Purification and properties of a cross-linked complex between cytochrome *c* and cytochrome *c* peroxidase

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Cytochrome *c* (horse heart) was covalently linked to yeast cytochrome *c* peroxidase by using the cleavable bifunctional reagent dithiobis-succinimidyl propionate in 5 mM-sodium phosphate buffer, pH 7.0. A cross-linked complex of molecular weight 48 000 was purified in approx. 10% yield from the reaction mixture, which contained 1 mol of cytochrome *c* and 1 mol of cytochrome *c* peroxidase/mol. Of the total 40 lysine residues, four to six were blocked by the cross-linking agent. Dithiobis-succinimidylpropionate can also cross-link cytochrome *c* to ovalbumin, but cytochrome *c* peroxidase is the preferred partner for cytochrome *c* in a mixture of the three proteins. The cytochrome *c* cross-linked to the peroxidase can be rapidly reduced by free cytochrome *c*-557 from *Crithidia oncopelti*, and the equilibrium obtained can be used to calculate a mid-point oxidation–reduction potential for the cross-linked cytochrome of 243 mV. Mitochondrial NADH–cytochrome *c* reductase will reduce the bound cytochrome only very slowly, but the rate of reduction by ascorbate at high ionic strength approaches that for free cytochrome *c*. Bound cytochrome *c* reduced by ascorbate can be re-oxidized within 10 s by the associated peroxidase in the presence of equimolar H₂O₂. In the standard peroxidase assay the cross-linked complex shows 40% of the activity of the free peroxidase. Thus the intrinsic ability of each partner in the complex to take part in electron transfer is retained, but the stable association of the two proteins affects access of reductants.

The mechanism of electron transfer between redox proteins has attracted considerable attention, but remains poorly defined. Often such proteins have buried redox centres, and areas of the molecular surface are believed to form binding sites for a reductase or an oxidase, these sites enclosing the point of entry or exit of the electron. For mitochondrial cytochrome *c* a large body of evidence now supports the idea that the binding surface for the physiological reductase and oxidase is shared and is formed by a number of basic amino acids surrounding the exposed edge of pyrrole ring II (Smith *et al.*, 1977; Pettigrew, 1978; Ferguson-Miller *et al.*, 1978; Kang *et al.*, 1978; Ahmed *et al.*, 1978; Rieder & Bosshard, 1978, 1980).

The complex of mitochondrial cytochrome *c* and yeast cytochrome *c* peroxidase affords a particularly attractive model for the study of interactions between redox proteins (Nicholls, 1974). The cyto-

chrome *c* peroxidase is water-soluble and readily purified (Yonetani, 1976). The tertiary structure of this enzyme has been determined by X-ray crystallography (Poulos *et al.*, 1980), and the structure of horse cytochrome *c* is known (Swanson *et al.*, 1977).

The present paper describes the formation of a covalently linked complex between horse cytochrome *c* and yeast cytochrome *c* peroxidase and the effects of formation of the complex on electron transfer. A preliminary account of some of this work was presented at the 14th FEBS Meeting, Edinburgh (Pettigrew & Seilman, 1981).

Materials and methods

Materials

Horse cytochrome *c* (grade VI), ovalbumin (grade VI), polyglutamate, *N*-ethylmaleimide, trinitrobenzenesulphonate, NADH and SDS were from Sigma Chemical Co., Poole, Dorset, U.K. Dimethyl

Abbreviation used: SDS, sodium dodecyl sulphate.

sulphoxide and acrylamide were from BDH Chemicals, Poole, Dorset, U.K. Dithiobis-succinimidylpropionate was from Pierce Chemical Co., Rockford, IL, U.S.A., and solutions in dimethyl sulphoxide were prepared freshly from a cold desiccated powder.

Cytochrome *c*-557 from *Crithidia oncopelti* was purified as described by Pettigrew *et al.* (1975). NADH-cytochrome *c* reductase (EC 1.6.99.3) was prepared from ox heart mitochondria as described by Hatefi & Stiggall (1978).

Yeast cytochrome c peroxidase (EC 1.11.1.5)

Cytochrome *c* peroxidase of yeast was purified by a modification of the method of Yonetani & Ray (1965). A 2 kg batch of pressed yeast (Distillers Co., Kirkliston, West Lothian, Scotland, U.K.) was crumbled on to the grille of a large vacuum desiccator and dried to about 1.3 kg. Cold ethyl acetate (200 ml) was added and the mixture was left overnight at 4°C. All subsequent operations were conducted at this temperature. The broken-cell suspension was diluted with 4 litres of cold water and stirred for 1 h. After centrifugation at 10000 *g* for 30 min (Beckman JA14 rotor, 6 × 250 ml), the slightly cloudy supernatant was passed through a Sephadex G-25 (coarse grade) column (7 cm diam. × 60 cm) equilibrated with 5 mM-sodium phosphate buffer, pH 6.1. Approx. 800 ml could be desalted in one pass through this column.

