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On the relationship between oxidation–reduction potential and biological activity in cytochrome *c* analogues

Results from four novel two-fragment complexes

Carmichael J. A. WALLACE and Amanda E. I. PROUDFOOT
Département de Biochimie médicale–Université de Genève, 9 avenue de Champel, 1211 Genève 4, Switzerland

We have confirmed the propensity of fragments of cytochrome *c* to form complexes that reproduce the structure and, in part, the functionality, of the native protein by preparing four novel complexes. We have used trypsin under three different sets of conditions in sequence to prepare a contiguous two-fragment complex (1–55)·(56–104). One of the intermediates is a stable overlapping complex (1–65)·(56–104). Conditions for limited acid hydrolysis of peptide bonds in cytochrome *c* have been developed that optimize the yield of fragments (1–50) and (51–104). These two fragments also form a stable association, as do (1–50) and (56–104). These complexes are potentially useful for the semisynthesis of analogues modified in the region of the cleavage sites, which include a number of highly conserved amino acid residues, and are being used for studies of protein folding, interactions with oxidase, cytochrome *c* immunogenicity and of artificially induced spontaneous resyntheses between complexing fragments. Like other known two-fragment complexes of cytochrome *c*, they exhibit normal visible spectra, including the presence of the 695 nm band, indicative of a functional haem crevice. Studies of their biological activities and redox potentials lead to a number of conclusions on structure–function relationships in cytochrome *c*. Most significantly there is a linear relationship between the logarithm of electron-transfer rates from cytochrome *c* reductase and redox potential in this series of analogues, indicating that such transfer is thermodynamically controlled. This discovery contributes to our understanding of the interaction of cytochrome and reductase. Since the relationship is obeyed by other types of analogues, except for those that involve modification of the active site of cytochrome *c*, we have a useful diagnostic for those residues that participate directly in electron transfer.

INTRODUCTION

Cytochrome *c* has a particular propensity for forming complexes from combinations of fragments derived from it. Such complexes, especially those formed from two contiguous peptides, essentially duplicate the conformation of the native protein and retain at least part of its functionality.

The reasons for this phenomenon may lie in the simplicity of the structure of the protein, in which the 104-residue polypeptide chain is economically wrapped around the haem prosthetic group in a succession of loops (Takano & Dickerson, 1981). There is no β-structure, nor are there disulphide bridges, and the relatively large hydrophobic haem group may act as a nucleating centre, facilitating the assumption of the active conformation from two or three pieces.

The first reported complex was that of two CNBr fragments, (1–65) and (66–104) (Corradin & Harbury, 1971); since then, others discovered include (1–38)·(39–104) (Harris & Offord, 1977), (1–53)·(54–104) (Hantgan & Taniuchi, 1977, 1978), (1–25)·(23–104) (Parr *et al.*, 1978), whereas (1–37)·(38–104) has been prepared by semisynthesis (Harris, 1979; Proudfoot *et al.*, 1986).

Besides the structure–function studies undertaken directly upon them, these complexes have been used for studies of protein folding (Hantgan & Taniuchi, 1978; Parr & Taniuchi, 1979), as intermediates in the preparation of semisynthetic complexes (Harris & Offord, 1977; Proudfoot *et al.*, 1986), in resyntheses of the whole protein or analogues of it (Corradin & Harbury, 1974; Wallace & Offord, 1979; Wallace & Rose, 1983) and in studies of cytochrome *c* antigenicity (Baumhüter *et al.*, 1987). For the furtherance of such studies, we have looked for new associations and have found two novel contiguous two-fragment complexes. One of these, (1–55)·(56–104), is prepared by a lengthy and complex procedure, including treatment with trypsin under three different sets of conditions, but which furnishes another, overlapping, complex [(1–65)·(56–104)] *en route*. The other, (1–50)·(51–104), is quite simply prepared. The haem fragment obtained in this preparation can be combined with the non-haem fragment of the other complex to give (1–50)·(56–104), with a five-residue gap.

