described by Tsou (1951). However, after 1 min of bubbling with CO, neither the reaction with cytochrome *c*-557 nor that with cytochrome *c*-558 was complete and slow changes were recorded over 1–2 h.

As an alternative to direct bubbling of CO, different volumes of previously equilibrated buffer containing CO could be added to the protein solution. Experiments were done in the presence of sodium dithionite, sodium ascorbate or with no reducing agent. CO was generated from H_2SO_4 and formic acid.

The ϵ^{peak} of the ferrocyanochrome-CO complex measured in 0.1M-NaOH was $11\text{mm}^{-1}\cdot\text{cm}^{-1}$ for cytochrome *c*-557 and cytochrome *c*-558.

Redox potential

Redox potentials were measured by using ferro-ferricyanide solutions and spectroscopic determination of the amounts of reduced and oxidized cytochrome. The thermodynamic parameters for the ferro-ferricyanide couple were taken from Hanania *et al.* (1967). The variation of the redox potential of cytochrome *c*-558 with pH was studied by using 1 mM-Tris-HCl and NaHCO_3 - Na_2CO_3 buffers between pH 8.3 and 11.3.

The thermodynamic parameters of the redox potential were measured essentially as described by Margalit & Schejter (1970). Ferricytochrome *c* was partially reduced with a known amount of potassium ferrocyanide and the changes in absorption of the α band with temperature were recorded between 12°C and 30°C in a water-circulated cell holder. The conditions used were 1 mM- KH_2PO_4 - K_2HPO_4 , pH 7.0, 0.5 mM-potassium ferrocyanide and approx. $15\mu\text{M}$ -cytochrome *c*. The ionic strength was 0.008 mol/l and E for the ferrocyanide-ferricyanide couple was taken as 379 mV (Hanania *et al.*, 1967). With cytochrome *c*-558, some autooxidation occurred during the experiment and therefore separate solutions were equilibrated at the various temperatures and ferrocyanide was added to each before quickly recording the redox state.

Complex-formation with imidazole

Reaction of cytochromes *c*-557 and *c*-558 with imidazole was studied as described by Schejter & Aviram (1969). Small volumes of 4M-imidazole (pH 7.0) were added to the cytochrome ($20\mu\text{M}$) in 0.1M- KH_2PO_4 - K_2HPO_4 , pH 7.0. Changes in the Soret region were followed in difference spectra with corrections being made for dilution, either of the total absolute absorption, or, if equal volumes of water were added to the reference cuvette, of the net difference absorption.

Formation of ferricytochrome-CN⁻ complexes and kinetics of their breakdown on reduction

Complex-formation of cytochromes *c*-557 and *c*-558 with CN^- (judged by the disappearance of the

695 nm absorption band) was achieved by incubation in 0.05M-KCN, 0.1M- KH_2PO_4 - K_2HPO_4 , pH 7.0.

Solid sodium dithionite was added and the spectrum was immediately recorded in the region of the α band.

In separate experiments, the kinetics of the breakdown of the ferrocyanochrome- CN^- complex were followed at appropriate wavelengths in the region of the Soret band [420 nm for cytochrome *c* (horse), 426 nm for cytochromes *c*-557 and *c*-558].

Heating and the 695 nm absorption band

Cytochrome solutions (approx. $50\mu\text{M}$) were heated, and the disappearance of the 695 nm absorption band was recorded. The effect of varying the ionic strength by adjusting phosphate or NaCl concentrations was investigated.

Haem-linked ionization

Alkaline haem-linked ionizations were investigated by the addition of small quantities of 0.5M- or 5M-NaOH to unbuffered cytochrome solutions (approx. $10\mu\text{M}$ in 0.066M- Na_2SO_4).

The pH was measured in the cuvette and the difference spectrum was then recorded between 470 and 360 nm against a reference solution of cytochrome at pH 7.0. Alternatively the change in the absolute absorption of the 695 nm band with pH was measured.

pH-jump experiments

pH-jump experiments were carried out in a Durrum stopped-flow apparatus and the kinetic changes were monitored at 406 nm. Experimental values of k_{obs} were obtained by jumping the pH of unbuffered cytochrome ($15\mu\text{M}$ in 0.066M- Na_2SO_4 , pH 7.0) to pH values between 8.9 and 10.5 by rapid mixing with 0.2M-glycine adjusted with 5M-NaOH. The ionic strengths of the buffers were adjusted to 0.2 mol/l by the addition of NaCl.

The rate constant for the back reaction (k_b) was determined by jumping unbuffered cytochrome in 0.066M- Na_2SO_4 at pH 10 to pH 6.8 with 0.1M- KH_2PO_4 - K_2HPO_4 .

The analysis of the experimental data is discussed below.

Reduction with dithionite at alkaline pH

Cytochrome ($10\mu\text{M}$), in buffer of the appropriate pH, was mixed in a stopped-flow spectrophotometer with 20 mM-sodium dithionite dissolved in the same buffer. [The buffers used are described in the legend of Fig. 11(a).]