The combined coloured eluate from the Sephadex G-25 column was passed through a DEAE-cellulose (Whatman DE-23) column (3 cm diam. × 5 cm) equilibrated with 5 mM-phosphate buffer, pH 6.1, and the adsorbed cytochrome *c* peroxidase was washed with 100 ml of 50 mM-phosphate buffer, pH 6.1, and then eluted with 20%-saturated (NH₄)₂SO₄. The brown eluate (approx. 100 ml) was dialysed overnight against 5 litres of 5 mM-phosphate buffer, pH 6.1, and then passed through a DEAE-cellulose (Whatman DE-52) column (1.5 cm diam. × 10 cm) equilibrated in the same buffer. The cellulose was washed with 50 ml of 50 mM-phosphate buffer, pH 6.1, and the adsorbed proteins were eluted with a linear gradient (600 ml) of 50–250 mM-phosphate buffer, pH 6.1. Three brown bands were usually observed on the column. The first, which was eluted at 60 mM-phosphate buffer, pH 6.1, showed no cytochrome *c* peroxidase activity and was discarded. The main band, eluted at 150 mM-phosphate buffer, pH 6.1, was retained as the cytochrome *c* peroxidase. A third band of variable amount, eluted at high concentrations of phosphate buffer, pH 6.1, also showed peroxidase activity, but was discarded.

At this stage the combined cytochrome *c* peroxidase fractions showed an A_{410}/A_{280} ratio of approx. 0.7. This solution was diluted with 3 vol. of water, and the peroxidase was re-adsorbed on a small

DEAE-cellulose (Whatman DE-52) column (1.5 cm diam. × 1.5 cm). After elution with 20%-saturated (NH₄)₂SO₄, the cytochrome *c* peroxidase solution (5–10 ml) was passed through a Sephadex G-75 column (3 cm diam. × 90 cm) and equilibrated in 5 mM-phosphate buffer, pH 6.1, and the A_{410}/A_{280} ratio of each collected fraction was measured.

Fractions with A_{410}/A_{280} ratio greater than 1 were combined, passed through a small DEAE-cellulose column and eluted with 20%-saturated (NH₄)₂SO₄ as described above. The concentrated solutions were dialysed against 5 mM-phosphate buffer, pH 6.1, and stored frozen at –20°C. Stored solutions had an A_{410}/A_{280} ratio of 1.05–1.2 and a concentration of 150–500 μM [based on $\epsilon_{410} = 93 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Yonetani & Ray, 1965)]. The yield of pure cytochrome *c* peroxidase was 0.5–2 μmol/kg.

A problem of variable magnitude has been the co-purification of a proteinase activity with the peroxidase. This contaminant is not evident from protein staining of purified peroxidase after gel electrophoresis but appears to be activated in the presence of SDS. Preparation of purified peroxidase for SDS/polyacrylamide-gel electrophoresis by incubation at 40°C for 30 min in 1% (w/v) SDS sometimes resulted in multiple degradation products of mol.wt. 10000–30000. If incubation was at 100°C for 2 min no degradation was observed. By comparing the self-digestibility of fractions from the Sephadex G-75 separation it was found that the proteinase was eluted just before, but incompletely separated from, the peroxidase peak. For this reason particular care was taken in combining fractions from this final chromatographic step.

Amino acid analysis

Protein samples (1–5 nmol) were hydrolysed in 6 M-HCl containing 0.03% (v/v) thioglycolic acid, and the amino acids were analysed by the method of Spackman (1963) on a Locarte analyser.

Polyacrylamide-gel electrophoresis

The method of Weber & Osborn (1969) was used in gel cylinders, 10% with respect to acrylamide and 0.23% with respect to methylenebisacrylamide. After electrophoresis in 0.1 M-sodium phosphate buffer, pH 7.0, containing 0.1% SDS, the gels were scanned at 410 nm (Gilford instrument) and then stained with Naphthalene Black.

Spectrophotometry

Spectra were recorded on a Pye Unicam SP. 1800 spectrophotometer with AR25 recorder. For redox potentiometry and reduction of the cross-linked cytochrome by ascorbate, NADH-cytochrome *c* reductase or *Crithidia* cytochrome *c*-557, an anaerobic cuvette was used that could be stirred and supplied with an argon atmosphere. The apparatus

was able to accept a combination platinum–Ag/AgCl electrode (Russell pH Ltd., Auchtermuchty, Fife, Scotland, U.K.) for measurement of redox potential.

