A new non-covalent complex of semisynthetically modified tryptic fragments of cytochrome *c*

Amanda E. I. PROUDFOOT,*† Carmichael J. A. WALLACE,*† David E. HARRIS†§ and Robin E. OFFORD*†

*Département de Biochimie Médicale, Centre Médical Universitaire, 1211 Genève 4, Switzerland, and †Laboratory of Molecular Biophysics, Department of Zoology, South Parks Road, Oxford OX1 3PS, U.K.

We have prepared a semisynthetic analogue of fully acetimidylated horse cytochrome *c*, a complex in which the peptide bond between residues glycine-37 and arginine-38 is lacking. In contrast with the complex that we have previously described [Harris & Offord (1977) *Biochem. J.* **161**, 21–25], in which the break in continuity is between residues arginine-38 and lysine-39, the new analogue has a nearly normal redox potential, and can more fully restore succinate oxidation to mitochondria depleted of cytochrome *c*. Studies of this and other analogues lead us to propose an explanation for the low biological activity of complex (1–38)–(39–104) and a role for the invariance of arginine-38.

INTRODUCTION

Eukaryotic cytochromes *c* are electron-transfer proteins of the respiratory chain associated with the mitochondrial inner membrane. They are small basic proteins composed of a single polypeptide chain wrapped around a covalently attached haem group (Dickerson & Timkovich, 1975). The modification of all 19 lysine residues of horse heart cytochrome *c* by acetimidylation (Wallace & Harris, 1984) leaves spectroscopic properties, redox potential and biological activity little changed (Harris & Offord, 1977; Boswell *et al.*, 1983; Wallace, 1984*a*). The blocking group may be removed from cytochrome *c* with retention of activity (Harris & Offord, 1977; Wallace, 1984*a*).

The cleavage of acetimidylated cytochrome *c* with trypsin occurs predominantly at a single peptide bond, forming two fragments, *N*- ϵ -acetimidyl fragment (1–38) and *N*- ϵ -acetimidyl fragment (39–104), in high yield. The protected fragments may be combined to form a stable complex (1–38)–(39–104) (the cytochrome *c*-T described by Harris & Offord, 1977) that exhibits physical properties similar to those of the intact protein but somewhat depressed redox potential and biological activity (Wallace, 1984*a,b*). Similar results have been obtained in studies on the formation and properties of the complex between the unmodified peptide fragments (1–38) and (39–104) (Parr *et al.*, 1978; Westerhuis *et al.*, 1979; Wallace, 1984*a,b*).

The complexes formed between fragments of cytochrome *c* have been used in studies on the residues involved in the biological activity of the protein (Wallace, 1984*a*), in studies on the spectroscopic properties of the protein (Wallace, 1984*b*) and in investigations of the residues involved in cytochrome *c* conformation and folding (Parr *et al.*, 1978; Juillerat & Taniuchi, 1982). The association of modified tryptic fragments has been used to prepare semisynthetic analogues of cytochrome *c* to investigate the role of residues at the site of cleavage on the formation and properties of peptide complexes (Harris & Offord, 1977; Proudfoot *et al.*, 1986). The

conclusions that can be drawn from these studies are necessarily limited by the fact that complex (1–38)–(39–104) does differ in properties from the native protein, and it would be desirable to re-form, after fragment modification, the missing peptide bond.

Reverse proteolysis has been effective for the resynthesis of peptide bonds in other two-fragment complexes (Homandberg *et al.*, 1978; Homandberg & Laskowski, 1979; Koryama *et al.*, 1980; Graf *et al.*, 1981), and has been reported for complex (1–38)–(39–104) (Juillerat & Homandberg, 1981). However, Proudfoot *et al.* (1984) were not able to demonstrate reverse proteolysis, though they did find that low concentrations of contaminants of the glycerol employed as organic co-solvent caused a cross-linking of the fragments that mimicked resynthesis. Efforts are continuing to find a chemical means of re-forming the bond between the fragments (A. E. I. Proudfoot, K. Rose & C. J. A. Wallace, unpublished work).