EXPERIMENTAL

Materials

Cytochrome *c* (horse heart, type III), and trypsin inhibitor (soya-bean, type I-S) were obtained from Sigma Chemical Co. (Munich, Germany); Trypsin [L-1-chloro-4-phenyl-3-tosylaminobutan-2-one('TPCK')-treated] from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); SP-Trisacryl from LKB; Sephadex G-50 and Sephadex G-25 from Pharmacia (Dübendorf, Switzerland). Other chemicals were obtained from Merck

(Darmstadt, Germany) and were of the highest grade available.

Fragment preparation

Fragments (1–38) and (39–104) were prepared by tryptic digestion of *N*-ε-protected cytochrome *c*, and subsequent deprotection, by the method of Proudfoot *et al.* (1984), and (1–65) was prepared by CNBr cleavage of the native protein (Corradin & Harbury, 1970).

Fragment (56–104) was a product of limited tryptic digestion of the reduced complex (1–38)·(39–104). The complex was dissolved in 0.1 M-potassium phosphate buffer, pH 7.0, at a concentration of 5 mg·ml^{-1} (0.4 mM), to which was added an excess of ascorbic acid to maintain the reduced state. After 5 min, sufficient trypsin was added to give a final enzyme/substrate ratio of 1:100, and the mixture left at room temperature for 1 h before gel filtration on Sephadex G-50 (fine grade) in 7% (v/v) formic acid. The major peak contains fragments (1–38) and (56–104) (Proudfoot *et al.*, 1986), which are resolved by ion-exchange chromatography as described below.

Tryptic treatment, under similar conditions, of the complex (1–65)·(56–104) was used to excise the overlap portion (56–65). An equimolar mixture of (1–65) and (56–104) was dissolved in 0.1 M-potassium phosphate buffer, pH 7.0, at 12.4 mg·ml^{-1} (1 mM). To each 10 ml of solution was added 1 mg of ascorbic acid, then, after 5 min, 10 μl of a 1% solution of trypsin in 1 mM-HCl (enzyme/substrate ratio 1:1250). The mixture was left for 1 h at room temperature; the reaction was stopped by a 5-fold excess of trypsin inhibitor over trypsin.

Fragments (1–50) and (51–104) were made by partial acid hydrolysis of cytochrome *c*. The protein was dissolved in 0.015 M-HCl at 10 mg·ml^{-1} and heated at 110 °C for 2 h. After cooling, fragments were separated from intact cytochrome by gel filtration on Sephadex G-50 (fine grade) in 7% formic acid.

Fragment purification and characterization

Cation-exchange chromatography was employed to purify individual fragments or to separate fragments from mixtures. We used SP-Trisacryl and a phosphate buffer system containing 7 M-urea to prevent association of fragments into complexes. The gradients used to elute fragments from the resin were either 10–100 mM-(for non-haem fragments or mixtures) or 20–200 mM-(for haem fragments) potassium phosphate/7 M-urea, pH 7.45 (uncorrected glass electrode), prepared by diluting the appropriate quantity of 1.6 M-potassium phosphate, pH 7.0, with 8 M-urea and water. The concentrated buffer was made by mixing 1.6 M solutions of K_2HPO_4 and KH_2PO_4 to obtain pH 7.0.

The structure of novel fragments was checked by amino acid analysis using standard methods.

Preparation and purification of complexes

Complexes were prepared by mixing equivalent quantities of fragments. These mixtures were chromatographed on Sephadex G-50 (fine grade) in non-denaturing solvent, normally 0.1 M-NH_4HCO_3. Peaks eluted at the cytochrome *c* position were freeze-dried and re-purified by ion-exchange under the non-denaturing conditions described by Wallace & Harris (1984).

Spectroscopy

U.v.–visible spectra in the range 750–250 nm were drawn with a Cary 210 spectrophotometer. The variation in intensity of the 695 nm band with pH over the range 6–11 was monitored as described by Wallace (1984*b*).

