Chemical modification of the haem propionate of cytochrome *c*

A re-evaluation of the reaction of cytochrome *c* with a water-soluble carbodi-imide

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Horse heart and tuna heart cytochromes *c* were treated with the water-soluble carbodi-imide 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide. When the reaction is followed spectroscopically two kinetic phases are apparent. Alteration of the reactivity of the proteins with such ligands as CO, however, occurs in a single phase identical with the faster phase detected spectroscopically. The modified proteins both show spectroscopic and redox properties identical with those described for the tuna heart cytochrome *c* derivative by Timkovich [Biochem. J. (1980) 185, 47–57]. The use of radiolabelled carbodi-imide identifies two or three sites of reactivity. However, the addition of glycine methyl ester to the reaction mixture leads to the addition of nine glycine moieties in the case of the horse protein and seven in the case of the tuna protein, indicating a larger number of reactive sites than previously reported. A further set of reaction sites was identified by peptide mapping of the modified proteins, and these sites take part in intramolecular reactions leading to internal cross-linking and the formation of an enzymically indigestible ‘core particle’. The haem group was identified as a site of reaction with the carbodi-imide, and is as a consequence covalently linked to the peptide by a bond in addition to the thioether bonds normally present. In the light of these findings, the alterations in the properties of the tuna protein, subsequent to reaction with the carbodi-imide, which have been previously explained in structural terms, must be re-evaluated. This study also highlights the importance of internal cross-link formation, which can occur by intramolecular nucleophilic attack, a process that has often been overlooked by investigators employing carbodi-imide modification of carboxylate groups in proteins.

INTRODUCTION

Many investigations, particularly those employing chemical modification, have been made in an effort to identify the mechanism whereby electrons from cytochrome *c* reductase can enter cytochrome *c* and then be transferred on to cytochrome *c* oxidase [1]. A range of evidence has been obtained suggesting that electron transfer occurs via the exposed haem edge in cytochrome *c* [2,3]. In particular, a set of studies on the effect of chemical modification of sites surrounding the exposed haem edge have been made [4–7], and these give support to the proposal that electron transfer involves the exposed periphery of the haem pyrrole rings II and III. Only one report has appeared in which a specific and selective modification of the haem edge itself was possible. It has been reported [8] that reaction of tuna heart cytochrome *c* with the water-soluble carbodi-imide EDC leads to the formation of a stable *N*-acylurea with the net addition of the 1-(3-dimethylaminopropyl)-3-ethyl-urea skeleton to the carboxylate moiety present at Asp-62 and the less buried haem propionate group. The protein formed by this reaction was reported to possess an unusual set of properties, the most significant, in terms of its electron-transfer properties, being the lack of a 695 nm band but the maintenance of ascorbate-reducibility. As such, studies of the electron-transfer activity of the modified protein might add important evidence to the controversy concerning the role of Met-80

ligation in electron-transfer reactions of cytochrome *c* [9–11].

Reaction of proteins with carbodi-imides can follow one or more of three major pathways. Initial reaction with a carboxylate group leads to the formation of an unstable *O*-acylurea. This can then undergo three major reactions: (1) hydrolysis back to a free carboxylate, associated with the release of an inert urea; (2) rearrangement to a stable *N*-acylurea; (3) intramolecular nucleophilic attack leading to internal cross-linking and the displacement of an inert urea [12]. In the initial report of the reaction of tuna cytochrome *c* with EDC Timkovich [8] identified the first two pathways as being of importance, although he did identify intermolecular nucleophilic attack leading to dimer and polymer formation [13]. Carbodi-imides have been specifically used to produce amide-bond cross-links between proteins [14–17]. In the present study we have investigated the reaction of both horse heart and tuna cytochromes *c* with EDC. Using h.p.l.c. analysis of peptide fragments, produced by a variety of proteolytic reactions, we have identified the formation of a significant number of intramolecular cross-links in both modified proteins. Further analysis has shown that the earlier reports of limited modification, which relied on radiolabelling, although reproducible, have been misinterpreted. We have also shown that the interpretation of a single reactivity for the haem group is incorrect, and that the haem group acquires another covalent attachment to the

Abbreviation used: EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide [‘1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide’].

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protein as a consequence of its reaction with EDC. Descriptions of the reactivity of EDC-modified cytochrome *c* in terms of the previously suggested structural alterations must therefore be re-evaluated. In a more general context it is clear that, although EDC and other carbodi-imides have been widely used in protein studies [16,18,19], the investigator should always identify internal cross-linking before assigning functional changes to specific modification of unique carboxylate groups.