Results and Discussion

The band corresponding to the 695 nm band of horse cytochrome *c* is found at 698 nm in cytochrome *c*-557 and 702 nm in cytochrome *c*-558. The observed shift to longer wavelengths is also found in the other spectral bands of the two protozoan cytochromes and is in keeping with the influence of the free vinyl group on the porphyrin transitions (Falk, 1964). Eaton (1967) has shown that the 695 nm absorption band of cytochrome *c* derives from a *z*-polarized transition. He argues that this transition is more probably iron to porphyrin than iron to axial ligand. Thus in cytochromes *c*-557 and *c*-558 the absorption peak and the structure of the prosthetic group are changed, although the arrangement of axial ligands (in cytochrome *c*-557) remains the same (Keller *et al.*, 1973). This tends to support Eaton's (1967) hypothesis that an iron-porphyrin transition is involved.

The opposite situation is found in cytochrome *c*₂ from *Rhodospirillum rubrum*, where the corresponding band is at 698 nm (G. Pettigrew & A. Schejter, unpublished work). The structure of the haem group of this cytochrome is identical with that of cytochrome *c* and the positions of other absorption bands are almost unchanged (Bartsch, 1968). Salemme *et al.* (1973) have commented that the methionine ligand in cytochrome *c*₂ is not exactly perpendicular to the plane of the porphyrin and this change in ligand orientation might be expected to perturb the 695 nm absorption.

For simplicity this absorption band will be referred to as the '695 nm band', in spite of these differences in absorption maxima.

Redox potential

The mid-point oxidation-reduction potentials of cytochromes *c*-557 and *c*-558 at pH 7.0 and 25°C are 254 mV and 244 mV respectively. A value for cytochrome *c* of 261 mV was found in agreement with the results obtained by Margalit & Schejter (1973). These authors found a variation of between 256 mV (tuna heart) and 264 mV (*Candida*) for different cytochromes *c*. Thus cytochrome *c*-557 has a very similar redox potential to the other cytochromes *c* studied, whereas that of cytochrome *c*-558 is appreciably lower. As shown by electrophoretic homogeneity, this lower redox potential was not due to the presence of modified or denatured protein.

Kusel *et al.* (1969) reported a redox potential of 280 mV for *Crithidia fasciculata* cytochrome *c*-555, which is closely related to *Crithidia oncopelti* cytochrome *c*-557 in sequence (G. C. Hill & G. W. Pettigrew, unpublished work). Perini *et al.* (1964) found a redox potential of 307 mV for *Euglena* cytochrome *c*-558. The reason for the discrepancy with our results is not known, although in some other features, such as a higher molecular weight, the Perini *et al.* (1964) preparation differs from ours.

Fig. 1. Variation of redox potential of cytochrome *c*-558 with pH

Reagent concns. were cytochrome *c*-558 (15 μ M) and potassium ferrocyanide (0.5 mM). Buffers are described in the text.

The variation of the redox potential of cytochrome *c*-558 with pH is shown in Fig. 1. By extrapolation of the slopes ($m = 0$ and $m = 75$ mV/pH unit) a pK of 9.8 was estimated. This pK value of the redox potential agrees with the alkaline pK values estimated from the spectroscopic titration and the pH-jump kinetic studies discussed below. Again this result does not agree with that of Perini *et al.* (1964), who found that the redox potential of cytochrome *c*-558 was stable up to pH 10.5.

Thermodynamic parameters of the redox potential

The results in Table 1 indicate that the thermodynamic parameters of the redox reaction are highly conserved. The ΔH and ΔS values for cytochromes *c*-557 and *c*-558 are nearly the same as for horse cytochrome *c*, and the lower redox potential of cytochrome *c*-558 involves a decrease of only 1.6 kJ/mol in the ΔG value. Thus the conclusions for cytochromes *c*-557 and *c*-558 are the same as those of Margalit & Schejter (1973) for the other cytochromes *c* studied. The constancy of the thermodynamic parameters of the redox reaction of eukaryotic cytochromes *c* presumably reflects closely related oxidized and reduced states and a highly conserved conformational change between these states.

Studies on ferricytochromes

Complex-formation with imidazole. To conserve material, titrations were originally performed in the region of the Soret band by using difference spectra. However, even for horse cytochrome *c*, the titration curve (Fig. 2) was complex, and although the initial

Table 1. *Physicochemical properties of horse cytochrome c, yeast iso-1-cytochrome c, cytochrome c-557 and cytochrome c-558*

References are as follows: (a) Margalit & Schejter (1973); (b) Margoliash & Frohwirt (1959); (c) Schejter & Aviram (1969); (d) Aviram (1968); (e) Greenwood & Wilson (1971); (f) Aviram & Schejter (1969); (g) Davis *et al.* (1974); (h) George & Schejter (1964). T_4 is the temperature at which half of the 695 nm band is lost. t_4 is the time for half-completion of the pseudo-first-order CO-binding reaction. $k_{25^\circ\text{C}}$ is the observed rate constant for the breakdown of the CN⁻ complex at 25°C. E_a is the activation energy and $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are the enthalpy and entropy of activation. K_H is the ionization equilibrium constant for the kinetic model described in the text. K_c is the equilibrium constant for the proposed conformational change with forward rate constant k_f and back rate constant k_b .

