Chemical Modification of the Haem Propionate of Cytochrome *c*

Russell TIMKOVICH

Department of Chemistry, Illinois Institute of Technology, Chicago, IL 60616, U.S.A.

(Received 29 May 1979)

The significance of the exposed haem edge in cytochrome *c* was directly probed by chemically modifying the partially exposed haem propionate in the crevice region around residues threonine-78 and threonine-49. Reaction of tuna heart cytochrome *c* with a water-soluble carbodi-imide at pH 3.7 in the absence of any added nucleophilic base leads to the covalent addition of substituted *N*-acylureas to the protein at two sites. One site has been shown to be a haem propionate by isotope-tracer and i.r.-spectral analysis on haem purified from the apoprotein. The other site is aspartic acid-62 on the back of the molecule. The modified cytochrome *c* demonstrates abnormal properties, including auto-oxidizability, a reduction potential of +105 mV, a reversible transition to a high-spin species below pH 5.3, no 695 nm charge-transfer band in the ferric state and abnormal binding to mitochondrial membranes. The derivative does react with cytochrome oxidase in deoxy-cholate-treated submitochondrial particles or in purified preparations with a specific activity of 43–65% compared with that obtained with native cytochrome *c*. The results are consistent with the view that an intact haem crevice is essential for normal values for physicochemical characteristics, but the significant residual enzymic activity suggests that the electron-transfer interface and/or the cytochrome oxidase-binding site cannot be localized solely in the region of the exposed haem propionate.

Cytochrome *c* has been the subject of extensive studies because of its essential function as the electron carrier between a reductase (cytochrome *c*₁) and a terminal oxidase (cytochrome *aa*₃) in the O₂ respiration systems of higher organisms. Current proposals for its mechanism of operation have focused on the role of the solvent-exposed haem prosthetic group edge (Salemme *et al.*, 1973; Timkovich & Dickerson, 1976). The most striking structural feature of cytochrome *c* is that the polypeptide folds around the haem so as to bury the iron centre and all but the periphery of pyrrole rings II and III (Dickerson & Timkovich, 1975). The invariant residues histidine-18 and methionine-80 complete the co-ordination sphere of the metal. It may be that the exposed ring structure, residing in what is called the haem-crevice region, is in some sense the 'active site' of cytochrome *c*.

Cytochrome *c* has been the subject of comprehensive chemical modification studies, including extensive probing of the iron inner co-ordination sphere. These have led to a systematic investigation of the contribution of specific functional groups towards cytochrome *c* structure and reactivity. A thorough literature review of cytochrome *c* chemical modifications has been given (Ferguson-Miller *et al.*, 1979). The following examples give a perspective on research on modifications near the haem edge. The ligand histidine-18 has been modified by Methylene Blue oxidation (Nakatani, 1960) or by diazonium tetrazole reaction (Horinishi *et al.*, 1965). Methionine-

80 has been chemically modified by oxidation (Ivanetich *et al.*, 1976a) and carboxymethylation (Schejter & Aviram, 1970; Margoliash *et al.*, 1973). There is disagreement as to whether the modified residue is retained as an axial ligand (Ivanetich *et al.*, 1976a; Schejter & Aviram, 1970) and as to the enzymic activity of this modified form (Margoliash *et al.*, 1973; Ivanetich *et al.*, 1976a; Schejter & Aviram, 1970). Although the formation of derivatives clearly affects the neighbouring geometry of the haem crevice, it more directly probes the significance of the axial ligands. Studies of the iron-depleted porphyrin-cytochrome *c* (Fisher *et al.*, 1973; Vanderkooi & Erecinska, 1975) and a cobalt haem (Dickinson & Chien, 1975) have accented the role of the iron itself. Lysine residues ring the haem-crevice region in what is now recognized as a characteristic structural feature of cytochrome *c*. Chemical modifications of these residues (Margoliash *et al.*, 1973; Wada & Okunuki, 1969; Aviram & Schejter, 1973; Ferguson-Miller *et al.*, 1978; Staudenmayer *et al.*, 1977) are revealing their role in the protein-interaction specificity of cytochrome *c* towards its oxidase and reductase. The haem ring itself has yet to undergo scrutiny by chemical modification. Probing the haem edge itself would provide direct information on its relation to the conformation of the molecule and on a possible role in determining specificity towards cytochrome *c* oxidase and reductase interactions.

The present paper describes the selective chemical

modification of cytochrome *c* from tuna heart muscle at two sites. One is the haem propionate side chain of the partially solvent-exposed pyrrole ring III, and thus is effectively a modification of the exposed haem ring itself. The other main site is aspartic acid-62 on what is traditionally called the bottom back of cytochrome *c*.

Modification has been accomplished through use of the water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide. This reagent reacts with carboxylate groups to form *O*-acylisoureas, which are highly labile towards attack by nucleophiles. If the nucleophile is an amine, the carbodi-imide backbone is displaced as an inert urea with the formation of an amide bond. However, water itself is a sufficient nucleophile to regenerate carboxylic acid plus the now inert urea. The present study has utilized a second reaction pathway available to the initially formed *O*-acylisourea. If there is no added strong nucleophile and/or if the *O*-acylisourea is sterically blocked towards solvolysis, it undergoes slow internal rearrangement to a stable *N*-acylurea, with the net addition of the carbodi-imide skeleton to the carboxylate through an amide bond. Further details of this reactivity behaviour have been described (Timkovich, 1977a).

This potential for chemical modification of proteins by carbodi-imide itself was first used by Riehm & Scheraga (1966), although at the time there was uncertainty as to the exact chemical reaction accompanying modification. Most chemical modifications promoted by carbodi-imides have instead relied upon their activating properties towards nucleophilic attack by other reagents. The present report demonstrates the type of derivative formation obtainable by the internal rearrangement pathway. The approach has also been used for selective modification of an acidic side chain in carboxypeptidase A (Riordan & Hayashida, 1970).