Oxidation–reduction potentials

Redox-potential determinations were made by a version of the 'method of mixtures'. Solutions of cytochrome *c* or its analogues, in 0.05 M-phosphate buffer, pH 7.0, were made redox buffers of known potential by adding various quantities of potassium ferro- and ferri-cyanide. The cytochrome redox equilibria were determined by measurements of 550 nm absorbance, by reference to fully oxidized and fully reduced samples of the protein solution. The ferrocytochrome–ferricytochrome redox equilibria are plotted against the ferrocyanide–ferricyanide ratios required to establish them. Extrapolation gives the midpoint potential (E'_0) at pH 7, given that the E'_0 of the ferrocyanide–ferricyanide couple is 0.43 V. Full details of the method were given by Wallace *et al.* (1986).

Biological activity

The complexes were tested for their ability to promote electron transfer in the succinate oxidase assay system of Jacobs & Sanadi (1960). Rat liver mitochondria were isolated by differential centrifugation. Their outer membranes were fractured by osmotic shock and became permeable to proteins. Cytochrome *c*, which is in loose electrostatic association with the inner membrane, may be removed by repeated washing with iso-osmotic solutions. The cytochrome *c*-depleted mitochondria exhibited only low levels of consumption of dissolved oxygen, possibly due to microsomal contamination. O_2 uptake, which was measured potentiometrically with a Rank Brothers oxygen electrode, in a buffer containing succinate as electron donor, and ADP, hexokinase and glucose (the mitochondrial inner membrane is intact, and electron transfer and phosphorylation remain coupled), increases up to 10-fold on incremental injection of cytochrome *c* into the chamber of the electrode and is plotted against the quantity of added haemochrome. It has been suggested that, in this system, the rate-limiting step is electron transfer from cytochrome reductase (cytochrome *bc*; complex III) to cytochrome *c*, so that the biological activity assayed is the productivity of the reductase–cytochrome *c* interaction (Wallace, 1984*a*).

RESULTS

Gel filtration of the limited digest of ferro(1–38)·(39–104) separates the fragments (1–38) and (56–104) from trypsin and small peptides, but the two fragments are not resolved (Proudfoot *et al.*, 1986). Separation is efficiently achieved on the ion-exchange system employed, since the peptides were eluted at very different ionic strengths (Fig. 1). The non-haem peptide has the expected amino acid composition of 56–104.

The progress of tryptic digestion of the complex formed by the (56–104) thus produced, and (1–65) from CNBr digests of the protein, was monitored by paper electrophoresis at pH 6.5 of portions of the digest. The only small peptide generated in the first few minutes was

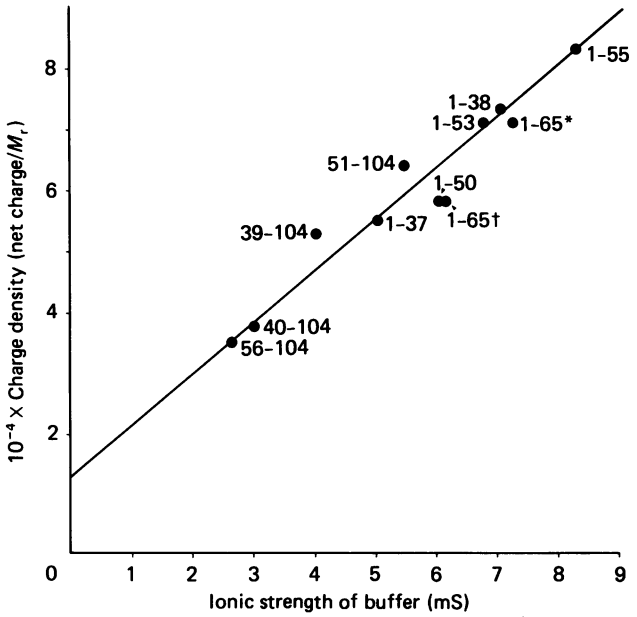

Fig. 1. Properties, on cation-exchange chromatography in denaturing conditions, of associating fragments of horse heart cytochrome *c*

Charge density (net charge divided by M_r) was plotted against the ionic strength necessary to elute it from a column (1 cm × 10 cm) of SP-Trisacryl (in phosphate/urea buffer, pH 7.0). The fragments studied are listed below. Net charges and molecular weights were calculated from the published sequence, with histidine residues counted as '½ charge' (Margoliash *et al.*, 1961).