Pyridine haemochrome

A sample of complex was made 90% (v/v) with respect to acetone containing 60 mM-HCl, left for 15 min and then centrifuged at 5000 *g* for 10 min. The supernatant, containing protohaem IX, was removed and dried under argon. The pellet and the dried supernatant were dissolved in 2 M-pyridine/0.2 M-NaOH, and the spectra were recorded between 530 and 570 nm and again after adding a few grains of Na₂S₂O₄.

The haem *c* concentration of the redissolved pellet was obtained by using $\epsilon_{550(\text{red.})} = 29.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and the concentration of protohaem IX in the acid/acetone extract by using $\epsilon_{557(\text{red.})} = 34.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Morton, 1958).

Determination of lysine

Protohaem IX was extracted from the cross-linked complex by addition of 4.5 ml of 60 mM-HCl in acetone to 10 nmol of the complex in 0.5 ml. The precipitated protein was recovered by centrifugation at 2000 *g* for 10 min and was redissolved in 0.25 ml of 0.12 M-NaHCO₃, pH 8.5, containing 0.2% SDS. The method of Pettigrew *et al.* (1976), based on that of Habeeb (1966), with trinitrobenzenesulphonate was used to determine the concentration of free lysine residues. The protein concentration was determined from the pyridine ferrohaemochrome spectrum or from parallel samples for amino acid analysis.

Peroxidase assay

The standard conditions described by Yonetani & Ray (1965) were used, and the reaction was initiated by adding the cross-linked complex or the H₂O₂. The cuvette contained 0.1 M-sodium phosphate buffer, pH 7.0, 1 mM-EDTA, 20 μ M reduced cytochrome *c*, 180 μ M-H₂O₂ and 3 nM-peroxidase or cross-linked complex in a total volume of 1 ml.

*Reduction of the cross-linked cytochrome *c* by NADH–cytochrome *c* reductase*

The conditions described by Hatefi & Stiggall (1978) were used, but under anaerobic conditions. The reaction was started by the addition of 75 μ g of the enzyme. The cuvette contained 0.2 M-potassium phosphate buffer, pH 8.0, 1.7 mM-NaN₃, 10 mM-NADH and 5 μ M-cytochrome *c* (either free or as the cross-linked complex) in a total volume of 3 ml, and was bubbled for 10 min with argon before the addition of the enzyme.

Results

Conditions for formation of complex

In order to determine reaction conditions suitable for formation of the complex in good yield, experiments were performed on a scale suitable for loading on to a gel for electrophoresis in the presence of SDS. Under these conditions the haem moiety of cytochrome *c* peroxidase is lost and therefore any bands on the gel that absorb light at 410 nm will be due to cytochrome *c*. Thus the presence of bands of higher molecular weight than the peroxidase can be detected, and the presence or absence of cytochrome *c* in these bands can be ascertained. The standard reaction conditions used were horse cytochrome *c* (3 nmol) and cytochrome *c* peroxidase (3 nmol) in 50 μ l of 5 mM-sodium phosphate buffer, pH 7.0. To this was added 5 μ l of fresh 4 mM-dithiobis-succinimidylpropionate in dimethyl sulphoxide. After incubation at 22–23°C for 10 min, 5 μ l of 1 M-ammonium acetate, pH 8.5, was added followed by 10 μ l of 0.6 mM-*N*-ethylmaleimide. Then 10 μ l of 10% SDS was added and the solution was boiled for 2 min. A 50 μ l portion of this solution (approx. 2 nmol of cytochrome *c*) was applied to the top of a gel.

Under these conditions the relative proportions of reagents shown in Table 1 apply. Thus there is enough dithiobis-succinimidylpropionate present to react with 33% of the lysine residues (assuming that an individual molecule of the reagent can always react with two lysine residues on either the same or different protein molecules). A gel separation obtained after these reaction conditions is shown in Fig. 1(b). The molecular weight of the band that appears as a result of treatment with dithiobis-succinimidylpropionate is 48 000 with reference to the mobilities of horse cytochrome *c* (*M_r* 12 000), cytochrome *c* peroxidase (*M_r* 34 000) and ovalbumin (*M_r* 43 000). This band contains cytochrome *c* (Fig. 1a). The band is therefore consistent with the formation of a 1:1 cross-linked complex between cytochrome *c* and cytochrome *c* peroxidase (predicted molecular weight 46 000) in approx. 10% yield.

Effect of varying the conditions on the formation of complex

Use of half as much dithiobis-succinimidylpropionate halved the amount of complex produced, but doubling the amount gave no increase in complex.