MATERIALS AND METHODS

Albacore tuna were caught on long lines off the East coast of New Zealand's South Island, and the hearts were quickly removed and stored in solid CO₂ until used. Cytochrome *c* was obtained from tuna hearts by a modification of the method reported by Brautigan *et al.* [20]. In the present preparation centrifugation replaced filtration in the early steps of the previously reported procedure. The crude cytochrome was then recovered by batchwise adsorption on CM-cellulose rather than adsorption on a column of Amberlite IRC-50. All other steps were as previously reported. Horse heart cytochrome *c* (type VI) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and deamidated and polymeric forms of the protein were removed by chromatography on CM-cellulose [20]. EDC hydrochloride was obtained from Sigma Chemical Co., and ¹⁴C-labelled EDC was prepared as described previously [21]. Chemical modification of both horse heart and tuna heart cytochromes *c* was performed exactly as previously described [8]. Monomeric protein was carefully separated from dimeric protein species resulting from EDC-promoted intermolecular cross-linking by passage down a Sephadex G-50 column (83 cm × 3.2 cm diam.) The cytochromes were also modified in the presence of glycine methyl ester. In this case the reaction was performed as indicated above except that the proteins were mixed with 0.5 M-glycine methyl ester hydrochloride at pH 4.75 before addition of EDC. After completion of the reaction, excess glycine methyl ester was carefully removed by exhaustive dialysis against 0.5 M-NaCl.

Concentrations of EDC in solution were determined by using the hydroxamate/Fe³⁺ reaction procedure previously reported [22].

Trypsin was obtained from Sigma Chemical Co. Trypsin hydrolysis was performed with a 2% (w/w) trypsin/protein ratio in 0.1 M-NH₄HCO₃, pH 8.5, containing 0.1 mM-CaCl₂. The reaction was carried out at 37 °C, usually for 6 h. *Staphylococcus aureus* endoprotease Glu-C (V8 proteinase) was obtained from Boehringer Mannheim (Sydney, N.S.W., Australia). V8-proteinase digests were carried out in 0.1 M-sodium phosphate buffer, pH 7.8, in the presence of 1.8 mM-EDTA, under which conditions the enzyme hydrolyses peptides C-terminal to glutamic acid and aspartic acid residues [23].

Peptide mapping was performed by using reverse-phase h.p.l.c. H.p.l.c. was performed with a Waters Associates system consisting of two solvent-delivery pumps, a sample injection valve fitted with a 2 ml loading loop, a system controller and an absorbance detector. Detection was at 214 nm. Separations were carried out at room temperature in a Radial Pak μ Bondapak C₁₈ column. The solvent system employed was composed of solvents A [0.1% (v/v) trifluoroacetic

Fig. 1. Time course of the reaction of tuna heart cytochrome *c* with EDC

The time course of the reaction of cytochrome with EDC was monitored at 623 nm (○) and 695 nm (□). The appearance of CO-binding capacity was monitored at 414 nm (△).

acid] and solvent B [0.1% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile]. For the injection, peptides were dissolved in solvent A and eluted from the column by using a linearly increasing gradient in solvent B as appropriate.

Peptides were hydrolysed with constant-boiling HCl in evacuated tubes at 110 °C for 12 h. Amino acid analysis was performed by using pre-modification of the amino acids with phenyl isothiocyanate, the phenylthio-carbamoyl-amino acid derivatives then being separated by reverse-phase h.p.l.c. [24].

Haem cleavage was achieved by using the Fisher modification [25] of the Paul procedure [26]. Free cysteine thiol groups were alkylated with iodo[2-³H]acetic acid by previously published methods [27].

Incorporation of ¹⁴C and ³H was determined by liquid-scintillation counting in a Packard 300-C liquid-scintillation spectrometer. Freeze-dried samples were treated with 0.3 ml of 30% (w/v) H₂O₂ for 2 h to destroy haem. A 4.7 ml portion of a standard Triton X-100/toluene scintillation fluid was then added.

Table 1. Incorporation of glycine methyl ester into carbodi-imide-activated cytochrome *c*

For experimental details see the text. nTHc and nHhc represent the native tuna and horse heart cytochromes *c* respectively and THc* and HHc* represent the proteins pre-modified with carbodi-imide. Native tuna cytochrome *c* contains 12 carboxylate groups/molecule and native horse cytochrome *c* contains 15.