	Horse cytochrome c	Yeast cytochrome c	Cytochrome c-557	Cytochrome c-558
Thermodynamic parameters of the redox potential	ΔE° 261 mV ΔH° -70 kJ·mol ⁻¹ ΔG° -25.1 kJ·mol ⁻¹ ΔS° -150 J·°K ⁻¹ ·mol ⁻¹	ΔE° 261 mV ΔH° -71.3 kJ·mol ⁻¹ ΔG° -25.1 kJ·mol ⁻¹ ΔS° -154 J·°K ⁻¹ ·mol ⁻¹	ΔE° 254 mV ΔH° -70 kJ·mol ⁻¹ ΔG° -24.6 kJ·mol ⁻¹ ΔS° -154 J·°K ⁻¹ ·mol ⁻¹	ΔE° 244 mV ΔH° -69.8 kJ·mol ⁻¹ ΔG° -23.5 kJ·mol ⁻¹ ΔS° -154 J·°K ⁻¹ ·mol ⁻¹
Ferriocytochromes				
Spectral maxima	410 nm, 529 nm, 695 nm (b)	412 nm, 531 nm, 698 nm	412 nm, 531 nm, 698 nm	412 nm, 529 nm, 702 nm
Equilibrium with imidazole	k_{obs} , 29 M ⁻¹ (c)	k_{obs} , 40 M ⁻¹	k_{obs} , 40 M ⁻¹	k_{obs} , 80 M ⁻¹
Heating (at $I = 0.2$ mol/l)	Biphasic loss of the 695 nm band, $T_4 = 61^\circ\text{C}$, no high-spin protein	Biphasic loss of the 698 nm band, $T_4 = 52^\circ\text{C}$, high-spin above 50°C	Biphasic loss of the 698 nm band, $T_4 = 52^\circ\text{C}$, high-spin above 50°C	First phase of loss of 702 nm band was small, $T_4 = 67^\circ\text{C}$, high-spin above 55°C
pK of haem-linked ionization	9 (e)	8.1 (f)	8.6 (but second pK between pH 11 and 12)	10.0 (but second pK between pH 11 and 12)
pK of change in redox potential	9 (a)			9.8
Kinetic and thermodynamic parameters of isomerization at alkaline pH	k_t , 6.1 s ⁻¹ ; k_b , 0.049 s ⁻¹ ; K_c , 124; K_H , 1.0×10^{-11} M; pK_H , 11; $p(K_H K_c)$, 8.9	k_t , 9.26 s ⁻¹ ; k_b , 0.078 s ⁻¹ ; K_c , 119; K_H , 2.45×10^{-11} M; pK_H , 10.6; $p(K_H K_c)$, 8.5	k_t , 9.26 s ⁻¹ ; k_b , 0.078 s ⁻¹ ; K_c , 119; K_H , 2.45×10^{-11} M; pK_H , 10.6; $p(K_H K_c)$, 8.5	k_t , 9.26 s ⁻¹ ; k_b , 0.078 s ⁻¹ ; K_c , 119; K_H , 2.45×10^{-11} M; pK_H , 10.6; $p(K_H K_c)$, 8.5
Ferrocytochromes				
Spectral maxima	416 nm, 521 nm, 550 nm (b)	419 nm, 523 nm, 557 nm	419 nm, 523 nm, 557 nm	421 nm, 525 nm, 558 nm
CO binding at pH 7	Not in native	20% reaction, t_4 400 s	20% reaction, t_4 400 s	90% reaction, t_4 400 s
Breakdown of CN ⁻ complex	$k_{25^\circ\text{C}}$ 4.27×10^{-3} s ⁻¹ E_a 92 kJ·mol ⁻¹ $\Delta H^\ddagger$ 89.1 kJ·mol ⁻¹ $\Delta S^\ddagger$ 8.8 J·°K ⁻¹ ·mol ⁻¹	$k_{25^\circ\text{C}}$ 4.45×10^{-3} s ⁻¹ E_a 122 kJ·mol ⁻¹ $\Delta H^\ddagger$ 120 kJ·mol ⁻¹ $\Delta S^\ddagger$ 112 J·°K ⁻¹ ·mol ⁻¹	$k_{25^\circ\text{C}}$ 2.66×10^{-3} s ⁻¹ E_a 123 kJ·mol ⁻¹ $\Delta H^\ddagger$ 121 kJ·mol ⁻¹ $\Delta S^\ddagger$ 107 J·°K ⁻¹ ·mol ⁻¹	$k_{25^\circ\text{C}}$ 6.6×10^{-3} s ⁻¹ E_a 116 kJ·mol ⁻¹ $\Delta H^\ddagger$ 113 kJ·mol ⁻¹ $\Delta S^\ddagger$ 100 J·°K ⁻¹ ·mol ⁻¹