The present paper reports a semisynthetic non-covalent complex, (1–37)–(38–104), that resembles the parent protein far more closely than does complex (1–38)–(39–104). A preliminary report of part of this work has been published (Harris, 1979).

MATERIALS AND METHODS

Materials

Cytochrome *c* (horse heart, type III), carboxypeptidase B (pig pancreatic, type I), trypsin inhibitor (soya-bean, type I-S), *N*-acetyl-L-methionine and L-arginine (free base) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K., and Munich, West Germany). Trifluoroacetic acid and *NN'*-dicyclohexylcarbodi-imide were obtained from Koch-Light Ltd. (Haverhill, Suffolk, U.K.). Di-*t*-butyl carbonate (purum) was from Fluka (Buchs, Switzerland). Trypsin (L-tosylphenylalanyl-chloromethane-treated) was obtained from Millipore (U.K.) Ltd. (London N.W.10, U.K.). Sephadex G-25, Sephadex G-50 and SP- (sulphopropyl)-Sephadex C-25

† To whom requests for reprints should be addressed.

§ Present address: Cambridge Research Biochemicals, Harston, Cambridge CB2 5NK, U.K.

were obtained from Pharmacia (Milton Keynes, Bucks., U.K.).

Methyl acetimidate hydrochloride was prepared by the method of Hunter & Ludwig (1962), and 1-hydroxybenzotriazole monohydrate was a gift from Dr. G. T. Young (Dyson Perrins Laboratory, University of Oxford). Other chemicals were obtained from Merck (Darmstadt, West Germany) and were of the highest available grade.

Amino group protection, cleavage of the protein and separation of fragments

Horse heart cytochrome *c* was made to react with methyl acetimidate hydrochloride by the method of Wallace & Harris (1984). The acetimidylated protein was digested with trypsin, and the tryptic peptides were separated by gel filtration on Sephadex G-50 (fine grade) as described by Harris & Offord (1977). The tryptic peptides *N*- ϵ -acetimidyl fragment (1–38) and *N*- ϵ -acetimidyl fragment (39–104) were each further purified by cation-exchange chromatography as described by Harris (1978). The peptides were desalted by gel filtration through a column (2.6 cm $\times$ 67 cm) of Sephadex G-25 in 1% (v/v) formic acid and were recovered from solution by freeze-drying.

Preparation of modified tryptic peptides

N- ϵ -Acetimidyl fragment (1–37) was prepared by carboxypeptidase B digestion of *N*- ϵ -acetimidyl fragment (1–38) (Harris, 1978). The fragment was purified by cation-exchange chromatography as described above, upon which it is eluted at a position corresponding to one less positive charge than the parent peptide.

Elongation of *N*- ϵ -acetimidyl fragment (39–104) was achieved by methods similar to those described for *N*- ϵ -acetimidyl fragment (81–104) (Wallace & Offord, 1979). *t*-Butoxycarbonylglycine was coupled via its *N*-hydroxysuccinimide ester, and the hydroxybenzotriazole ester of *t*-butoxycarbonylarginine was formed before coupling as described by those authors.

Products of coupling were purified after deprotection on cation-exchange chromatography as described above. Confirmation of successful couplings of the two purified peptide products was obtained by amino acid compositions and end-group determinations.

Formation of acetimidyl non-covalent complexes

Haem-containing peptides were dissolved in dilute neutral phosphate buffers, and non-haem peptides were dissolved in water or 1 mM-HCl. The peptides were mixed in equivalent quantities, and the non-covalent complexes thus formed were usually characterized without further treatment.

However, to ensure absolute equimolarity of the fragments in samples used for redox potential determinations, the complexes were gel-filtered on a Sephadex G-50 (fine grade) column (1.6 cm $\times$ 150 cm) equilibrated in 0.1 M-NH₄HCO₃.