If the phosphate buffer was replaced with 20 mM-triethanolamine/HCl buffer, pH 8.0, a similar amount of the 1:1 complex was observed, but the gel electrophoresis also showed the presence of a second cross-linked band of molecular weight 59 000, indicative of a complex containing 2 molecules of cytochrome *c* and 1 of the peroxidase (predicted molecular weight 58 000). Small amounts of this

Table 1. *Conditions for cross-linking cytochrome c and cytochrome c peroxidase*

	Concn. in reaction mixture
Horse cytochrome <i>c</i>	54 μ M
Yeast cytochrome <i>c</i> peroxidase	54 μ M
Sodium phosphate buffer, pH 7.0	5 mM
Dithiobis-succinimidylpropionate	360 μ M
Dimethyl sulphoxide	9% (v/v)
Lysine residues of cytochrome <i>c</i>	1.03 mM
Lysine residues of cytochrome <i>c</i> peroxidase	1.14 mM

Fig. 1. *Detection of a cross-linked complex between cytochrome c and cytochrome c peroxidase*

Cross-linking and gel electrophoresis were performed as described in the Materials and methods section. The two gels contained 2 nmol each of cytochrome *c* and cytochrome *c* peroxidase, for gel (b) after exposure to cross-linking conditions and for gel (c) without treatment with the cross-linking reagent. The scan (a) is at 410 nm for gel (b) before the staining with Naphthalene Black.

species were also obtained in the presence of phosphate buffer if the molar ratio of cytochrome *c* to the peroxidase was raised to 2:1.

If oxidized cytochrome *c* was replaced with reduced cytochrome *c* no increase in the yield of complex was observed. Reaction of cytochrome *c* with dithiobis-succinimidylpropionate before addition of the peroxidase resulted in no formation of the cross-linked complex.

Formation of complex between cytochrome c and ovalbumin

Cytochrome *c* becomes covalently linked to ovalbumin in the presence of dithiobis-succinimidylpropionate. The yield is similar to that observed for cytochrome *c* and cytochrome *c* peroxidase (Fig. 2). Unlike the case of cytochrome *c* and cytochrome *c* peroxidase, no non-covalent complex was detected

when cytochrome *c* was mixed with ovalbumin and passed down a Sephadex G-57 column equilibrated in 5 mM-sodium phosphate buffer, pH 7.0.

If the cytochrome *c* cross-linking reaction was performed in the presence of both ovalbumin and cytochrome *c* peroxidase, the complex with the peroxidase is the favoured product (Figs. 2a and 2c).

Inhibition of the formation of complex

When the reaction was performed in 0.2 M-sodium phosphate buffer, pH 7.0, cross-linking was less extensive.

In the presence of 300 μ M-polyglutamate cross-linking was also inhibited and cytochrome *c* dimer was detected.

Purification of complex

With the use of the reaction concentrations given in Table 1 the cross-linking was performed on 500 nmol each of cytochrome *c* and cytochrome *c* peroxidase. The reaction was stopped after 10 min by addition of 0.83 ml of 1 M-ammonium acetate and the reaction mixture was passed through a Sephadex G-75 column (3 cm diam. $\times$ 90 cm) equilibrated in 5 mM-sodium phosphate buffer, pH 7.0, at 4°C. Selected fractions (0.2 ml) were subjected to SDS/polyacrylamide-gel electrophoresis, and the 1:1 complex was found to be associated with the leading edge of the peroxidase peak but was not separated from it. The entire peak was combined and the solution was passed through a DEAE-cellulose (Whatman DE-52) column (1.5 cm diam. $\times$ 10 cm) equilibrated in 5 mM-sodium phosphate buffer, pH 7.0. The column was washed with 50 ml of 50 mM-sodium phosphate buffer, pH 7.0, and then developed with a linear gradient of 50–250 mM (600 ml total) of the same phosphate buffer. The absorbance at 410 nm of the eluted fractions and the SDS/polyacrylamide-gel electrophoresis of selected fractions (0.2 ml) are shown in Fig. 3. The reduced and oxidized spectra of fraction 35 showed the presence of both the peroxidase and cytochrome *c*, whereas those of fraction 80 showed only the peroxidase. Fractions were combined on the basis of

Fig. 2. Detection of a cross-linked complex between cytochrome *c* and ovalbumin

Cross-linking conditions and gel electrophoresis were as described in the Materials and methods section. Gels were loaded with the following mixtures: (b) 2 nmol each of ovalbumin, cytochrome *c* peroxidase and cytochrome *c* with no cross-linking; (c) as for (b) but exposed to cross-linking conditions; (d) 2 nmol each of cytochrome *c* peroxidase and cytochrome *c* exposed to cross-linking conditions; (e) 2 nmol each of ovalbumin and cytochrome *c* exposed to cross-linking conditions. (a) is a scan of absorbance at 410 nm before the staining of gel (c).