Fig. 2. Titration of cytochrome *c* (horse) with imidazole in the region of the Soret band

ΔE is the difference in the extinction at 403 nm between uncomplexed and partially complexed cytochrome. Arrows indicate concentrations at which half of the 695 nm band and all of the 695 nm band have disappeared (from Schejter & Aviram, 1969).

slopes of Hill plots of $\log [\alpha/(1-\alpha)]$ against $\log [\text{imidazole}]$ were near 1, the slopes at higher concentrations of imidazole were greater. This complex titration in the Soret region was not found by Aviram & Schejter (1969) for the 695 nm band. It may indicate that the Soret band is sensitive to further changes in the protein after the 695 nm band has disappeared. The same phenomenon was found to occur in two quite distinct *c*-type cytochromes, cytochrome *c*₂ from *Rhodospirillum rubrum* and cytochrome *f* from *Euglena* (G. W. Pettigrew & A. Schejter, unpublished work). The possibility that at high concentrations imidazole acts as a denaturing agent or a protein side-

Fig. 3. Hill plots of imidazole titration results in the region of the 695 nm band

$\alpha/(1-\alpha)$ is the ratio of cytochrome-imidazole complex to free cytochrome. ●, Cytochrome *c* (horse); ○, cytochrome *c*-557; Δ, cytochrome *c*-558.

chain ligand has been discussed by Schejter & Aviram (1969).

Because of these considerations, the titrations recorded as Hill plots in Fig. 3 were done in the 695 nm region. The values for $K_{\text{obs.}}$ are 29 M^{-1} for horse cytochrome *c* (Aviram & Schejter, 1969), 41 M^{-1} for cytochrome *c*-557 and 80 M^{-1} for cytochrome *c*-558. The corresponding value for yeast iso-1-cytochrome *c* is 260 M^{-1} (Aviram, 1968). These values are calculated on the basis of unprotonated imidazole concentration ($\text{p}K = 7.0$ at 23°C).

Thus the equilibrium constant for cytochrome *c*-557 is very similar to that for horse cytochrome *c*, whereas that of cytochrome *c*-558 is larger, indicating a weaker crevice. Yeast iso-1-cytochrome *c* has a much weaker crevice, in agreement with the conclusions from other properties of the protein (Aviram & Schejter, 1969).

Table 2. Effect of heating on the 695 nm band of horse cytochrome *c* and cytochrome *c*-557

The thermodynamic parameters of the low-temperature phase are shown. Ionic strength was varied by adjusting potassium phosphate or NaCl as given in parenthesis. $T_{\frac{1}{2}}$ is the temperature at which half of the total absorption change had occurred.

<i>I</i> (mol/l)	<i>k</i> _{26°C}	ΔH (kJ·mol ⁻¹)	ΔG (kJ·mol ⁻¹)	ΔS (J·°K ⁻¹ ·mol ⁻¹)	<i>T</i> _{$\frac{1}{2}$} (°C)
Cytochrome <i>c</i> (horse)					
0.01 (phosphate)*	0.059	61	7.2	181	—
0.1 (phosphate)	0.024	92	9.0	276	58
0.4 (phosphate)	0.035	69	8.4	197	63
1.0 (phosphate)	0.063	47.2	6.9	134	66
0.1 (NaCl)	0.025	85	9.2	250	59
1.0 (NaCl)	0.043	57.7	7.9	167	66
Cytochrome <i>c</i> -557					
0.1 (phosphate)	0.069	101	6.7	320	46

* Data from Schejter & George (1964); $T_{\frac{1}{2}}$ could not be determined because of precipitation of the protein.

Fig. 4. *van't Hoff* plots of changes in 695 nm absorption in horse cytochrome *c*

K is the ratio of 'hot' to 'cold' forms. ●, 0.5M- KH_2PO_4 - K_2HPO_4 buffer, pH 7.0; ○, 0.05M of the same buffer.

Heating and the 695 nm band. The results of heating experiments with different cytochromes and at different ionic strengths are shown in Table 2.

(a) Cytochrome *c* (horse). At $I = 0.01$ mol/l, thermodynamic parameters were obtained similar to those found by Schejter & George (1964). Precipitation occurred at about 60°C. At ionic strengths of 0.1 mol/l or greater it was possible to abolish completely the 695 nm band without precipitation of protein. The *van't Hoff* plots of these results, particularly at higher ionic strength, show two distinct phases (Fig. 4). The slope of the second or higher-temperature phase was quite constant for various ionic strengths. The 'hot' form of cytochrome *c* obtained in these high-temperature experiments contained no high-spin protein, judging by the absence of 620 and 490 nm absorption bands. The complete change was reversible on cooling.

The biphasic plots for the heating experiments should be compared with the similar results of Kaminsky *et al.* (1973). These workers showed that, in the presence of various concentrations of ethanol,

Fig. 5. Change in the spectrum of cytochrome *c*-557 with temperature
For details see the text.