Fragment	Charge	M_r
1–37	+2½	4630
1–38	+3½	4802
1–50	+3½	6068
1–53	+4½	6382
1–55	+5½	6625
1–65*	+5½	7794
1–65†	+4½	7812
56–104	+2	5759
51–104	+4	6216
40–104	+3	7434
39–104	+4	7582

* Homoserine lactone form (on Figure too).
† Homoserine form (on Figure too).

identified as (56–65). It gives a yellow stain with ninhydrin/cadmium, characteristic of *N*-terminal glycine, is uncharged at pH 6.5 in the lactone form, and has a mobility relative to aspartic acid of +0.23 (predicted +0.23) in the homoserine form. Excision was complete at 1 h, since there was no further increase in the yellow ninhydrin colour in the neutral zone, at which time a small quantity of another peptide was observed that had the properties of Asn-Lys (54–55). It gave an orange–red colour with ninhydrin/cadmium and had a mobility of −0.63 relative to aspartic acid (the predicted mobility of Asn-Lys is −0.62).

Ion-exchange of a 1 h digest of (1–65)·(56–104) revealed two haem-containing fragments, both well separated from (56–104) (Fig. 1). The major peak is

Table 1. p*K* of the alkaline transition in cytochrome *c* and its analogues

Values were determined as described by Wallace (1984*b*) in 0.1 M-potassium phosphate buffer.

Cytochrome or complex	pK_{695}
Horse cytochrome *c*	9.30
(1–50)·(56–104)	7.60
(1–38)·(39–104)	7.10
(1–50)·(51–104)	7.35
(1–55)·(56–104)	7.25
Acetimidyl-cytochrome *c*	9.90
Acetimidyl-(1–38)·(39–104)	8.15
Acetimidyl-(1–37)·(38–104)	8.10

Table 2. Midpoint oxidation–reduction potentials (E_0') of cytochrome *c* and derived two-fragments complexes

Redox equilibria were determined spectrophotometrically by the 'method of mixtures' as described by Wallace *et al.* (1986). Data for the acetimidyl-protein and complexes are taken from Proudfoot *et al.* (1986).

Cytochrome or complex	E_0' (mV)
Horse cytochrome *c*	260
(1–50)·(56–104)	160
(1–50)·(51–104)	166
(1–55)·(56–104)	193
(1–65)·(56–104)	184
Acetimidyl-horse cytochrome *c*	245
Acetimidyl-(1–38)·(39–104)	150
Acetimidyl-(1–37)·(38–104)	216

(1–55), the minor (1–53). The latter is the principal product of limited digestion of the overlap complex (1–65)·apo-(1–104) reported by Hantgan & Taniuchi (1977). The identities of these fragments were confirmed by amino acid analysis.

Limited acid cleavage yielded two fragments that were barely resolved on gel filtration but which could be separated by ion exchange (Fig. 1) under denaturing conditions. The two techniques, used sequentially, serve to rid the fragments of uncleaved cytochrome and other contaminants, among which the product of additional cleavage at Asp-2, (3–50), is noticeable. Ion exchange of the mixture of the (1–50) and (51–104) thus produced, under non-denaturing conditions, permits resolution of the complex from any excess of one or other fragment. Samples of peaks resolved by gel filtration in 7% formic acid on a long (1.6 cm × 150 cm) gel-filtration column were used for amino acid analysis, and identified as (51–104) and (1–50), which were eluted in that order.

Purified complexes showed u.v.–visible absorption spectra typical of cytochrome *c*, including a fully-developed 695 nm band, diagnostic of an intact haem crevice (Dickerson & Timkovitch, 1975). The disappearance of this band with rising pH signals the transition from state III to state IV and occurs with a p*K* of about 9.3 in the native protein at moderate ionic strength. The p*K* values determined for these and previously reported complexes (Proudfoot *et al.*, 1986) are noted in Table 1.