Spectrophotometry

U.v.- and visible-absorption spectra were recorded with a Unicam SP800A or Cary 210 spectrophotometer. The concentrations of the proteins, non-covalent complexes and peptides used in this study were determined by absorbance measurements at appropriate maxima by using published absorption coefficients for the haem-containing materials (Margoliash *et al.*, 1959; Harris &

Offord, 1977) and a value of 12 mM⁻¹·cm⁻¹ for the molar absorption coefficients at 277 nm of the *N*- ϵ -acetimidyl fragments (38–104), ([Gly³⁸]38–104) and (39–104) (Harris, 1977).

Biological assays

The ability of cytochrome *c* and analogues to restore succinate oxidation to cytochrome *c*-depleted mitochondria (Jacobs & Sanadi, 1960) was determined as previously described (Harris & Offord, 1977). The method has been discussed by Wallace (1984a).

Oxidation-reduction potentials

Redox potentials were measured by the method of Wallace *et al.* (1986).

Formation and tryptic digestion of non-acetimidylated complexes (1–38)-(39–104) and (1–37)-(38–104)

Unprotected complex (1–38)-(39–104) was prepared as described by Proudfoot *et al.* (1984). Of the two routes described by these authors, that using methanesulphonyl-ethoxycarbonyl protection was employed. Fragment (1–37) was formed by carboxypeptidase B treatment of methanesulphonyl-ethoxycarbonyl fragment (1–38) in the same way as was described above for *N*- ϵ -acetimidyl fragment (1–37), with subsequent deprotection. Fragment (38–104) was obtained by deprotection of *N*- ϵ -acetimidyl fragment (38–104) by the method of Wallace & Harris (1984).

Tryptic digestion of cytochrome *c*, complex (1–38)-(39–104) and complex (1–37)-(38–104) was performed under the conditions described by Parr *et al.* (1978) for the overlapping complex of fragments (1–38) and apo-(1–104).

RESULTS

Preparations of pure tryptic fragments of *N*- ϵ -protected cytochromes *c* gave fragment (1–38) in 60–70% yield and fragment (39–104) in 30–40% yield. Recovery of fragment (1–37) from carboxypeptidase A truncation of fragment (1–38) was nearly quantitative, whereas that of the elongation reactions was approx. 30%.

The u.v.- and visible-absorption spectra of all non-covalent complexes were essentially identical, in both oxidized and reduced states, with those of the intact protein in the range 250–750 nm. The complexes exhibited a blue-shift in the Soret band of 3 nm and a well-developed absorption band at 695 nm in the ferric state.

The complexes studied fall into three groups on the basis of their biological activities (Fig. 1). The complexes (1–37)-(39–104) and (1–37)-([Gly³⁸]38–104), in which arginine-38 is missing or replaced, show only 13–17% of the activity of the parent protein, similarly to semi-synthetic analogues in which arginine-38 has been modified with a bulky substituent (Wallace & Rose, 1983). The presence of the arginine residue in complex (1–38)-(39–104) causes an increase in biological activity, to 20–30%, but it is complex (1–37)-(38–104), with the arginine present in the non-haem fragment, that has the highest activity (55–70% of that of the native protein).

The results of redox potential determinations are set out in Table 1. Complex (1–37)-(38–104) shows a decrease of approx. 20 mV in redox potential, whereas for complex (1–38)-(39–104) the change is at least

Fig. 1. Biological assays of acetimidylated non-covalent complexes compared with 19-*N*- ϵ -acetimidylated cytochrome *c*

Cytochrome *c*-depleted mitochondria were suspended in a succinate-, ADP- and O_2 -containing buffer in the chamber of a Rank Brothers oxygen electrode. Successive injections of cytochrome *c* or analogues were made and the O_2 uptake was measured polarographically. ●, Cytochrome *c*; ○, complex (1-37)-(38-104); ■, complex (1-38)-(39-104); □, complex (1-37)-(39-104); ▲, complex (1-37)-[Gly³⁸]-104; △, fragment (1-38).

100 mV (Table 1): most of the determinations for this complex gave values near 150 mV. The potentials of the other complexes assayed, in which arginine-38 is missing or replaced, are obviously even lower, since no meaningful result could be obtained by using the ferrocyanide/ferricyanide couple in the method of mixtures.