Fig. 6. Change in the spectrum of cytochrome *c*-558 with temperature
For details see the text.

Fig. 7. Changes in the absorbance of the 695 nm band with temperature

●, Horse cytochrome *c*; ○, cytochrome *c*-557; △, cytochrome *c*-558. All in 0.1M-KH₂PO₄-K₂HPO₄ buffer, pH7.0.

a complete abolition of the 695nm band can be achieved and that the van't Hoff plots are clearly biphasic. They concluded that the disruption of the methionine-iron bond proceeds via different mechanisms at high and low temperatures. This is essentially the same conclusion as that reached by Urry (1965), who examined the changes in the optical-rotatory-dispersion spectrum with temperature.

However, our results on the effects of ionic strength do not agree with those of Kaminsky *et al.* (1973), who found that increasing the concentration of NaBr lowers the temperature at which the 695nm band is lost. In the present work exactly the opposite was found. The temperature at which the 695nm band is half abolished, the end-point temperature and the temperature of the break in the van't Hoff isochores all became higher as the salt concentration was raised. The same effect was observed whether phosphate or NaCl was used to increase the ionic strength.

(b) Cytochromes *c*-557 and *c*-558. Cytochrome *c*-557 lost its 695 nm band at lower temperatures than did horse cytochrome *c*, but not at the much lower temperatures found by Aviram & Schejter

(1969) for yeast cytochrome *c*. The thermodynamic parameters of the initial gradual phase were very similar to those of horse cytochrome *c* (Table 2). Like horse cytochrome *c* there was a steep transition at higher temperatures, but for cytochrome *c*-557, this was accompanied by the formation of high-spin bands at 620 and 490nm (Fig. 5).

For cytochrome *c*-558 in 0.1M-KH₂PO₄-K₂HPO₄, pH 7.0, there was very little change until above 60°C, then high-spin material appeared in parallel with the loss of the 695nm band (Fig. 6). In Fig. 7, the changes in the 695nm absorption for cytochromes *c*-557 and *c*-558 are compared with those in horse cytochrome *c*.

These results for the three cytochromes may be summarized and interpreted in the following way. Horse cytochrome *c* undergoes two phases of conformational change on heating (which may or may not reflect two different types of conformational change). Part of the 695nm absorption is lost in an initial and gradual change at lower temperatures. The remainder of the 695nm band is lost in a small temperature range, the value of which is influenced by the ionic strength. Neither of these two types of change gives rise to high-spin protein.

With cytochrome *c*-557 there is an initial gradual loss of the 695nm band with increasing temperature, which has similar thermodynamic parameters to the corresponding change in horse cytochrome *c* and results in a low-spin species. At higher temperatures there occurs a sharp decrease in the 695nm absorption, but this is accompanied by formation of high-spin material. The transition is thus more complex than for horse cytochrome *c* and the spectra of Fig. 6 do not show isosbestic points. The extinction of the 620nm band after the 695nm band has been abolished is lower than for cytochrome *c*-558, probably indicating that some 'hot', low-spin protein is still present.

With cytochrome *c*-558 it is difficult to decide whether a low-spin, 'hot' species can exist. Almost all the 695nm band is lost in a steep transition to a high-spin species and the spectra show good isosbestic points. Thus it is probable that the low-spin, 'hot' species, if it exists at all, is very unfavoured, and the

predominant forms are the 'cold', low-spin and the 'hot', high-spin species.

It is possible that the formation of 'hot' high-spin species in both cytochromes *c*-557 and *c*-558 is a result of the increased flexibility of the haem attachment which makes the 'hot', low-spin state unstable. Another low-spin species of cytochromes *c*-557 and *c*-558 which lacks the 695nm band, the alkaline isomer, also appears to be unstable. This is further discussed below.

Haem-linked alkaline ionization. The p*K* values obtained for cytochromes *c*-557 and *c*-558 were 8.6 and 10.0 respectively compared with 9.1 for cytochrome *c*. The slopes of the Hill plots were near unity. This result for cytochrome *c*-558 agrees with that reported by Stellwagen & Cass (1974). These authors performed titrations in the region of the 695nm band and found that above pH 10.5, isosbestic points were lost and a new peak at 620nm was formed.

We found that above pH 11 further spectral changes occurred in both cytochromes *c*-557 and *c*-558. Table 3 shows the spectral characteristics of the new species of cytochrome *c*-558 formed between pH 11 and 12. For cytochrome *c*-557 the same type of change was found, but it occurred at higher pH values. Horse cytochrome *c* in 1M-NaOH showed very little change in spectrum compared with that at pH 11.

The appearance of the shoulder at 610nm, and the loss of the 530nm band, indicates that at least some high-spin material is formed above pH 11 in both cytochromes *c*-557 and *c*-558.