Fig. 3. Chromatography of the cross-linked complex on DEAE-cellulose

Fractions from the Sephadex G-75 elution that contained the cross-linked complex were combined and passed through DEAE-cellulose as described in the Results section. After elution of the adsorbed proteins with a phosphate gradient, portions (0.3 ml) of selected fractions were taken for SDS/polyacrylamide-gel electrophoresis. On the basis of these electrophoresis results, fractions containing only the cross-linked complex were combined (in this case fractions 15–55). The fractions containing both cross-linked complex and cytochrome *c* peroxidase were combined, and the DEAE-cellulose chromatography was repeated. No separation was achieved, indicating that more than one ionic form of the cross-linked complex is present in the reaction mixture and not all can be separated from the peroxidase by the ion-exchange chromatography employed.

the gel electrophoresis (15–58), and the combined volume was diluted with 4 vol. of water and passed through a small DEAE-cellulose (Whatman DE-52) column (1.5 cm diam. $\times$ 1.5 cm) equilibrated in 5 mM-sodium phosphate buffer, pH 7.0. The complex was removed from the column with 0.5 M-sodium phosphate buffer, pH 7.0, and then passed through a Sephadex G-25 (fine grade) column (1.5 cm diam. $\times$ 1.5 cm) equilibrated in 5 mM-sodium phosphate buffer, pH 7.0. It was stored at -20°C in this buffer.

Properties of the purified complex

Purity and reversibility. SDS/polyacrylamide-gel electrophoresis of the purified cross-linked complex is shown in Fig. 4. After staining with Naphthalene Black, minor contaminants with mobilities that correspond to the peroxidase, cytochrome *c* dimer and cytochrome *c* are observed, each of which probably constitutes less than 2% of the protein present (Fig. 4). Some of this apparent contamination may be due to the intrinsic instability of the cross-linking agent under the conditions used for

preparation of the sample (Wang & Richards, 1974).

The cross-linked complex is almost completely dissociated by incubation with 0.03 M-dithiothreitol in 1% (w/v) SDS (Fig. 4).

Fig. 4. Purity of the cross-linked complex and dis-association with dithiothreitol

SDS/polyacrylamide-gel electrophoresis of the purified cross-linked complex (1.4 nmol) was performed as described in the Materials and methods section, and the gel was stained with Naphthalene Black (scan b). A second portion of the cross-linked complex was exposed to 30 mM-dithiothreitol before SDS/polyacrylamide-gel electrophoresis and staining (scan c). Scan (a) shows the separation of a standard protein mixture containing 1 nmol each of ovalbumin (1), cytochrome *c* peroxidase (2) and cytochrome *c* (3). Because of the higher absorbance at 610 nm of the stained bands in comparison with that at 410 nm for the unstained proteins, the background noise is much less than in Fig. 1, and the small peaks represent clearly visible bands.

Spectra. The spectra of the oxidized and reduced forms of the complex closely resemble those of an equimolar mixture of cytochrome *c* and cytochrome *c* peroxidase. By using the isosbestic points of 421 nm

and 431.5 nm for cytochrome *c* peroxidase and cytochrome *c* respectively, the concentration of each in a mixture can be calculated ($\epsilon_{\text{Cyt. } c, 421(\text{red.}-\text{ox.})} = 42 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; $\epsilon_{\text{Cyt. } c \text{ peroxidase}, 431.5(\text{red.}-\text{ox.})} = 51 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

By using this method on three different preparations of the cross-linked complex, values of 0.76–0.78 mol of the peroxidase/mol of cytochrome *c* were obtained. A parallel analysis on an equimolar mixture of cytochrome *c* and cytochrome *c* peroxidase gave a molar ratio of 0.91:1.00.

If the protohaem IX moiety of the complex was extracted into acidified acetone followed by determination of the haem *c* and protohaem IX by their pyridine ferrohaemochromes, a value of 0.6 mol of the peroxidase/mol of cytochrome *c* was obtained. A similar determination on an equimolar mixture of the two proteins gave a value for this ratio of 0.83:1.00.

Amino acid analysis. The amino acid composition of the purified cross-linked complex showed good agreement with that of an equimolar mixture of the two components. No value for an amino acid differed by more than 13%, and most values were within 8% of each other.

Extent of lysine modification. Values of 34 and 36 mol of free lysine residues/mol of protein were obtained after treatment of two preparations of the cross-linked complex with trinitrobenzenesulphonate. An equimolar mixture of cytochrome *c* and cytochrome *c* peroxidase incorporated 39 mol of trinitrophenyl groups/mol. This is in good agreement with the theoretical value of 40 mol of lysine residues/mol, and indicates that only four to six lysine residues in the complex have been modified by the cross-linking agent. If in each case the bifunctional imido ester has modified two lysine residues, only two or three cross-links (inter- and intra-molecular) will be present in the complex.