Experimental

Materials

Cytochrome *c* was prepared from albacore-tuna (*Thunnus alalunga*) heart muscle by the method of Margoliash & Walasek (1967) and crystallized as described by Takano *et al.* (1973) or purchased as a freeze-dried powder from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and rechromatographed on Sephadex G-75. Before use samples were dialysed to remove potential inhibitory ions (Smith *et al.*, 1973). Non-labelled 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide hydrochloride was purchased from Calbiochem (Los Angeles, CA, U.S.A.), and the ^{14}C -labelled form was prepared as described previously (Timkovich, 1977a). Mitochondria (Low & Vallin, 1963), electron-transport particles (Lee & Ernster, 1967) and cytochrome *c*-depleted mito-

chondria (Jacob & Sanadi, 1960) were prepared by standard procedures. Solubilized cytochrome oxidase was prepared by the method of Hartzell & Beinert (1974). Chromatography resins were supplied by Bio-Rad Laboratories (Richmond, CA, U.S.A.) and Pharmacia (Uppsala, Sweden). All other materials were reagent grade.

Determination of cytochrome *c* and derivatives

Native cytochrome *c* concentrations were determined from the 550 nm band of the reduced protein by using an absorption coefficient of $27.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The derivative was determined by its pyridine haemochromogen spectrum (Bartsch, 1971), iron content (Cameron, 1965) or amino acid content, the results obtained with all methods being in agreement. The determination of incorporated carbodi-imide has been described in detail (Timkovich, 1977a).

Structural chemistry

Isoelectric migration in 7.5% gels containing Bio-Rad Biolytes (pH range 8–10), Brinkman Phisolytes or LKB Ampholines (pH 8–10) plus the free base arginine (1.4 mg/ml) was allowed to proceed towards focusing equilibrium, but stopped after maximum resolution of components. This technique (L. Smith & G. McLain, personal communication) eliminated some of the severe end-of-gel effects that occur during the focusing of very basic cytochromes. Enzymic digestions of native and derivative cytochrome *c* with either trypsin alone or trypsin followed by chymotrypsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) were patterned after those used for the original sequence determination of tuna heart cytochrome *c* (Kreil, 1963). Pepsin digestion utilized 3.75 mg of freeze-dried cytochrome *c*/ml of aq. 5% formic acid containing pepsin at 0.1 mg/ml for 3 h. Reaction was terminated by addition to a Dowex 50 ion-exchange column. Peptide fractionation, peptide 'mapping' and amino acid analysis were performed by contemporary techniques (Timkovich *et al.*, 1976).

Activity assays

Cytochrome oxidase activity measurements were patterned after the spectroscopic assay method of Smith and co-workers (Smith & Conrad, 1956; Smith *et al.*, 1974, 1976). Control experiments consisted of concurrent activity measurements on native tuna cytochrome *c* under identical conditions. Because of auto-oxidizability of the reduced derivative, the following protocol was adopted. Cytochrome *c* in buffer composed of 50 mM-Tris and 50 mM-cacodylic acid, pH 7.2, was reduced anaerobically by adding 10 μl of a fresh NaBH_4 solution

(40mg/ml of water) into a solution that had been deoxygenated by repeated purging with vacuum and argon. The solution was allowed to stand with occasional degassing for 1h, during which excess borohydride decomposed. On another arm of the cuvette, a stopcock-valved reservoir was filled with an equivalent volume of air-saturated buffer containing the preparation of either mitochondrial membrane fragments or purified cytochrome oxidase. Reaction was initiated by quickly opening and then closing the stopcock, causing the pressure difference to mix the solutions efficiently. Auto-oxidation rates for the derivative were determined in the same manner, with cytochrome oxidase absent, and subtracted from those for the enzyme-catalysed reactions. This protocol and the auto-oxidation of the derivative restrict the concentration range for the assays to 0.5–30 μ M-cytochrome *c*. Kinetic data were analysed as described by Errede and co-workers (Errede *et al.*, 1976; Errede & Kamen, 1978). The binding assays reported below followed the procedure of Vankerkooy *et al.* (1973a), with the substitution of freshly prepared bovine mitochondria for pigeon mitochondria.

Physical studies

Formal reduction potentials were determined by redox buffer-mediated titrations by the methods of Brandt *et al.* (1966) with ferricyanide/ferrocyanide and Davenport & Hill (1952) with ferric/ferrous EDTA. Visible-u.v.-absorption spectra were measured on a Cary 15 spectrometer and c.d. spectra on a Cary 60 spectropolarimeter.

Removal of haem

The covalently attached haem was removed for some experiments by a modification of the Ag_2SO_4 method (Paul, 1951) in which BaCl_2 was used to precipitate gravimetrically excess reagent after cleavage. Other haem-removal procedures (Ambler, 1963) proved to be inapplicable because of the acquired zwitterionic character of the derivative haem.

Reaction of cytochrome *c* and carbodi-imide

Cytochrome *c* solutions were made 0.4mM in distilled water either by dissolving freeze-dried protein or diluting dissolved dialysed crystals, and the pH was adjusted to 3.7 with 1M-HCl. Solid carbodi-imide (30mg/ μ mol of protein) was added with stirring, with constant monitoring of pH. A rapid initial rise in pH of about 1 unit occurred, owing to proton uptake on formation of active *O*-acylisourea adducts and to solvent interaction. The pH was back-titrated to 3.7 with 1M-HCl. Over a span of 2h the pH drifted downward, owing to proton release on hydrolysis or rearrangement of the *O*-acylisoureas

and to solvent hydrolysis, but was constantly adjusted to 3.7 with 1M-NaOH. After the pH had stabilized for 15min, a second portion of carbodi-imide was added and the process repeated.