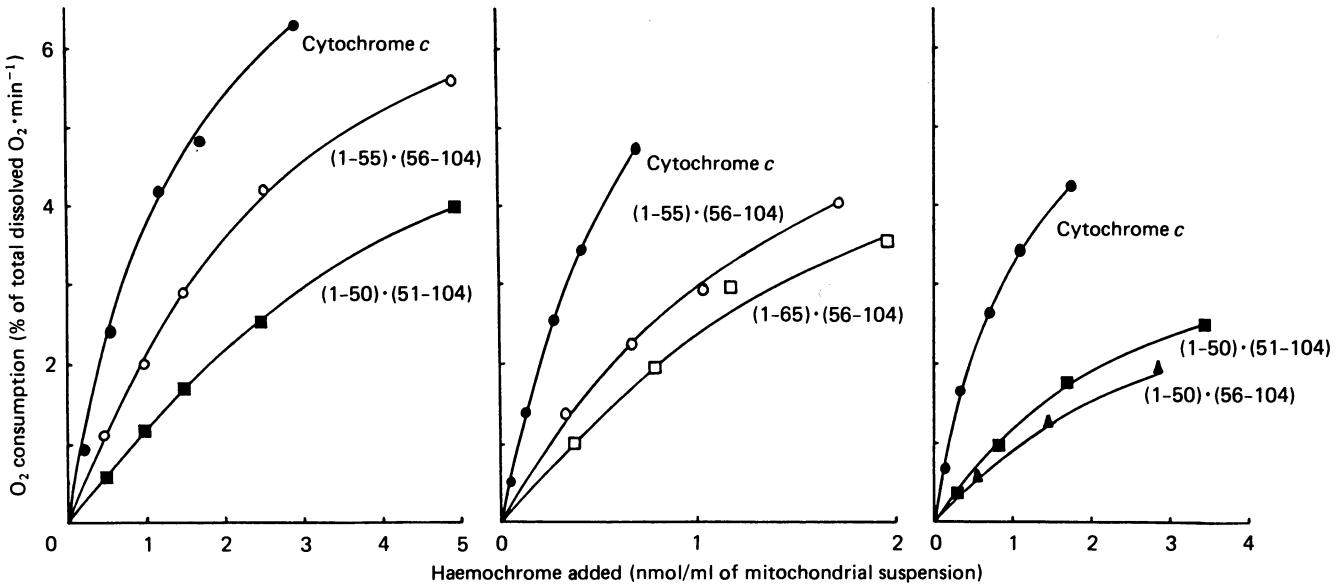

Fig. 2. Biological activities of the two-fragment complexes compared with intact cytochrome *c*

The proteins were assayed for their ability to stimulate O_2 uptake in cytochrome *c*-depleted mitochondria. The curves represent the result of incremental additions of cytochrome *c* or analogues to depleted mitochondria derived from 0.2 g of rat liver suspended in 1 ml of 0.3 M-sucrose and mixed with 4.1 ml of buffer in the chamber of an oxygen electrode. The assay buffer is 300 mM-sucrose, 75 mM-glucose, 9.42 mM-$MgCl_2$, 12.5 mM-phosphate, 18.75 mM-succinate and 1.87 mM-ATP, adjusted to pH 7.0 by the addition of KOH. Each 4.1 ml portion contained 10 units of hexokinase. The yield of mitochondria per g of rat liver was variable; the likely amount present was 10–20 mg of mitochondrial protein per ml of suspension, representing approx. 1–2 nmol of oxidase, and 0.5–1 nmol of reductase. The Figure shows data obtained with three separate mitochondrial preparations at room temperature.

The redox potentials determined for the complexes were all lower, to various extents, than that of cytochrome *c*, a characteristic of complexes attributed by Proudfoot *et al.* (1986) to a looser, more solvent-accessible, structure at the bottom of the haem crevice. It is also possible that if structural change in the complexes modified the hexacyanide-binding properties of the protein, since the method of determination uses a ferricyanide titration, then such change could be responsible for the altered potentials. However, known hexacyanide-binding sites are distant from the bottom loop (Eley *et al.*, 1982). The state of occupancy of these sites does not appear to influence potential, since identical values are obtained with methods that do not employ hexacyanides. Nor does the binding of other anions elsewhere on the surface produce variations of greater than 20 mV (B. E. Corthésy & C. J. A. Wallace, unpublished work). The creation of a new site is conceivable, though the evidence of Proudfoot *et al.* (1986) suggests that this region is mobile in the complexes. Furthermore, Moore *et al.* (1984) obtained a value for the redox potential of acetimidyl-(1–38)·(39–104) of 160 mV (cf. 150 mV in Proudfoot *et al.*, 1986) using another method, so it appears most likely that the observed changes are consequences of the nick in the polypeptide chain and dependent in magnitude on the site of the nick.