Thus the low-spin alkaline conformations of cytochromes *c*-557 and *c*-558 appear to be unstable, and undergo a further conformational change as the pH is raised. This is like the situation found in the heating experiments discussed above, where the 'hot' low-spin state was unstable and gave rise to a 'hot' high-spin state. Again it is possible that this is due to the increased flexibility of the haem attachment.

pH-jump studies. The values of p*K*_{obs} for both cytochromes *c*-557 and *c*-558 differed from that of cytochrome *c*. pH-jump studies were therefore undertaken to investigate the origin of the changes.

The model of Davis *et al.* (1974) described the alkaline isomerization of cytochrome *c* in terms of an

Table 3. Comparison of the spectral maxima of ferricytochrome *c*-558 at alkaline and neutral pH values

Values in parentheses are millimolar extinction coefficients. An unbuffered solution at pH 7.0 was adjusted to pH 11 and 12.5 by addition of 10M-NaOH and the spectra were recorded.

pH 7	364nm* (30)	412nm (107)	529nm (11.7)	560nm* (8)
pH 11	358nm* (29)	408nm (119)	531nm (10.6)	560nm* (8)
pH 12.5	344nm (39)	408nm (78)	533nm (7.4)	572nm* (6) 610nm* (4)

* Denotes shoulders.

ionization (K_H) followed by a conformational equilibrium (K_c):

The kinetic equation describing this model is:

$$\frac{1}{k_{\text{obs.}} - k_b} = \frac{1}{k_f} + \frac{1}{k_f K_H} [\text{H}^+]$$

[eqn. (7) of Davis *et al.* (1974)]

and thus experimental determination of $k_{\text{obs.}}$ and k_b enables derivation of the parameters k_f and K_H (terms are defined in the legend to Table 1).

Graphical treatments of the results for cytochromes *c*-557 and *c*-558 are shown in Figs. 8 and 9 respectively. The derived parameters are summarized in Table 1.

For cytochrome *c*-558 the experimental points do not fall on a straight line and thus the simple model of an ionization followed by a conformational change does not apply in this case. Modified models in which two or more ionizations precede the conformational change give rise to higher-degree equations. For example, for two ionizations preceding the conformational change the equation will be:

$$k_{\text{obs.}} = k_f \cdot \frac{K_{H_1} K_{H_2}}{K_{H_1} K_{H_2} + K_{H_1} [\text{H}^+] + [\text{H}^+]^2} + k_b$$

This leads to:

$$\frac{1}{k_{\text{obs.}} - k_b} = \frac{1}{k_f} \left(\frac{K_{H_1} K_{H_2} + K_{H_1} [\text{H}^+] + [\text{H}^+]^2}{K_{H_1} K_{H_2}} \right)$$

Fig. 8. Stopped-flow pH-jump experiments on the alkaline isomerization of cytochrome *c*-557

The line is a least-squares line of all points except the two at $10^{10} \times [\text{H}^+] = 14.8$. S.D. of the intercept is ± 0.06 . Points at higher values of $[\text{H}^+]$ tend to be more susceptible to error as k_f approaches k_b .

and analogous forms for three or more ionizations. For all these functions when $[\text{H}^+]$ is zero:

$$\frac{1}{k_{\text{obs.}} - k_b} = \frac{1}{k_f}$$

This allows us to estimate $1/k_f$ by extrapolation of the curve in Fig. 9. k_f is approx. 5, so that K_c is 4.

Thus for cytochrome *c*-557, the kinetic and thermodynamic parameters do not differ greatly from those of horse cytochrome *c*, K_c is of similar magnitude and the lower $pK_{\text{obs.}}$ appears to be due to an increase in K_H . The pK_H for cytochrome *c*-557 is 10.6 compared with 11 for horse cytochrome *c*.

Cytochrome *c*-558 shows more complex alkaline isomerization kinetics, which may be due to two ionizations. In this protein tyrosine-67 is replaced by phenylalanine, and it is possible that the absence of this residue, which is intimately concerned in the pattern of hydrogen and hydrophobic bonding in the region of the sixth iron ligand, affects the alkaline isomerization. The value of k_b for cytochrome *c*-558 is much higher than that for horse cytochrome *c*, whereas the estimate of k_f is similar. Thus the conformational equilibrium K_c is shifted in favour of the neutral species.

Studies on ferrocyclochromes

Autoxidizability and CO binding. Both cytochromes *c*-557 and *c*-558 were more autoxidizable than horse cytochrome *c*. For $5 \mu\text{M}$ protein in $5 \text{ mM-KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$, pH 7.0, at 25°C , the rate of autoxidation of cytochrome *c*-558 was approx. 1.8 nmol/h , and for cytochrome *c*-557 0.3 nmol/h . In this time-scale, autoxidation of horse cytochrome *c* was negligible.

Fig. 9. Stopped-flow pH-jump experiments on the alkaline isomerization of cytochrome *c*-558

Experimental values clearly did not fit a straight line. An extrapolation by eye of the curve gave an intercept of approx. 0.2. The justification for deriving k_f from this is discussed in the text.

The preparation of cytochrome *c*-558 of Perini *et al.* (1964) was described as being rapidly autoxidizable and unreactive with CO.