As discussed below under 'Physicochemical properties', at this pH the formation of a cytochrome *c* derivative modified at the haem moiety is accompanied by the appearance of a 625nm absorption band as well as incorporation of radioactive label on to the haem. This allows monitoring of the extent of haem modification with time. The kinetics of the reaction are dominated, not by the haem reactivity, but by the largely unproductive turnover of the carbodi-imide as it reacts with acids or solvent to form activated *O*-acylisoureas, which are then hydrolysed to inert urea and regenerate the acidic functions. For purposes of kinetic characterization, it was imperative that no native cytochrome *c* remain, and hence the second addition and incubation were necessary to drive the reaction with the haem moiety to completion.

At the end of the second stage, 4M-sodium acetate, pH3.7, was added to a final concentration of 0.8M to act as a quenching agent for remaining carbodi-imide. After 30min the pH was raised to 7.9 with solid Tris base, and the solution immediately chromatographed on a Sephadex G-75 column (5.0cm $\times$ 100cm) eluted with 0.1M- NH_4HCO_3 . The gel filtration separated a monomeric fraction from covalent polymeric fractions, which have been previously discussed (Timkovich, 1977b). In early experiments the monomer fraction was subjected to chromatography on Bio-Rex 70 (a strong cation-exchanger) or CM-Sephadex C-50 by using NaCl gradients from 0 to 0.6M in sodium phosphate buffer. Both procedures failed to resolve any components. At this stage the extent of incorporated covalent label as measured by the observed ^{14}C radioactivity, the known specific radioactivity of [^{14}C]carbodi-imide and the known amount of protein was 2.2 ± 0.2 mol of label/mol of cytochrome *c*. Gel isoelectric focusing gave two bands. The main one accounted for 80% of applied material and was approx. 0.50pH unit more basic than native cytochrome *c*, and the secondary band accounted for 20% of applied material and was 0.65pH unit more basic than native cytochrome *c*. No native cytochrome *c* was observed in the derivative preparation, and the resolution of bands of native and derivative cytochrome *c* on the gels was confirmed by co-electrofocusing prepared mixtures. From the product stoichiometry and the gel experiments it was concluded that the product was a mixture of two species, with the main component containing two covalently bound *N*-acylurea groups and the minor component containing three.

Partial fractionation of this mixture was achieved by ion-exchange chromatography on CM-Sephadex C-50 eluted with a constant-ionic-strength buffer. Fig. 1 displays a typical chromatogram. The ion-

Fig. 1. Ion-exchange chromatogram of the carbodi-imide derivative of cytochrome *c* on CM-Sephadex

The column (1.5 cm × 35 cm), at 4°C, was eluted at 36 ml/h with constant-ionic-strength buffer composed of 0.15 M-NaCl in 50 mM-sodium 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate adjusted to pH 7.55 with 1 M-HCl. Sample (approx. 20 mg) was loaded in the above buffer not containing NaCl. Fractions of volume 5.5 ml were collected and the absorbance at 410 nm was read. Only the column fractions that contained haem protein are shown. The void volume was approx. 30 ml.

exchange chromatographic properties of cytochrome *c* are largely determined by the preponderance of basic side chains on the molecule. Since the modification affected acidic residues, which do not interact directly with the support, it is perhaps not surprising that the column resolution was incomplete. The fractions were pooled as indicated. Fraction I was found to contain 2 mol of label/mol of cytochrome *c* and was concluded to be the two-site derivative. The kinetic and spectral properties of fractions I and II were compared and found to be indistinguishable. Both components were labelled at the same two main sites, but fraction II was modified at a third site, though the additional site did not seem to impart any significantly unique properties to the molecule.

Additional acidic functions on the molecular surface were also modified by a procedure in which glycine methyl ester, glycine amide or semicarbazide was added as an attacking nucleophile. Cytochrome *c* (0.4 mM) was allowed to react with 0.1 M-1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide and 0.5 M-nucleophile at pH 4.75 for 2 h, followed by quenching with an equivalent volume of 4 M-sodium acetate. The material was dialysed and chromatographed on Sephadex G-75 to remove covalent polymers.

Results

The derivative obtained after reaction of cytochrome *c* with carbodi-imide in the absence of other

nucleophiles is modified at two sites. It is known that in proteins aspartic acid, glutamic acid and, to a much limited extent, cysteine and tyrosine are the only residues reactive with carbodi-imides (Hoare & Koshland, 1967). Tuna cytochrome *c* has no free cysteine residues. Reactions of tyrosine residues may be reversed by hydroxylamine treatment (Carraway & Koshland, 1968), yet such treatment applied to the derivative did not diminish incorporation of radioactivity. Hence only acidic residues were modified in cytochrome *c*. In experiments in which one of the nucleophiles glycine methyl ester, glycine amide or semicarbazide hydrochloride was present, covalent attachment of the carbodi-imide moiety was still observed, in addition to the expected amidation with exposed acidic residues.

Cytochrome *c* that had reacted with glycine methyl ester plus carbodi-imide was analysed for amino acid content. The increase in glycine content shown in Table 1 indicated that three exposed aspartic acid/glutamic acid residues had been modified by the added nucleophile. Tuna cytochrome *c* contains ten potentially reactive acidic functions (four aspartic acid residues plus five glutamic acid residues plus one

Table 1. Amino acid compositions of modified cytochrome *c*

Results are expressed as mol/mol of protein or peptide observed after 24 h hydrolysis in 6 M-HCl, with theoretical integral stoichiometry in parentheses. Values for threonine and serine were not corrected for degradation. Proline and cysteine were not determined. When present in a sample, only small, variable amounts of tryptophan survive the acid hydrolysis. The detection of some tryptophan in the chromatogram is indicated by +.