Fig. 2 compares the relative efficiencies of the complexes at promoting electron transfer, and hence oxygen consumption, in depleted mitochondria. All analogues have activities lower than that shown by native cytochrome *c*, but, once again, there are considerable individual differences. It is to be noted that activity decreases in the same order in which redox potential decreases.

DISCUSSION

Peptide bonds formed by aspartic acid residues are known for their sensitivity to hydrolysis in acid conditions (Schultz, 1967). Cytochrome *c* contains only three free aspartate residues, at positions 2, 50 and 93. Apparently the 93–94 bond, Asp–Leu, is less labile than either of the others, Asp–Val or Asp–Ala, since peptide (94–104) seen on gel filtration in denaturing conditions is present only in very low yields relative to other fragments. The extent of recovery of fragment (3–50) upon ion-exchange chromatography suggests that the 2–3 and 50–51 bonds are equally labile.

The fragments of cytochrome *c*, including those described for the first time in the present study, illustrate well the generality of a direct relationship between the affinity for an ion-exchanger and the charge density of the substance bound (Fig. 1). Novel fragments appear as capable as previously reported examples of forming associations, but, like them, the new complexes are less than fully competent.

The complexes (1–50)·(51–104), (1–65)·(56–104), (1–55)·(56–104) and (1–50)·(56–104) all exhibit, like the other reported examples, redox potentials more negative than that of the native protein. Since a high potential requires, among other factors, that the haem be shielded from solvent (Stellwagen, 1978), Proudfoot *et al.* (1986) have attributed the lower oxidation–reduction potentials of complexes (1–37)·(38–104) and (1–38)·(39–104) to a more open, solvent-accessible,