Reducibility of cytochrome *c* in the cross-linked complex. The cytochrome *c* partner of the cross-linked complex could not be reduced by ascorbate under aerobic conditions, but when oxygen was removed ascorbate completely reduced the cytochrome in a pseudo-first-order reaction. The half-times for ascorbate reduction at two ionic strengths compared with those for free cytochrome are shown in Table 2. SDS/polyacrylamide-gel electrophoresis of the reduced product showed that ascorbate had not cleaved the disulphide cross-link.

The reducibility of the cytochrome *c* by NADH-cytochrome *c* reductase was also measured, and this again had to be conducted anaerobically. In aerobic conditions this reaction has been shown to proceed with complex kinetics involving a zero-order portion followed by a first-order portion (Errede, 1976). We found that under anaerobic conditions the progress curve could be analysed as a single pseudo-first-order reaction for both free cytochrome *c* and

Table 2. Rate of reduction of cytochrome *c* (a) by ascorbate and (b) by NADH–cytochrome *c* reductase
(a) The cuvette contained 1 mM-EDTA, 1 mM-sodium phosphate buffer, pH 7.0, and 4 μ M-cytochrome *c* or cross-linked complex in a total volume of 3 ml. The concentration of NaCl was either 10 mM or 0.2 M. The cuvette contents were stirred for 10 min with argon bubbling, and then 10 μ l of 0.1 M-sodium ascorbate was added. (b) The cuvette contained 0.2 M-potassium phosphate buffer, pH 8.0, 1.7 mM-Na₂S₂O₃, 5 μ M-cytochrome *c* or cross-linked complex and 10 mM-NADH in a total volume of 3 ml. The cuvette contents were stirred for 10 min with argon bubbling, and then 5 μ l of a 15 mg/ml solution of NADH–cytochrome *c* reductase was added.

	$t_{1/2}$ for reduction rate (s)		
	With ascorbate		With NADH– cytochrome <i>c</i> reductase
	10 mM-NaCl	0.2 M-NaCl	
Horse cytochrome <i>c</i> , free	20	72	26
Horse cytochrome <i>c</i> , in cross-linked complex	300	140	1080

Fig. 5. Reduction of the cytochrome *c* cross-linked to cytochrome *c* peroxidase by *Crithidia* cytochrome *c*-557
(a) Absorption spectra of the reduced and oxidized forms of horse cytochrome *c* (—) and *Crithidia* cytochrome *c*-557 (---). The isosbestic points at 557 nm for the former and at 546 nm for the latter are indicated by arrows.
(b) A 2.8 ml sample of the cross-linked complex (4 μ M) in 1 mM-EDTA/2 mM-sodium phosphate buffer, pH 7.0, was made anaerobic by bubbling with argon for 10 min. Then 0.2 ml of reduced *Crithidia* cytochrome *c*-557 (to give a final concentration of 4 μ M) was added and the spectrum was recorded (curve B). The combined spectrum of the two fully oxidized components was calculated and is shown as a broken line (curve C). Full reduction was achieved by addition of 10 μ l of 100 mM-sodium ascorbate (curve A). The arrows indicate the isosbestic points at 546 nm for cytochrome *c*-557 alone and at 557 nm for cross-linked complex alone. From absorbance values at these wavelengths the midpoint potential of the cytochrome *c* cross-linked to the peroxidase can be calculated.

Fig. 6. Reduction by ascorbate of the cytochrome *c* cross-linked to cytochrome *c* peroxidase followed by re-oxidation by H_2O_2

The anaerobic cuvette contained 1 mM-sodium phosphate buffer, pH 7.0, 10 mM-NaCl, 1 mM-EDTA and 5 μM cross-linked complex. After the mixture had been bubbled with argon for 10 min, 10 μl of 100 mM-ascorbate was added (A). After complete reduction was achieved ($t_{1/2}$ 5 min), 3 μl of 4.5 mM- H_2O_2 was added (B), and then a further 3 μl (C). These additions gave final concentrations in the cuvette of 4.5 μM and 9 μM . An 80% oxidation of the cytochrome *c* was achieved with the first addition of H_2O_2 and a further 7% oxidation with the second addition. Further additions did not oxidize the cytochrome completely (not shown). After the H_2O_2 has been used up, re-reduction of the cytochrome by excess ascorbate becomes evident.

cytochrome *c* bound to the peroxidase, and the corresponding half-times for these reactions are shown in Table 2.