Protein	Glycine content (mol/mol of protein)	Glycine methyl ester incorporated (mol)
nTHc	20.1	7.1
THc*	17.1	4.1
nHhc	21.1	9.1
HHc*	14.9	2.9

Fig. 2. Time course of the reaction of horse heart cytochrome *c* with EDC studied by peptide mapping

Tryptic peptide maps were obtained as described in the text at 0 min (a), 50 min (b) and 6 h (c). -----, Elution gradient of solvent B in solvent A.

RESULTS

Modification of the protein

In all the experiments performed horse and tuna heart cytochromes *c* behaved identically unless otherwise stated. Modification of both cytochromes with EDC lead to marked changes in the spectra and chemical reactivity of the proteins. In particular, reaction with EDC led to the loss of the 695 nm band, the appearance of a band at 623 nm and the appearance of reactivity of the reduced protein with CO. Each of these changes was

monitored in order to follow the progress of the reaction (Fig. 1). Both the absorption-band alterations occurred in two phases, with the predominant faster phase having a rate of approx. 0.16 min^{-1} and the slower phase having a rate of approx. 0.03 min^{-1} . The appearance of CO binding to the ferrous form of the protein was associated with a rate of 0.15 min^{-1} . In order to exclude the possibility that this biphasic character might arise from changes in the EDC concentrations due to hydrolysis during the reaction, the rate of EDC hydrolysis in the presence of protein was determined. The rate of decay of the EDC due to hydrolysis was found to be 0.014 min^{-1} ,

Fig. 3. Gel filtration of tryptic digests of native and EDC-modified horse heart cytochrome *c*

Tryptic digests of native (a) and EDC-modified (b) horse heart cytochrome *c* were chromatographed on Sephadex G-50. The elution was monitored at 214 nm (—) and 410 nm (-----). Pooled fractions were obtained as illustrated, and these fractions were then subjected to h.p.l.c. analysis.

with only one kinetic phase being observed. Attempts to separate any components present in the finally modified species proved unsuccessful. Chromatography on CM-cellulose with either constant-ionic-strength buffer or a pH gradient produced only a very broad single band. Isoelectric focusing in the range of pH 9–11 yielded a single broad band with pI approx. 11. Non-denaturing polyacrylamide-gel electrophoresis at pH 9.1 produced a broad band that migrated faster than native cytochrome *c*, indicating that the modified proteins are a complex mixture of species possessing different net charges. The measurement of a range of physicochemical properties of both EDC-modified cytochromes *c* showed them to be identical with the EDC-modified tuna heart protein previously reported by Timkovich [8]. The protein had the same spectra as previously reported and showed a high-spin-low-spin transition with a pK of 5.5 ± 0.4 (compared with 5.3 previously reported [8]). The reduced protein was found to react with O_2 , as previously reported, and, in addition, to bind CO. The oxidized protein was found to be ascorbate-reducible, as reported by Timkovich [8].

Number of reactive sites

The number of sites reacting with EDC was measured by two different techniques. Reaction with [^{14}C]EDC followed by quantification of the incorporated label yielded a value of two to four modification sites (exact values could not be obtained owing to some chemical contamination of the labelled EDC and radiolytic degradation of the synthesized labelled compound). Quantification of the number of sites reacting with EDC was also carried out by performing the reaction in the presence of a large excess of glycine methyl ester. Under these conditions reaction of carboxylate groups with EDC leads to formation of *O*-acylureas, which rapidly undergo nucleophilic displacement by the large excess of added ester. Quantification of the increase in glycine content of the protein then provides a measure of the number of reacting sites. This process was carried out for native cytochrome *c* and for cytochromes *c* that had previously reacted with EDC. The difference in glycine content of the native and pre-EDC-modified products should then give a minimum estimate of the number of EDC-modified carboxy groups present in the EDC-premodified protein. The results are presented in Table 1.

Both of these methods are insensitive to the formation of intramolecular cross-links formed by nucleophilic substitution of the *O*-acylurea intermediate by an intramolecular nucleophilic species, and so recourse was made to peptide mapping.