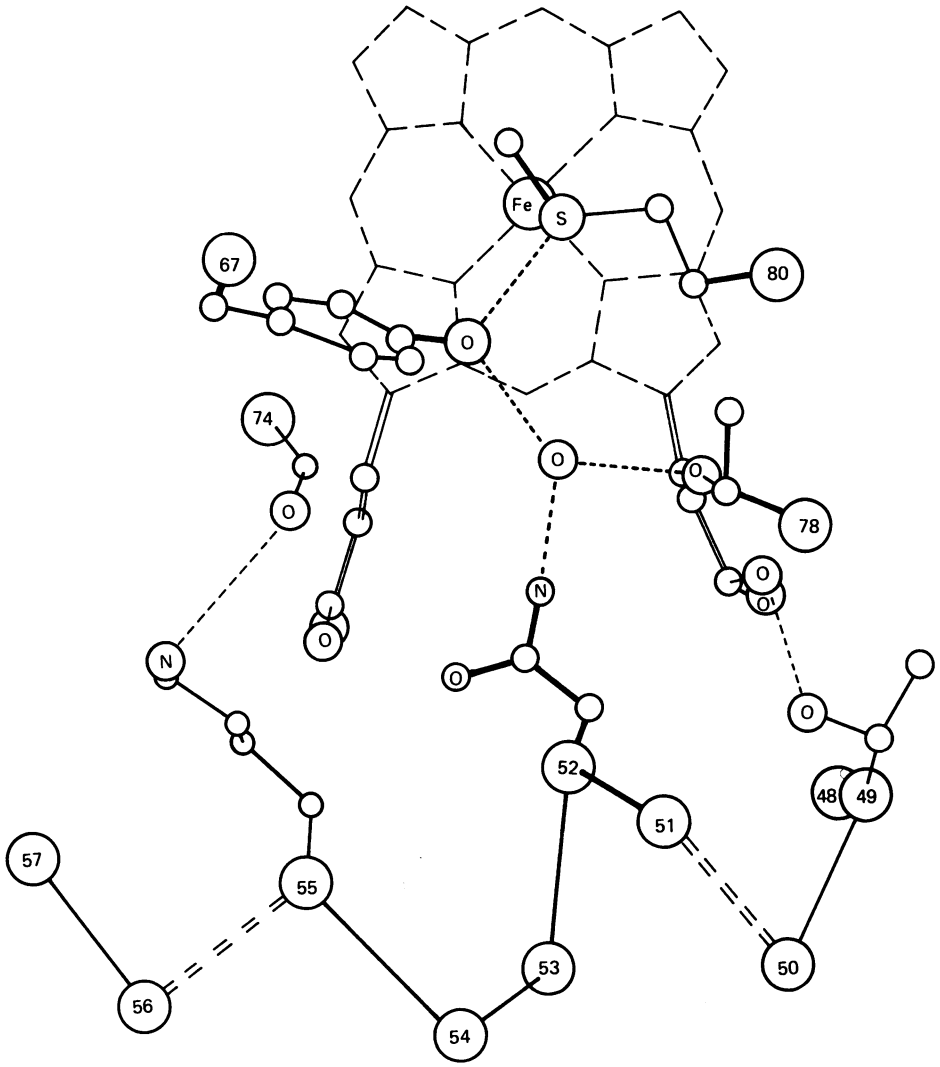

Fig. 3. Structure of the ferricytochrome *c* haem crevice

A network of hydrogen bonds (------) links the methionine sulphur atom, sixth ligand to the haem iron, with the hydroxy groups of Tyr[67] and Thr[78], the amide of Asn[52] and a central water molecule. Additionally, the stretch of bottom loop illustrated is linked to the bulk of the molecule by two further hydrogen bonds between Lys[55] and Tyr[74], and between Thr[49] and the haem outer propionate group. The bonds marked --- are missing in the complexes presently under discussion.

structure obtaining in the (conventional) bottom loop of the cytochrome structure after rupture of the polypeptide chain.

The variability observed between one complex and another was ascribed to a greater or lesser disruption of the normal tight structure, depending on the site of the chain break. The approx. 70 mV difference between (1–37)·(38–104) and (1–38)·(39–104) can be explained by the retention on the loop in (1–37)·(38–104) of the stabilizing residue arginine-38 (Proudfoot *et al.*, 1986).

Although there is no similarly compelling explanation of the 30 mV difference between (1–50)·(51–104) and (1–55)·(56–104), it is conceivable that, in the latter, the presence at the *C*-terminus of that portion of the loop that runs directly beneath the bottom edge of the haem (residues 37–48), of three residues that form hydrogen bonds to the remainder of the molecule, rather than just one in the (1–50)·(51–104) case, decreases solvent access to the haem crevice. This proposition is illustrated in Fig. 3, and is supported by the observation that the removal of residues 51–55 in the (1–50)·(56–104) complex has little further effect.

Although the complexes exhibit spectra nearly identical with that of the native protein at pH 6, there is a pronounced difference in the p*K* values observed for the change from spectral state III to state IV, the 'alkaline transition', of ferricytochrome *c* (Theorell & Akesson, 1941), in which the 695 nm band is lost. Values determined for these and other complexes (Table 1), which all incorporate their cleavage site in the conventional bottom loop, are very close to one another. Thus, although the stability of the complexes, as expressed by their relative redox potentials, varies with the precise position of the nick, resistance to the alkaline denaturation is diminished indiscriminately by chain breaks in this region. Gel-filtration experiments at alkaline pH have shown that the change is not a consequence of dissociation of the complex.

The transition accompanies a ligand exchange at the sixth position, though what replaces methionine-80 is