	Amino acid composition (mol/mol)	
	Derivative obtained after carbodi-imide-promoted nucleophilic attack by glycine methyl ester	Isolated labelled peptide from the carbodi-imide-only derivative
Asp	10.30 (10)	2.79 (3)
Thr	6.86 (7)	1.17 (1)
Ser	3.20 (4)	—
Glu	10.03 (9)	0.02
Gly	15.92 (13)	0.01
Ala	7.21 (7)	—
Val	6.53 (6)	0.89 (1)
Met	1.96 (2)	0.09
Ile	3.97 (4)	0.92 (1)
Leu	6.34 (6)	1.20 (1)
Tyr	4.11 (5)	0.04
Phe	2.59 (3)	0.08
Lys	15.26 (16)	0.09
His	2.09 (2)	0.04
Arg	2.34 (2)	—
Trp	+ (2)	+ (1)

exposed propionate group). Five of these remained unmodified by either carbodi-imide itself or glycine methyl ester. It is possible that the unmodified groups are involved with hydrogen-bonding or ionic interactions with other residues or solvent ions, and so protected from reaction. X-ray-crystallographic analysis has shown that glutamic acid-69 makes a strong hydrogen bond to arginine-91 and that glutamic acid-90 bonds to lysine-13 (Takano *et al.*, 1977). In addition, proximity relations exist for the following pairs, although no bonding was reported: glutamic acid-21 and lysine-25, aspartic acid-50 and lysine-53, and aspartic acid-93 and lysine-5. Aspartic acid-62 is identified below as a carbodi-imide-addition site. The remaining residues appear to have no interacting partners and hence are the prime candidates for nucleophilic addition: aspartic acid-2, glutamic acid-44 and glutamic acid-66. As discussed below, the extensively modified cytochrome *c* was kinetically least interesting, so further evidence for these assignments was not obtained. The glycine addition was repeated in the presence of 7.5M-urea. The product was highly insoluble in the absence of urea and tended to occlude urea and glycine methyl ester. The increase in glycine content was variable and ranged from 13 to 20mol/mol of protein. This presumably reflects modification of all acid residues after loss of structure plus a variable content of occluded glycine methyl ester.

Identification of the attachment sites for the carbodi-imide derivative of cytochrome *c*

One of the haem propionic acid side chains was established as a major modification site by several lines of evidence. Haem isolated from the apoprotein was found to contain 0.98 ± 0.02 mol of [^{14}C]-N-acylurea/mol of haem iron. Haem obtained from the modified cytochrome *c* demonstrated markedly different solubility properties from native haem, in keeping with the acquisition of zwitterionic character due to one of the two carboxylate groups being replaced by a moiety containing a tertiary amine. The derivative haem was not soluble in non-polar organic solvents or extractable from aqueous solutions under conditions that solubilized or extracted native haem. Comparative thin-layer or paper electrophoresis at neutral pH indicated mobilities for the derivative haem and the derivative haem peptide (residues 14–25) consistent with a net positive charge increase. Elemental analysis on haems purified on silica-gel columns by pyridine elution confirmed an increased C and N content over iron for the derivative haem. The i.r. spectrum in Fig. 2 shows the loss from the derivative haem of one of the carboxylate peaks in the 1720cm^{-1} region (Granick, 1961) and the appearance of a strong amide type I absorption.

The second major site of carbodi-imide attachment

Fig. 2. Comparative i.r. spectra of haem isolated from native cytochrome *c* (a) and the carbodi-imide derivative (b). Dried haem (approx. 1mg) was mixed with KBr (250mg) and pressed into a 1.0mm pellet. The peak at 1720cm^{-1} for native cytochrome *c* is an incompletely resolved doublet of carboxylic acid carbonyl groups, as assigned by Granick (1961). In the derivative, this is replaced by the strong amide absorbance at 1650cm^{-1} .

Fig. 3. Ion-exchange chromatogram for the peptic peptides of the carbodi-imide derivative of cytochrome *c* on Dowex 50 (X2; 200–400 mesh)

Chromatography conditions were as follows: $0.9\text{cm} \times 100\text{cm}$ column; 50°C ; flow rate 36ml/h; 3ml/fraction; buffers as indicated. Radioactivity was measured on 0.5ml samples in a Triton/toluene scintillation 'cocktail'; all buffers were pyridine/acetate at the indicated molarity of pyridine; 0.2M, pH 3.1; 2.0M, pH 5.0; 4M, pH 5.6; 8M, pH 5.6.

was identified by peptide fractionation of enzymic hydrolysates of radioactively labelled derivative. Attempts to reproduce in detail the peptide separations and peptide 'maps' described in the original sequence determination by Kreil (1963) were not successful. In part this may be due to some ambiguity in the brief original report of experimental detail or to significant differences in separation media now

commercially available. Tryptic or combined tryptic-chymotryptic digestions of the apoprotein gave chromatograms showing a single ^{14}C -containing fraction. However, ninhydrin analysis of fractions and t.l.c. showed that the radioactive fraction consisted of a mixture of peptides. These proved difficult to resolve by subsequent gel-filtration chromatography or preparative t.l.c. Yields became so low that it was not possible to monitor adequately the ^{14}C content of various fractions. Peptic digestion yielded a different single ^{14}C -labelled fraction, which was shown by t.l.c. to be a single peptide (see Fig. 3). Fractions with radioactivities greater than twice the background were pooled and studied quantitatively by amino acid analysis and scintillation counting. The amino acid composition is given in Table 1 and identifies the peptide as:

The cleavage locations are *N*-terminal to isoleucine-57 and methionine-65, which is consistent with pepsin specificity. The total yield of recovered peptide was 22% on the basis of mol of protein applied to the column and 25% on the basis of recovered net radioactivity. Although somewhat low, this is not atypical for recovery of a major peptide fragment from Dowex 50 (Margoliash & Smith, 1962).

Physicochemical properties

Table 2 summarizes some of the characteristics of the carbodi-imide derivative. Especially noteworthy is the diminution in reduction potential from the usual value of 260 mV. The loss of the 695 nm band in the oxidized form would be traditionally interpreted as indicating loss of the co-ordination of the methionine-80 ligand. In addition, Fig. 4 shows that there is a reversible pH-induced transition, in the oxidized form, from a typical low-spin type of visible-absorption spectrum to a high-spin type with a transition *pK* of 5.3. Such behaviour is also atypical of normal methionine ligation.