After bubbling solutions of ferrocyanochrome for 1 min with CO, both cytochrome *c*-557 and *c*-558 showed less than 5% reaction. However, further slow changes took place until approx. 90% of cytochrome *c*-558 and approx. 20% of cytochrome *c*-557 had reacted.

The approach to equilibrium in each case followed first-order kinetics with $t_{1/2} = 400$ s for both cytochromes *c*-558 and *c*-557. However, at one-fifth of the above CO concentration, only 50% of cytochrome *c*-558 reacted and $t_{1/2}$ was 1200 s. Thus the reaction is pseudo-first-order and depends on the CO concentration. In both cytochromes the binding was slowly reversible after removal of the CO in solution and was rapidly reversible by exposing the solution to strong light for a few seconds.

This type of slow reaction with CO is not unknown in research on cytochrome *c*. Kaminsky *et al.* (1972) found that treatment of horse cytochrome *c* with ethanol resulted in CO binding at a very slow rate. They showed that this CO binding was not necessarily accompanied by a tendency to autoxidation. Dupre *et al.* (1974) have shown that one of the phases observed in the reaction of CO with cytochrome *c* dimers is very slow. In this case, however, the rate is independent of CO concentration and therefore consistent with the existence of conformational equilibrium between a closed and a more open crevice form, with the latter reacting with CO.

Therefore in horse cytochrome *c* CO binding is not a property of the native protein but of variously modified or denatured forms (Margoliash & Lust-

garten, 1962). For this reason the objection may be raised that we are dealing with a denatured form of cytochrome *c*-558. However, the 695 nm band is still present with a normal extinction coefficient, the protein is monomeric as shown by gel filtration of this preparation on Sephadex G-75 [in confirmation of the results of Meyer & Cusanovich (1972)], and the reaction with CO occurred to the same extent and rate at early stages in the purification of the protein. It thus appears that CO binding is a property of native cytochrome *c*-558 and to a lesser extent of cytochrome *c*-557.

Formation of ferrocyanochrome-CN⁻ complexes and kinetics of breakdown. The spectra of the changes in the α band after reduction of ferricytochrome-CN⁻ complexes are shown in Fig. 10. In both cytochromes *c*-557 and *c*-558 the ferrocyanochrome-CN⁻ complexes have symmetrical α bands shifted to 561 nm. van't Hoff isochores for the breakdown of the complexes were plotted and the derived parameters of activation are shown in Table 1.

Thus the activation energies (E_a) of the non-mammalian cytochromes are several kilojoules/mol more positive than that of horse cytochrome *c*, whereas the entropies of activation ($\Delta S^\ddagger$) are also higher by more than $83.6 \text{ J}^\circ\text{K}^{-1} \cdot \text{mol}^{-1}$. As a result, the observed rates at 25°C are very similar.

This complementary behaviour of energies and entropies of activation near 300°K for a group of homologous proteins suggests that in the dissociation of the ferrocyanochrome-CN⁻ complex the phenomenon of water compensation (Lumry & Rajender, 1970) plays an important role. Therefore the contribution of solvent effects to the entropy changes of ferrocyanochrome *c* reactions cannot be disregarded, and

Fig. 10. Spectra of native and CN⁻-bound ferrocyanochrome *c*

(a) Horse cytochrome *c*; (b) cytochrome *c*-557; (c) cytochrome *c*-558. Curves marked 1 are spectra of the native proteins. Those marked 2 are spectra of the CN⁻ adducts recorded immediately after addition of dithionite. Ferrocyanochrome-CN⁻ complexes were formed in 0.05 M-KCN, 0.1 M-KH₂PO₄-K₂HPO₄ buffer, pH 7.0.

Fig. 11. Reduction of cytochrome *c*-558 with dithionite at alkaline pH values

(a) In curves (1)–(4), cytochrome *c*-558 (20 μ M) was dissolved in 0.1 M-glycine adjusted to pH 8.9 (1), 9.5 (2), 10.0 (3) and 10.5 (4) respectively with 5 M-NaOH. In curve (5) cytochrome was in 0.1 M- K_2HPO_4 adjusted to pH 11.0 with 5 M-NaOH. These solutions were mixed with 20 mM-sodium dithionite in the same buffer. The traces are oscilloscope records of the changes in transmission at 558 nm, on a vertical scale setting of 5 mV (800 mV = full scale, 100% transmission). (b) Instrument settings were as for (a) except that the time base was altered to 2 s/division. The pH was 11.0.

previous analyses of these changes, based exclusively on considerations of protein conformation (George & Schejter, 1964), should be revised. Obviously such a revision cannot be undertaken without information on the hydration of ferrocycytochrome *c* and its CN^- complex.

A discussion of the contribution of water compensation to entropy terms for the redox reaction of cytochrome *c* has been published (Margalit & Schejter, 1973). From the arguments used by these workers the results in Table 1 imply that on formation of their CN^- complexes the non-mammalian ferrocycytochromes bind three molecules of water less than horse cytochrome *c*.