Peptide mapping

Tryptic digestion of native horse and tuna heart cytochromes *c* gave a set of well-resolved tryptic peptides, which were analysed for their amino acid composition and correlated with the published amino acid sequence of both proteins [28,29] (all possible tryptic peptides were identified in both cases). In the case of the tuna protein three peptides were eluted from the h.p.l.c. column with identical amino acid compositions, corresponding to the tryptic peptide Glu⁶⁶-Tyr-Leu-Glu-Asn-Pro-Lys⁷². It is possible that two of these peptides represent the native peptide and a species deamidated at Asn-70. However, the existence of a third peptide with

Fig. 4. H.p.l.c. analysis of the molecular-mass fractions obtained from Sephadex chromatography of the tryptic digest of EDC-modified horse heart cytochrome *c*

The molecular-mass fractions obtained as shown in Fig. 3 were subjected to h.p.l.c. analysis as described in the text. (a), (b) and (c) show the elution profiles obtained for fractions I, II and III respectively of the Sephadex elution. -----, Elution gradient of solvent B in solvent A. Subfractions Ia, Ib, IIIa and IIIb (hatched) were collected and subjected to amino acid analysis.

Fig. 5. H.p.l.c. analysis of V8-proteinase digests of native and EDC-modified tuna heart cytochrome *c*

Native tuna heart cytochrome *c* (a) and its EDC-modified derivative (b) were digested with V8 proteinase as described in the text and then subjected to h.p.l.c. analysis -----, Elution gradient of solvent B in solvent A.

this amino acid composition is difficult to explain. It is possible that the original sequence data [28] are incorrect and that one of Glu-66 or Glu-69 is in fact a glutamine residue. Partial deamidation of the native protein could thus lead to the production of three differently eluted tryptic peptides with seemingly identical amino acid compositions after acid hydrolysis.

In the case of the modified proteins the nature of the h.p.l.c. elution profile depended on the extent of modification (Fig. 2). As the reaction progressed fewer discrete peptides were observed in the tryptic peptide map and a very broadly eluted band appeared late in the elution profile. The extent of this broad feature was not diminished by increasing the length of the tryptic digestion to 24 h, increasing the trypsin concentration to 10%, adding additional portions of trypsin or pre-denaturing the protein with ethanol [29].

In order to characterize further this unusual feature observed by h.p.l.c., trypsin-digested protein was passed down a Sephadex G-50 column (Fig. 3), and the various molecular-mass fractions were then subjected to analysis by h.p.l.c. Various samples recovered from the h.p.l.c. runs were subjected to amino acid analysis (Fig. 4). The native protein was digested into its constituent peptides

and the haem peptide was retarded on the Sephadex column. Analysis of the two native fractions yielded peptide maps consisting of all the non-haem peptides for the early-eluted fraction and haem peptide for the late-eluted fraction. In the case of the modified proteins three major fractions were apparent in the elution profile from the Sephadex column. These three fractions, when subjected to h.p.l.c. analysis (Fig. 4), yielded three distinct profiles. H.p.l.c. analysis of the earliest Sephadex fraction showed a broadly eluted band and small amounts of discrete peptides. Subfractions of the broad peak (Ia and Ib) were subjected to amino acid analysis and were found to contain a mixture of many amino acids in approximately the proportions expected for a whole protein. The second fraction eluted from the Sephadex column produced an h.p.l.c. profile with well-resolved peptides that could be identified as tryptic fragments of the native protein. The last fraction to be eluted from the Sephadex column, when subjected to h.p.l.c., showed the presence of one major band (IIIb) which had the amino acid composition of the haem peptide. It should be noted, however, that in the case of

Fig. 6. H.p.l.c. analysis of CNBr digests of native and EDC-modified tuna heart cytochrome *c*

Native tuna heart cytochrome *c* (a) and its EDC-modified form (b) were treated with CNBr as described in the text and subjected to h.p.l.c. analysis. -----, Elution gradient of solvent B in solvent A.

Fig. 7. Gel filtration of native and horse heart cytochrome *c* subsequent to haem cleavage

Native horse heart cytochrome *c* (a) and its EDC-modified derivative (b) were treated with silver salts as described in the text in order to cleave the haem thioether linkages. The proteins were then subjected to chromatography on Sephadex G-25. The elution was monitored at 280 nm (—) for protein and at 410 nm (-----) for the presence of haem.

the modified proteins most of the haem was present not in the later fraction but in fact in the first fraction to be eluted from the Sephadex column. In order to rule out the possibility that the broad unresolved feature seen in the h.p.l.c. profile of the trypsin-digested modified proteins was not arising from altered trypsin specificity, the modified proteins were also digested with V8 proteinase and with CNBr. In both experiments the native proteins were susceptible to cleavage by these reagents, yielding a small number of discrete peaks in the h.p.l.c. elution profiles as expected, whereas the modified proteins gave one major unresolved peak (Figs. 5 and 6).