Table 2. Characteristics of the carbodi-imide derivative of cytochrome *c*

Absorption maxima	
Reduced	417, 522, 550 nm
Oxidized above pH 5.3	406, 525 nm
Oxidized below pH 5.3	401, 495 (shoulder), 525, 558 (shoulder), 625 nm
Reduction potential, pH 7	105 ± 10 mV
Isoelectric point	11.0–11.2*
Auto-oxidation rate (at O_2 concn. $120 \mu\text{M}$)	$0.2 \times 10^{-2} \text{ s}^{-1}$
Typical reductants	Dithionite, ascorbate, NaBH_4

* Only approximate for reasons discussed in the text.

Fig. 4. Spectral titration for the carbodi-imide derivative of cytochrome *c*

Spectra were obtained on samples that were $25 \mu\text{M}$ for the 525 nm (Δ) and 623 nm curves ($\blacksquare$) and $5 \mu\text{M}$ for the 410 nm curve ($\bullet$). The buffer was a mixture of sodium citrate and sodium phosphate, each 50 mM, adjusted to the lower pH with 1 M-HCl. Titrations proceeded with the addition of NaOH, and all absorbances were corrected for slight dilution. The changes at 525 nm and 410 nm result from band shifts (see Table 2) monitored by absorbance changes at these wavelengths. The 623 nm plot monitors the appearance of a new band characteristic of a low-spin to high-spin transition below pH 5.5. Within experimental error, the three spectral changes occur with equivalent pH behaviour.

The c.d. spectra of native cytochrome *c* and its carbodi-imide derivative are compared in Fig. 5.

Kinetics and binding

The carbodi-imide derivative shows a high reactivity with cytochrome oxidase in the concentration range $0.5\text{--}30 \mu\text{M}$. With cytochrome *c*-depleted bovine mitochondria or submitochondrial electron-transport particles, the derivative demonstrated 45% of the activity of the native cytochrome *c* when activities were compared at $10 \mu\text{M}$ -cytochrome *c*. Various independent preparations of the derivative and cytochrome oxidase gave a range of activities from 43% to 51%. Identical comparisons made with bovine cytochrome oxidase (prepared by the method of Hartzell & Beinert, 1974) gave activities from 63% to 65%.

The specificity of the derivative towards interaction with the mitochondrial membrane was investigated by measuring binding to cytochrome *c*-depleted mitochondria by the methods of Vanderkooi *et al.* (1973a). Fig. 6 compares Scatchard-type plots for the binding of native cytochrome *c* and its derivative. The curve for native cytochrome *c* was a control and essentially reproduced the data of Vanderkooi *et al.*

Fig. 5. *C.d. spectra for native ferricytochrome c (●) and its carbodi-imide derivative (■)*
Samples were in 50mM-sodium phosphate buffer, pH7.2. Path length was constant at 10mm. Concentrations were adjusted so that each sample had an absorbance of 1.0 at the following band maxima: 220nm (a); 280nm (b); 409nm (c). Repetitive spectral scans were obtained and then averaged at the points indicated.

(1973a), although the experiment did not extend as far into the dilute concentration range. It showed the asymptotic approach to a high-affinity curve, which has been interpreted as the binding of two molecules of cytochrome c to two highly specific sites in the cytochrome oxidase complex (Vanderkooi *et al.*, 1973a; Ferguson-Miller *et al.*, 1976). The displacement of the curve for the derivative relative to that for native cytochrome c indicated more heterogeneous binding at multiple sites on the membrane. The concentration range accessible with available equipment was not adequate for conclusions to be drawn

Fig. 6. *Binding of the carbodi-imide derivative of cytochrome c to cytochrome c-depleted bovine heart mitochondria*
Ferricytochrome c was added to centrifuge tubes, in increasing amounts from 0.3 to 25 μM, to suspensions of freshly prepared bovine cytochrome c-depleted mitochondria (3.4mg of total mitochondrial protein/2.55 ml of 50mM-mannitol/50mM-sucrose). After 1 h of incubation, the mitochondria were removed by centrifugation at 8000g and 4°C for 10min and the free cytochrome c concentration of the supernatant was determined from absorbance readings at 409nm and 550nm after dithionite reduction. The curve for native cytochrome c (●) parallels the determination made by Vanderkooi *et al.* (1973a), after which this experiment is patterned. The displacement and curvature of the curve for the carbodi-imide derivative (■) indicate multiple binding sites for that derivative.

Table 3. *Minnaert mechanistic constants for cytochrome c and its carbodi-imide derivative*

The Minnaert mechanistic constants are as defined and discussed by Errede & Kamen (1978), and the notation is consistent with that reference. The complete mechanism is more complex than that shown. However, over the concentration range experimentally open for the carbodi-imide derivative, the observed data reflect only the constants k_1 , k_2 and k_3 . The values for γ and δ refer to reaction with the purified (Hartzell & Beinert, 1974) cytochrome oxidase at pH7.2, in 50mM-Tris/cacodylate buffer.

Relevant mechanistic steps:

Definitions:

$$\begin{aligned} \gamma &= \frac{k_1 k_3}{(k_2 + k_3)} \quad \delta = \frac{k_1}{k_2} \\ &\text{(catalytic)} \quad \text{(binding)} \\ &\text{transfer) } \end{aligned}$$

	$\gamma \text{ (M}^{-1} \cdot \text{s}^{-1})$	$\delta \text{ (M}^{-1})$
Native cytochrome c	8.0×10^6	0.7×10^6
Carbodi-imide derivative	3.2×10^6	0.4×10^6

Fig. 7. Comparison of reaction rates with purified cytochrome oxidase as a function of total cytochrome *c* concentration