The cytochrome *c* bound to the peroxidase could also be reduced by free cytochrome *c*. This could not easily be shown with free horse cytochrome *c*, but *Crithidia* cytochrome *c*-557 is sufficiently different in spectrum to allow analysis of the mixed spectrum obtained after addition of reduced *Crithidia* cytochrome *c*-557 to the oxidized complex under anaerobic conditions. The reaction was too fast to follow with the method used, and gave rise to an equilibrium (Fig. 5) from which the mid-point potential of the cross-linked cytochrome *c* could be calculated.

This value was confirmed by a conventional redox titration in Fe^{3+} /EDTA with Fe^{2+} as a titrant. These methods gave a value of the mid-point potential at pH 7.0 of 243 mV, only 20 mV lower than that for free horse cytochrome *c* measured by the same two methods.

Peroxidase activity of the cross-linked complex. The complex could oxidize free reduced cytochrome *c* in the presence of H_2O_2 at a rate approx. 40% that of the free peroxidase.

After reduction of the cytochrome *c* by ascorbate under anaerobic conditions, addition of H_2O_2 equimolar with the complex produced very rapid oxidation of the cytochrome at rates too fast to measure in the spectrophotometer. After the H_2O_2

had been used up, the slow reduction by excess ascorbate was once more evident, and the experiment could be repeated after full reduction had again been achieved (Fig. 6). The rapid phase of re-oxidation of the cytochrome by the H_2O_2 involved approx. 80% of the total cytochrome, and this proportion was not increased by addition of more H_2O_2 .

Discussion

Conditions for cross-linking cytochrome *c* and cytochrome *c* peroxidase

Conditions were investigated, based on those described by Briggs & Capaldi (1977), which would introduce the minimum of ionic complexity into the cross-linked product of a reaction involving modification of lysine residues, and the maximum yield. The conditions selected result in the isolation of a complex in 10% yield in which four to six of the 40 lysine residues have been modified. Raising the concentration of dithiobis-succinimidylpropionate does not improve the yield of cross-linking, probably because modification of a free cytochrome on its binding surface for the peroxidase inhibits formation of the complex. This is supported by the fact that prior modification of cytochrome *c* with dithiobis-succinimidylpropionate followed by addition of the peroxidase produces no covalent complex.

The interaction between cytochrome *c* and cytochrome *c* peroxidase is known to be electrostatic in nature and inhibited by high salt concentrations and the presence of polyglutamate (Yonetani, 1976; Mochan, 1970). This is borne out by the inhibition of cross-linking under these conditions.

Cross-linking ovalbumin and cytochrome c

Ovalbumin has the same number of lysine residues as cytochrome *c* peroxidase and a similar isoelectric point. It does not, however, form a complex with cytochrome *c* that is detectable after molecular-exclusion chromatography on Sephadex G-75, nor does it protect cytochrome *c* from lysine-residue modifications (Pettigrew, 1978). It is somewhat surprising therefore to find that ovalbumin is as productive as cytochrome *c* peroxidase in forming a cross-linked complex with cytochrome *c*. This result must provoke caution in the interpretation of specific binding sites on the peroxidase and cytochrome revealed by this type of modification.

In the presence of both ovalbumin and the peroxidase, however, cross-linking of cytochrome *c* with the latter is clearly preferred (Fig. 2), and no appearance of a complex including all three proteins was detected.

Purification of cross-linked complex

The major cross-linked product is a species of molecular weight 48000 that can be purified from unreacted cytochrome *c* and cytochrome *c* peroxidase by molecular-exclusion chromatography on Sephadex G-75 and ion-exchange chromatography on DEAE-cellulose. This molecular weight, the reversal of cross-linking to yield the peroxidase and cytochrome *c* and the amino acid analysis are consistent with this being a 1:1 complex of cytochrome *c* and cytochrome *c* peroxidase. However, the spectroscopic analysis and the quantitative determination of haem *c* and protohaem IX by formation of pyridine ferrohaemochrome suggest that some protohaem IX has been lost from the peroxidase during the reaction.

We do not claim that the isolated complex is a single ionic species. Indeed, electrophoresis of the complex under non-denaturing conditions produces a very broad diffuse band, indicating ionic complexity. This is to be expected because of varying extents of lysine-residue modification and will introduce complexity into an analysis of cross-linked peptides.

Properties of the cross-linked complex

We can pose three main questions about the ability of the cross-linked complex to perform electron transfer. Can the cross-linked cytochrome *c* be reduced? Is the cross-linked peroxidase still

active? And is the cross-linked cytochrome *c* competent to donate electrons to the peroxidase?