Characterization of haem binding

As identified in Fig. 3, tryptic digestion of the modified proteins gave a 'core particle', which contained almost all of the haem (fraction I), rather than a discrete haem peptide. The nature of the attachment of the haem to the protein was investigated by means of conventional

haem-cleavage techniques. Attempts at haem extraction by using the method of Timkovich [8] were not successful. The thioether linkages that attach haem *c* to the protein cytochrome *c* can be cleaved by the action of silver salts [25]. Treatment of the native proteins with silver salts followed by Sephadex chromatography yielded early-eluted apoprotein and late-eluted free haem (Fig. 7). When the modified protein was treated with silver salts, chromatography showed that the haem remained attached to the protein (Fig. 7). In order to discount the possibility that in the case of the modified protein treatment with silver salts did not cleave the thioether bonds, the silver-salt-treated protein was subsequently treated with dithiothreitol in order to convert the silver-thiol complex into reduced thiols [25]. After removal of dithiothreitol the protein was treated with iodo[2- 3 H]acetic acid. The presence of free thiol was then detected by the incorporation of radioactivity. A high degree of labelling was found in the case of the 'apo' modified proteins, clearly showing that successful cleavage of the protein-haem thioether bonds had occurred in these cases.

DISCUSSION

The physicochemical characterization of the modified proteins produced in this study clearly shows the modified proteins to be identical with the EDC-modified tuna protein described previously [8]. The biphasic characteristic of the modification process implies that the initial reaction leads directly to a disruption of the haem crevice environment, so allowing reaction with CO. The second phase of the modification has not been associated with any functional changes for the proteins, although it is tempting to propose that this phase represents the formation of intramolecular cross-links. Indeed, some support for this is given by the data in Fig. 2, which show that significant cross-linking occurs only after the first 15 min of the reaction (the period during which CO reactivity reaches its maximum). The peptide mapping studies clearly identify the formation of intramolecular cross-links, and the haem-protein thioether-bond-cleavage experiments show that within the 'core particle' formed by cross-linking the haem group acquires other covalent attachment to the protein.

These data bring into question the validity of the previously published interpretation of the reactivity of EDC with cytochrome *c* [8]. From the above data it would appear that, owing to the relatively poor resolving power of separation techniques for peptides used before the advent of h.p.l.c., the full extent of chemical modification of this protein has been overlooked. In the previously reported study [8] the isolation techniques employed specifically led to the isolation of either small discrete peptides or free haem from any mixture. The large core particle was therefore not observed. This, together with the reliance put on the radiolabelling studies, which are, by their nature, unable to detect intramolecular cross-links, thus led to an underestimate of the degree of modification of this protein by EDC and an overestimate of the specificity of the reaction. Timkovich's [8] inability to reproduce peptide maps by using the classical techniques, and the isolation of only very low yields of haem, obviously arise from his overlooking the occurrence of intramolecular nucleophilic attack on the initially formed *O*-acylureas.

Thus, although it has been stated that 'The cytochrome *c* modification illustrates that carbodiimide may be used in the absence of nucleophiles to effect limited modification of acidic residues in proteins . . . ' [8], it is clear that the degree of modification is more extensive than previously thought, and that the modified proteins are a mixture of species containing different degrees of carboxy-group modification.

We therefore propose that, in the light of these new data, the very interesting properties exhibited by the modified cytochromes, namely the maintenance of ascorbate-reducibility in the absence of a 695 nm band, and reactivity with the native oxidase together with the ability to bind CO and O₂ in the ferrous oxidation state, derive from a disruption of the normal Met-80 ligation state. This altered ligation state is clearly different to that which is found in carboxymethylated cytochrome *c* [30, 31] and denatured forms of cytochrome *c* that lack the 695 nm band, as these species are not reducible by ascorbate [32,33]. It is possible that this difference in reactivity is brought about by the rigidity of the protein structure induced by the intramolecular cross-linking process.

We thank Dr. D. L. Christie for several useful discussions, and also thank Dr. D. M. Gibson, Mr. D. M. Shirer and Mr. G. Voss of The Fisheries Research Division of the Ministry of Agriculture and Fisheries (N.Z.) for providing the tuna hearts. A.J.M. thanks the University Grants Committee (N.Z.) for a postgraduate scholarship.

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Received 24 February 1986/16 June 1986; accepted 25 July 1986