All assays were performed at 25°C in 50mM-Tris/cacodylate buffer, pH 7.2, at an effective O₂ concentration of 120–130 μM by the procedure described in the Experimental section. Cytochrome *c* concentrations (●, native cytochrome *c*; ■, carbodi-imide derivative) ranged from 0.5 to 10 μM. Above 2.5 μM reactions were monitored at both 418 nm and 550 nm, but below 2.5 μM only at 418 nm. The cytochrome oxidase concentration (expressed in terms of a unit containing two *a*-type haem moieties and two Cu atoms) was 17.8 nM in all experiments. The modified Minnaert mechanism described by Errede & Kamen (1978) predicts that the observed first-order rate constants will vary as:

$$k = \gamma[aa_3] - \delta k[\text{cyt}]$$

where γ and δ are further defined in the text and in Table 3. The straight lines represent the least-squares fit to the data points.

concerning high-affinity binding to cytochrome oxidase. This was instead approached through kinetic measurements, as described below. However, it was possible to conclude that the derivative interacted with more sites on the mitochondrial membrane than did native cytochrome *c*.

The carbodi-imide derivative retained affinity for the active cytochrome oxidase site or sites, as shown by its ability to act as an inhibitor of the normal reaction between native cytochrome *c* and the mitochondrial cytochrome oxidase. Rates of reaction between mitochondrial cytochrome oxidase and native tuna ferrocytochrome *c* at concentrations between 3 and 12 μM were measured in the presence of various amounts of the derivative, and the data were analysed by Dixon plots (Bergmeyer, 1978) to give a co-operative inhibition constant of $2 \pm 1 \mu\text{M}$. Scatter in the data and the resultant error limits on

the inhibition constant were due to electron transfer between native ferrocytochrome *c* and derivative ferricytochrome *c* and the subsequent auto-oxidation plus enzymic oxidation of the derivative ferrocytochrome *c*. Rates of non-enzymic oxidation of mixtures of native and derivative cytochromes were subtracted from observed rates with added enzyme. However, the observed rate could not be separated into a rate due to turnover of the native ferrocytochrome *c* and a rate due to turnover of derivative ferrocytochrome *c* produced by haem *c*-haem *c* electron exchange.

The molecular parameters that characterize the reaction between cytochrome oxidase and the carbodi-imide derivative were determined by analysis according to a modified Minnaert scheme, as established by Errede & Kamen (1978). Observed first-order rate constants were obtained at cytochrome *c* concentrations between 0.5 and 10 μM, which was an appropriate range for study of the highest-affinity cytochrome oxidase site. Plots of k versus $k \times$ concentration of cytochrome *c* were linear for both native cytochrome *c* and its derivative. From the slope and limiting intercept at zero cytochrome *c* concentration, values for the limiting rate of reaction and the binding affinity were determined (Table 3 and Fig. 7).

The glycine methyl ester derivative showed an extremely high auto-oxidation rate. At approx. 60 μM-O₂ concentration auto-oxidation was faster than the experimental mixing dead-time, corresponding to a pseudo-first-order rate in excess of 0.5 s⁻¹. Since this is faster than typically measured enzymic rates, it precluded further kinetic analysis.

Discussion

The cytochrome *c* modification illustrates that carbodi-imide may be used in the absence of nucleophiles to effect limited modification of acidic residues in proteins. Unfortunately, the selectivity cannot be controlled. Those residues partially shielded from solvolysis are susceptible to the rearrangement to stable *N*-acylurea, whereas the general class is not, but it is not universally possible to predict if a residue has the favourable microenvironment. However, functionally important acid groups may frequently be found shielded in active sites and this type of chemical modification becomes feasible.

The physical properties of the carbodi-imide cytochrome *c* derivative indicate that its conformation is perturbed from that of native cytochrome *c*. Loss of the 695 nm band in the ferric state suggests loss of methionine ligation, although some authors question the correlation of the two properties (Ivanetich *et al.*, 1976a,b). The c.d. spectra indicate conformational changes that suggest a loosening from a relatively rigid compact structure (Kaminsky *et al.*,

1972; Myer, 1968). Unfortunately, no quantitative correlation can be made from the spectra as to the exact degree of structural change. The diminution in reduction potential and the observation of decreased stability during long-term storage also reflect structural change. The residues that have been modified are known by the nature of the modification reaction not to be freely exposed on the molecular surface. Therefore the net amidation reaction would be expected to introduce a perturbation. Most of the properties mentioned above reflect the haem environment, and therefore it seems reasonable to ascribe their appearance to modification of the haem propionate. Aspartic acid-62 is on the (so-called) back of the molecule, opposite the haem-crevice region; it is not known to be involved in any extraordinary structural arrangement in the X-ray-crystallographic determination (Takano *et al.*, 1977). The nearby methionine-65 has been selectively chemically modified by other workers without perturbation of the native structure, insofar as this has been checked (Azzi *et al.*, 1972; Vanderkooi *et al.*, 1973b; Brown & Wuthrich, 1977). These methionine-65 modifications have been reported to affect cytochrome *c* binding to mitochondrial or artificial membranes. The abnormal membrane interaction (Fig. 6) of the carbodi-imide derivative modified at aspartic acid-62 is consistent with and therefore supports the conclusions of the previous studies that the back of the molecule interacts with membranes.