Reduction with dithionite at alkaline pH values. When cytochrome *c* is reduced with dithionite above pH 8.5, a second kinetic phase, which is independent of the dithionite concentration, is observed after the initial fast reduction (Lambeth *et al.*, 1973).

The second phase of the reduction of cytochrome *c*-558 at alkaline pH with dithionite is shown in Fig. 11(a). The rate constants are very similar to those found by Lambeth *et al.* (1973) for horse cytochrome *c* (2.2 s $^{-1}$ for cytochrome *c*-558 at pH 10.0 and 2 s $^{-1}$ for horse cytochrome *c* at pH 11). The approximate pK value derived from the extent of the second phase was 9.8, in agreement with the kinetic and thermodynamic studies described above. A third and very slow phase was observed for cytochrome *c*-558 at above pH 11. The rate constant was 0.023 s $^{-1}$ and the absorption change was smaller than that of the second phase and in the opposite direction (Fig. 11b).

In the Cary spectrophotometer this change ap-

peared as an increase of 1–2% in the absorbance of the α peak and this change was not observed in horse cytochrome *c*. Thus in ferrocycytochrome *c*-558 a second change of an unknown nature takes place at pH values above 11, when the protein is reduced by dithionite. The [dithionite]-independent slow phase of horse cytochrome *c* is thought to reflect replacement of lysine-79 by methionine-80 as the sixth iron ligand (Lambeth *et al.*, 1973). If this interpretation is valid, then the third phase of cytochrome *c*-558 may in turn involve replacement of the methionine residue. As discussed above, the titration of the ferricytochrome *c*-558 was also atypical in this high-pH region.

Thus the replacement of the cysteine at residue 14 has not resulted in radical changes in cytochromes *c*-557 and *c*-558, although some differences in the physicochemical properties from those of horse cytochrome *c* have been found. A summary of the properties studied is shown in Table 1. The study of properties has of course been highly selective. Different workers have chosen various experimental systems to compare cytochromes *c*. Morgan & Riehm (1973), for example, in their investigation of *Humicola* cytochrome *c*, studied the titration and solvent perturbation of tyrosine residues and circular-dichroism and optical-rotatory-dispersion spectra. The difficulty in choosing experimental systems for comparison, and the problems of interpreting what are large and small, important and unimportant differences in the properties studied, are measures of our almost complete ignorance of the relationship between properties, structure, physiological function and natural selection in cytochrome *c*.

Of the two cytochromes studied, cytochrome *c*-557 is very close to horse cytochrome *c* with respect to the thermodynamic parameters of the redox potential, the ligand binding and the characteristics of the alkaline isomerization. The absence of a thioether bond may be reflected in the instability of the low-spin state to high pH values and to heating. This instability to high pH values and temperature is even more pronounced in cytochrome *c*-558. In the current interpretation (Davis *et al.*, 1974; Wilgus & Stellwagen, 1974), the alkaline isomerization may involve replacement of methionine-80 by lysine-79 as the sixth ligand, and it is possible that this may also apply to the 'cold'-'hot' transition. Thus in cytochromes *c*-557 and *c*-558 the lysine-79 co-ordination may be unstable.

The alkaline isomerization of cytochrome *c*-558 appears to follow a different kinetic path from that of horse cytochrome *c* and cytochrome *c*-557. As Stellwagen & Cass (1974) emphasize, the fact that isomerization does occur in cytochrome *c*-558 tends to eliminate tyrosine-67 as a possible trigger for the process. However, the kinetic results indicate that a simple model involving the ionization of lysine-79, followed by a conformational change, does not apply to cytochrome *c*-558.

It is fair to say that the evidence for lysine-79 co-ordination at alkaline pH in ferricytochrome *c* is not conclusive. The problem is of interest because it is possible that *in vivo*, a low-spin form, lacking the 695 nm band, may function during some stage of electron transfer. Wilgus & Stellwagen (1974) consider that the invariant lysine residues in cytochrome *c* and cytochrome *c*₂ support the hypothesis that lysine-79 is the residue involved. However, neither cytochrome *f* from algae (Laycock, 1972; Ambler & Bartsch, 1975) nor cytochrome *c*₂ from *Rhodospseudomonas capsulata* (R. P. Ambler, personal communication) have a lysine residue immediately preceding the methionine residue, although these proteins undergo the same type of alkaline transition to a low-spin species as occurs in cytochrome *c* (Ben Hayyim & Schejter, 1974; G. W. Pettigrew & A. Schejter, unpublished work). Cytochrome *c*₂ from *Rhodospirillum rubrum* also shows a complex behaviour at alkaline pH values, which is not easily explained by a simple methionine-lysine change (Pettigrew & Schejter, 1974).

Thus the model of a lysine-79-methionine-80 side-chain-displacement mechanism, operating after ionization of the lysine residue at alkaline pH, may not be the final word, nor be generally applicable to the low-spin *c*-type cytochromes.

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