Unlike the case of free cytochrome *c*, the rate of reduction by ascorbate was greater at higher ionic strength, and the rate approached that of free cytochrome *c*. This suggests that the ascorbate site on the cytochrome is relatively inaccessible in the cross-linked complex at low ionic strength, but that salt disrupts electrostatic linkages and allows access of the ascorbate. The reduction by ascorbate under all conditions followed a simple first-order progress curve, suggesting that only one orientation of the cytochrome on the peroxidase may be present.

This was also so for reduction by NADH–cytochrome *c* reductase, but the rates were much lower than for free cytochrome *c* and were not increased by high ionic strength. We conclude that access to the site of reduction on the cross-linked cytochrome by this large multienzyme complex is much more restricted than for ascorbate.

In view of this it is noteworthy that reduction of the cross-linked cytochrome *c* by free cytochrome *c* proceeds very quickly. We have not measured the rate of this reduction, since it is essentially over before it can be observed in the spectrophotometer. But the speed of reduction in comparison with the results obtained for ascorbate and NADH–cytochrome *c* reductase is perhaps surprising, and suggests that an alternative route of electron entry to the cytochrome *c* haem may exist.

A major concern in the present work has been the question of whether cytochrome *c* can be reduced by more than one route. A large body of evidence has suggested that oxidases and reductases share a common binding site on cytochrome *c* that encloses the exposed haem edge (reviewed by Rieder & Bosshard, 1980), but Hill & Nicholls (1980) have presented evidence that cytochrome *c* in a non-covalent complex with cytochrome *c* oxidase can still accept electrons from *NNN'*-tetramethyl-*p*-phenylenediamine, but not from ascorbate, implying that separate routes of electron transfer within the cytochrome *c* molecule do exist. Our results showing reduction of the cross-linked complex with free cytochrome *c* support this conclusion.

The mid-point potential of a cytochrome is an indicator of modification of the haem environment, and the fact that the midpoint potential of the cross-linked cytochrome *c* is only 20 mV lower than that of free cytochrome *c* suggests that the cross-linking process has not greatly altered the cytochrome. However, the autoxidizability is not a property of the free cytochrome. This could be due to an oxidase action by the attached peroxidase in the absence of H_2O_2 , or an alternative is that the haem crevice of the cytochrome is opened slightly on binding to the peroxidase and that this allows attack by O_2 . The latter alternative would imply a

conformational change on binding of the cytochrome to the peroxidase.

The peroxidase activity of the cross-linked complex is 40% that of the free peroxidase, in contrast with the 10% obtained by Waldmeyer *et al.* (1980) for their isolated complex. The discrepancy may be due to the lower ionic strength of their reaction mixture, which would tend to favour tight binding of the interaction areas of the cytochrome and the peroxidase in the cross-linked complex. In the peroxidase assay we do not know whether the free cytochrome is donating electrons directly to the peroxidase or via the cross-linked cytochrome. We can, however, say that, in the absence of free reduced cytochrome *c*, the cross-linked cytochrome is able to donate electrons to the associated peroxidase in a fast reaction. Approx. 80% of the cross-linked cytochrome *c* can be oxidized in this way, and we propose that this corresponds to the 80% of the molecules of the cross-linked complex that were found to contain protohaem IX. This suggests that most of the cytochrome *c* in the cross-linked complex is correctly orientated for electron transfer to the peroxidase, and our expectation is that cross-linking has simply stabilized a 'front face' binding of the cytochrome on the peroxidase. This is the type of binding that was established by chemical-modification studies on the non-covalently linked complex (Pettigrew, 1978; Kang *et al.*, 1978).

The complex of cytochrome *c* and cytochrome *c* peroxidase is an attractive model for studying the nature of an electron-transfer reaction. Lewis & Allison (1978) showed that *N*-5-azido-2-nitrobenzylsuccinimide could be used to cross-link the two proteins, and Waldmeyer *et al.* (1980) used dithiobis-succinimidylpropionate under conditions similar to those described in the present paper to isolate a cross-linked complex.

These investigations and the present work showing the isolation of a correctly orientated cross-linked complex between cytochrome *c* and cytochrome *c* peroxidase, competent in many of the electron-transfer processes that have been tested, opens up the possibility of studying an electron-transfer reaction in detail. The complex may be amenable to crystallization and X-ray-diffraction analysis. Establishment of the lysine residues involved in just one cross-link between the cytochrome *c* molecule and the peroxidase, in combination with knowledge of the binding surface of cytochrome *c* (Pettigrew, 1978; Kang *et al.*, 1978), would allow the correct orientation of the known structures of the two proteins to be compared with the model structure proposed by Poulos & Kraut (1980) on the basis of complementary charge interactions. We are hopeful that such an analysis will not be complicated by the presence of a heterogeneous population

of complexes with different orientations of the two partners, because the kinetic results show simple progress curves consistent with the presence of only one type of complex.

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