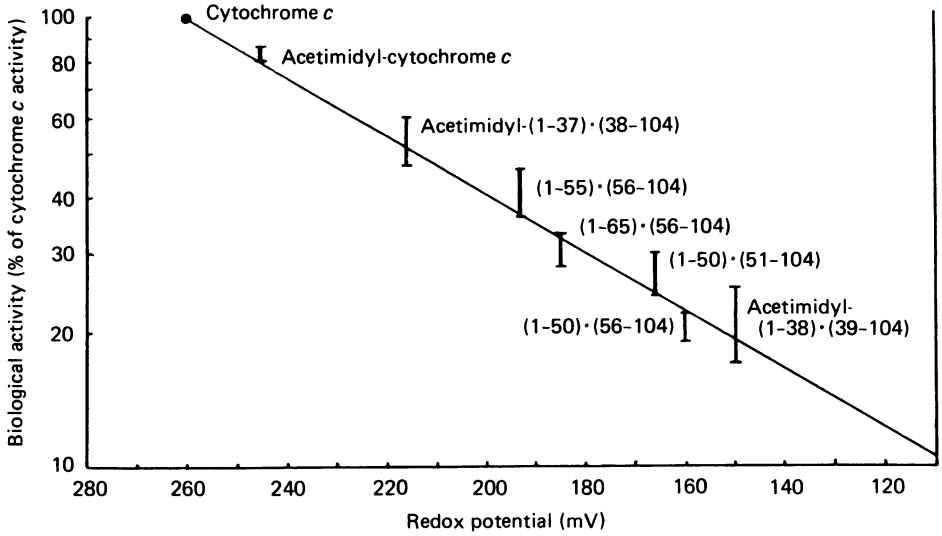

Fig. 4. Relationship between biological activity in a succinate oxidase system and oxidation–reduction potential at pH 7.0, for six two-fragment complexes, cytochrome *c* and acetimidyl-cytochrome *c*

The redox potentials used are average values and the biological-activity axis shows the range of values determined for tangents to the curves of which those of Fig. 2 are examples.

unclear. From our results it seems likely that this propensity to ligand exchange is resisted by the presence of an intact bottom loop, and any break is highly detrimental, shifting the p*K* of the transition by about 2 pH units. It can easily be imagined that a break in the bottom loop would facilitate a conformational rearrangement.

The biological activity of the complexes varies considerably. We have, in the past (Proudfoot *et al.*, 1986), remarked on the apparent correlation between the redox potentials of complexes and their analogues and their ability to stimulate electron transfer in cytochrome *c*-depleted mitochondria. We now have a sufficient variety of two-fragment complexes that it is possible to quantify this correlation. We find that there is a linear relationship between redox potential and the logarithm of biological activity (Fig. 4).

This linearity suggests to us that, in this system, the electron-transfer rate is under purely thermodynamic control. The notion that the limiting step is a reductase-to-cytochrome *c* transfer is supported by the observed decrease in activity with lower potential. Measurements of the individual components of the respiratory chain show that, normally, cytochrome *c* and its reductase are roughly equipotential. With a falling redox potential for cytochrome *c* in the cytochrome *c*–cytochrome reductase complex, in which equilibrium is established between the two haem centres, the electron would spend progressively more time at the c_1 haem. Thus upon dissociation of the complex, proportionately fewer cytochrome *c* molecules will be reduced and capable of electron transfer to oxidase. This model implies that association and dissociation are not influenced by oxidation state. The results of Bill & Azzi (1984) suggest that, although the affinity for oxidase differs between the two states, there is no difference in the case of the reductase.

That redox potential should be the major determinant of the rate of electron transfer to these analogues is unsurprising in view of the fact that the point of cleavage, and of structural perturbation, is distant from the active site of cytochrome *c*. Indeed, other analogues that do not involve modification of the active site, such as those specifically *o*-acetylated at certain tyrosine residues, also obey this relationship. Conversely, a direct modification of active site residues, as in 19-*N*-ε-methanesulphonylethyloxycarbonyl-cytochrome *c*, leads to extreme divergence from this pattern (C. J. A. Wallace, unpublished work).

Fitting the values determined for a cytochrome *c* derivative to the slope thus provides a way of deciding whether a particular residue is directly involved in electron transfer or not. The technique will be of particular value in investigations of the residues of the hydrophobic stretch lining the haem edge (Poulos & Kraut, 1980; Pielak *et al.*, 1985; Wallace *et al.*, 1986).

The unmodified complexes should also find use in studies of the interaction of cytochrome *c* with other physiological partners in the mitochondrion, and the site of the cleavage points makes them potentially of great value as intermediates in the semisynthesis of analogues of the protein modified in the 50–60 region, which includes a number of invariant or highly conserved residues.

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