The most important conclusion is that the perturbed structure is still a suitable substrate for cytochrome oxidase. Indeed, it efficiently inhibits the reaction with native ferrocycytochrome *c* with a K_i of $2\mu\text{M}$. The kinetic behaviour of the derivative shows all the characteristics of native cytochrome *c* oxidation, i.e. a time course of oxidation strictly first-order with respect to ferrocycytochrome *c*, non-saturation of rates and increasing observed Smith-Conrad first-order rate constants with decreasing initial concentration. The derivative fits into the kinetic scheme that Errede & Kamen (1978) proposed for native cytochrome *c* reacting with its oxidase. Although reactive, the derivative does show decreased reactivity when compared with native tuna cytochrome *c*. Both the limiting rate [$k_1k_3/(k_2+k_3)$] and cytochrome oxidase affinity (k_1/k_2) of the modified Minnaert mechanism decrease to approximately half the values for native tuna cytochrome *c*. Errede & Kamen (1978) have noted the general trend in the reactions of different cytochromes from different species that these parameters seem to increase or decrease in a concerted manner. They concluded that binding (k_1/k_2) dominates over transfer rate in determining reactivity. The derivative shows a concerted decrease in both parameters, and the simplest interpretation would be that the derivative

has abnormal decreased binding to cytochrome oxidase. The decrease in reactivity of the carbodi-imide derivative might be compared in extent with decreased reactivity with lysine-13 modifications, where trifluoroacetylation decreases affinity for cytochrome oxidase 5-fold (Staudenmayer *et al.*, 1977) and carboxydinitrophenylation decreases it 100-fold (Ferguson-Miller *et al.*, 1978). The approximately 50% decrease for the carbodi-imide derivative supports the hypothesis that the bottom of the haem crevice is less critical for cytochrome oxidase specificity than is the top region around lysine-13.

An important caveat to this interpretation is that it cannot be proved that the derivative reacts with cytochrome oxidase by the same mechanism as does native cytochrome *c*. The kinetic data are consistent with but not unambiguous proof for a common mechanism. The perturbations in physical properties especially need to be examined to gauge the probable extent of conformational change.

Although the derivative has lost the 695 nm band, other chemically modified forms of cytochrome *c* devoid of the band have been reported to retain reactivity with cytochrome oxidase. Methionine-80 sulphoxide derivatives have been reported to be 82–100% as reactive as native cytochrome *c* towards cytochrome oxidase (Ivanetich *et al.*, 1976a), and a methionine-80 carboxymethyl derivative at least 90% as reactive when measured at $10\mu\text{M}$ or higher (Margoliash *et al.*, 1973). The lowered reduction potential of 105 mV can be compared with the 85 mV potential of formylated cytochrome *c* modified at tryptophan-59, which has 60% of the maximal velocity obtained with native cytochrome *c* in a cytochrome oxidase O_2 -uptake assay system (Erecinska, 1975). Kassner (1972) has correlated reduction potentials with environment in cytochrome *c*, and has noted that solvent-exposed haem (as in haem peptides) has a reduction potential in the range -50 mV to -200 mV . The degree of disruption around the haem crevice can also be gauged by ligand attack. The auto-oxidation rate for the carbodi-imide derivative is $2 \times 10^{-3}\text{ s}^{-1}$, whereas that of native cytochrome *c* is essentially zero. However, extensively denatured cytochrome *c*, as in the derivative with all acid groups modified by carbodi-imide plus nucleophile, has rates of auto-oxidation that are faster by more than 250-fold. It has also been shown that cyanide binds to the derivative with a rate 8-fold greater than with native cytochrome *c* at 50 mM-cyanide, but cyanide binds to random-coil cytochrome *c* with rates 10^3 -fold greater than with native cytochrome *c* (Dyer *et al.*, 1979). The conclusion is that carbodi-imide modification has not fully opened the haem crevice and a large degree of structural integrity is preserved.

The carbodi-imide derivative shares some properties with other cytochromes, chemically modified by

nitration at tyrosine-67 (Sokolovsky & Aviram 1970), oxidation or carboxymethylation at methionine-80 (Ivanetich *et al.*, 1976a; Schejter & Aviram, 1970), formylation at tryptophan-59 (Erecinska, 1975) and iodination at tyrosine-67 and tyrosine-74 (Morton, 1973). All the mentioned derivatives show a pH-linked low-spin to high-spin transition with a transition pK of approx. 5.5: 5.3 for the carbodi-imide derivative (Fig. 4), 5.9 for the nitrated tyrosine-67 derivative (Sokolovsky & Aviram, 1970), 5.2 for the oxidized methionine-80 derivative (Ivanetich *et al.*, 1976a), 5.5 for the tryptophan-59 derivative (calculated from data in Erecinska, 1975) and 5.9 for the iodinated derivative (Morton, 1973). In the cases of nitrated tyrosine-67 and the iodinated derivative, this has been interpreted as being due to a pH titration of modified tyrosine-67. The presence of the same transition pK in derivatives with other chemical modifications argues against the assignment, but does not suggest an obvious alternative.

Similarities among different derivatives suggest that there exists for cytochrome *c* a conformational state near in total free energy to the native state, which the molecule may assume after slight perturbations at various and different structural regions, but all having some contact with the haem crevice region. The state is characterized by loss of the 695 nm band, a pH-linked low-spin to high-spin transition with pK between 5 and 6, some lowering of the reduction potential, retention of ascorbate reducibility and significant reactivity with cytochrome oxidase.

This work was supported by grant GM 23869 from the National Institutes of Health.

References

- Ambler, R. P. (1963) *Biochem. J.* **89**, 349–378
- Aviram, I. & Schejter, A. (1973) *FEBS Lett.* **36**, 174–176
- Azzi, A., Tamburro, A. M., Farnia, G. & Gobbi, E. (1972) *Biochim. Biophys. Acta* **256**, 619–624
- Bartsch, R. G. (1971) *Methods Enzymol.* **13**, 344–363
- Bergmeyer, H. U. (1978) in *Principles of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 35–40, Verlag Chemie, New York
- Brandt, K. G., Parks, P. C., Czerlinski, G. H. & Hess, G. P. (1966) *J. Biol. Chem.* **241**, 4180–4185
- Brown, L. R. & Wuthrich, K. (1977) *Biochim. Biophys. Acta* **468**, 389–410
- Cameron, B. F. (1965) *Anal. Biochem.* **11**, 164–169
- Carraway, K. L. & Koshland, D. E. (1968) *Biochim. Biophys. Acta* **160**, 274–276
- Davenport, H. E. & Hill, R. (1952) *Proc. Soc. London Ser. B.* **139**, 335–337
- Dickerson, R. E. & Timkovich, R. (1975) *Enzymes 3rd Ed.* **11**, 397–547
- Dickinson, L. C. & Chien, J. C. W. (1975) *Biochemistry* **14**, 3526–3534
- Dyer, C., Schubert, A., Timkovich, R. & Feinberg, B. A. (1979) *Biochim. Biophys. Acta* **579**, 253–268
- Erecinska, M. (1975) *Arch. Biochem. Biophys.* **159**, 199–208
- Errede, B. & Kamen, M. D. (1978) *Biochemistry* **17**, 1015–1027
- Errede, B., Haight, G. P. & Kamen, M. D. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 113–116
- Ferguson-Miller, S., Brautigan, D. L. & Margoliash, E. (1976) *J. Biol. Chem.* **251**, 1104–1115
- Ferguson-Miller, S., Brautigan, D. L. & Margoliash, E. (1978) *J. Biol. Chem.* **253**, 149–159
- Ferguson-Miller, S., Brautigan, D. L. & Margoliash, E. (1979) in *The Porphyrins* (Dolphin, D., ed.), vol. 7, pp. 150–240, Academic Press, London and New York
- Fisher, W. R., Taniuchi, H. & Anfinsen, C. B. (1973) *J. Biol. Chem.* **248**, 3188–3193
- Granick, S. (1961) *J. Biol. Chem.* **236**, 1168–1172
- Hartzell, C. R. & Beinert, H. (1974) *Biochim. Biophys. Acta* **168**, 318–338
- Hoare, D. G. & Koshland, D. E. (1967) *J. Biol. Chem.* **242**, 2447–2453
- Horinishi, H., Kurihara, K. & Shibata, K. (1965) *Arch. Biochem. Biophys.* **111**, 520–524
- Ivanetich, K. M., Bradshaw, J. J. & Kaminsky, L. S. (1976a) *Biochemistry* **15**, 1144–1153
- Ivanetich, K. M., Bradshaw, J. J. & Fazakerley, G. V. (1976b) *Biochem. Biophys. Res. Commun.* **72**, 433–439
- Jacob, E. E. & Sanadi, D. R. (1960) *J. Biol. Chem.* **235**, 531–534
- Kaminsky, L. S., Yang, F. C. & King, T. E. (1972) *J. Biol. Chem.* **247**, 1354–1359
- Kassner, R. J. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2263–2267
- Kreil, G. (1963) *Hoppe-Seyler's Z. Physiol. Chem.* **334**, 154–166
- Lee, C. P. & Ernster, L. (1967) *Methods Enzymol.* **10**, 543–548
- Low, H. & Vallin, I. (1963) *Biochim. Biophys. Acta* **69**, 361–374
- Margoliash, E. & Smith, E. L. (1962) *J. Biol. Chem.* **237**, 2151–2160
- Margoliash, E. & Walasek, O. F. (1967) *Methods Enzymol.* **10**, 339–348
- Margoliash, E., Ferguson-Miller, S., Tulloss, J., Kang, C. H., Feinberg, B. A., Brautigan, D. L. & Morrison, M. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3245–3249
- Morton, R. A. (1973) *Can. J. Biochem.* **51**, 472–475
- Myer, Y. P. (1968) *Biochemistry* **7**, 765–776
- Nakatani, M. (1960) *J. Biochem. (Tokyo)* **48**, 633–635
- Paul, K. G. (1951) *Acta Chem. Scand.* **5**, 389–405
- Riehm, J. P. & Scheraga, H. A. (1966) *Biochemistry* **5**, 99–115
- Riordan, J. F. & Hayashida, H. (1970) *Biochem. Biophys. Res. Commun.* **41**, 122–127
- Salemme, F. R., Kraut, J. & Kamen, M. D. (1973) *J. Biol. Chem.* **248**, 7701–7716
- Schejter, A. & Aviram, I. (1970) *J. Biol. Chem.* **245**, 1552–1557
- Smith, L. & Conrad, H. (1956) *Arch. Biochem. Biophys.* **63**, 403–406
- Smith, L., Nava, M. E. & Margoliash, E. (1973) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S. & Morrison, M., eds.), pp. 629–636, University Park Press, Baltimore

- Smith, L., Davis, H. C. & Nava, M. (1974) *J. Biol. Chem.* **249**, 2904–2910
- Smith, L., Davis, H. C. & Nava, M. E. (1976) *Biochemistry* **15**, 5828–5831
- Sokolovsky, M. & Aviram, I. (1970) *J. Biol. Chem.* **245**, 1552–1557
- Staudenmayer, N., Ng, S., Smith, M. B. & Millet, F. (1977) *Biochemistry* **16**, 600–604
- Takano, T., Kallai, O. B., Swanson, R. & Dickerson, R. E. (1973) *J. Biol. Chem.* **248**, 5234–5255
- Takano, T., Trus, B. L., Mandel, N., Mandel, G., Kallai, O. B., Swanson, R. & Dickerson, R. E. (1977) *J. Biol. Chem.* **252**, 776–785
- Timkovich, R. (1977a) *Anal. Biochem.* **79**, 135–143
- Timkovich, R. (1977b) *Biochem. Biophys. Res. Commun.* **74**, 1463–1468
- Timkovich, R. & Dickerson, R. E. (1976) *J. Biol. Chem.* **251**, 4033–4046
- Timkovich, R., Dickerson, R. E. & Margoliash, E. (1976) *J. Biol. Chem.* **251**, 2197–2206
- Vanderkooi, J. M. & Erecinska, M. (1975) *Eur. J. Biochem.* **60**, 199–204
- Vanderkooi, J. M., Erecinska, M. & Chance, B. (1973a) *Arch. Biochem. Biophys.* **145**, 219–229
- Vanderkooi, J. M., Erecinska, M. & Chance, B. (1973b) *Arch. Biochem. Biophys.* **157**, 531–540
- Wada, K. & Okunuki, K. (1969) *J. Biochem. (Tokyo)* **66**, 249–262