The consequences of tryptic treatment of ferrocycytochrome *c* and complexes were examined by a combination of gel filtration under non-denaturing and denaturing conditions, ion-exchange separation and amino acid analyses of the fragments recovered. Ferrocycytochrome *c* is quite unaffected by these mild conditions, and the products of digestion of the complexes are still stable two-fragment complexes. However, complex (1-38)-(39-104) had been completely converted into complex (1-38)-(56-104), one of the products of the digestion of the overlapping complex reported by Parr *et al.*

(1978) and indicative of specific quantitative cleavage at lysine-55. In the case of complex (1-37)-(38-104), by contrast, substantial quantities of the original complex were recovered upon identical treatment, as well as some of the products of limited digestion.

A much greater resistance to the action of trypsin is thus indicated for the (1-37)-(38-104) complex.

DISCUSSION

The spectral properties show that the haem crevice structure in all the analogues studied is retained, yet biological activity is diminished, in some cases greatly. It is likely that these observed differences in electron-transfer capacity reflect the degree to which the complexes differ in structure from the active conformation of cytochrome *c*.

One of the principal factors governing redox potential is solvent accessibility to the haem redox centre (Stellwagen, 1978). Our values for the potentials of the complexes suggest that the structure of complex (1-38)-(39-104) permits greater exposure of the haem to solvent than that of the native protein, and may be thought of as looser. Complex (1-37)-(38-104), in contrast, must have a relatively tight structure.

Such a conclusion is supported by the results of trypsin treatment of the reduced protein and complexes. Under the conditions employed, the native ferrocycytochrome resists digestion completely, despite the 19 lysine residues and two arginine residues present in the sequence. This property is considered to be a consequence of the protein's compact and stable structure (Dickerson & Timkovitch, 1975). The property is partially shared by complex (1-37)-(38-104), but not by complex (1-38)-(39-104), implying a looser structure for the latter. Moreover, since the digestion of complex (1-38)-(39-104) occurs specifically at lysine-55, we may conclude that it is in the region of residues 39-56 that such destabilization occurs.

In terms of primary structure, the two complexes are very similar, both differing from the parent protein by a single nick in the polypeptide main chain. In one, complex (1-38)-(39-104), the nick is immediately on the C-terminal side of arginine-38, whereas in the other, complex (1-37)-(38-104), the nick is immediately on the N-terminal side of arginine-38. This arginine residue is invariant among eukaryotic cytochromes *c*. In the tuna protein crystal structure, its side chain is hydrogen-bonded to the main-chain carbonyl group of leucine-35, to the main-chain carbonyl group of tryptophan-33 (histidine-33 in the horse protein) and to the inner haem propionate

Table 1. Oxidation-reduction potentials of cytochrome *c* and its analogues

Midpoint potentials were determined by the method of mixtures described by Wallace *et al.* (1986) in 0.05 M-phosphate buffer, pH 7.0. The value for acetimidyl fragment (1-38)-(39-104) is approximate, since the potential is at the limit of the range that can be determined by this method.

Cytochrome	Redox potential	Reference
Horse cytochrome <i>c</i>	258 $\pm$ 2	Wallace (1984a)
Acetimidyl-cytochrome <i>c</i>	242 $\pm$ 3	Wallace (1984a)
Acetimidyl-(1-37)-(38-104)	220 $\pm$ 5	Present work
Acetimidyl-(1-38)-(39-104)	110-150	Present work

Fig. 2. The conventional bottom loop of cytochrome *c* (residues 36–59) and its relationship with the prosthetic group and residues 28–35, as viewed from the methionine-80 side

The loop is stabilized by seven intra-loop hydrogen bonds (37–59, 40–57, 41–52, 42–40, 46–43, 52–49 and 59–38). Of the 11 hydrogen bonds linking the loop to the remainder of the molecule, the three provided by the guanido group of arginine-38 are shown. The others are 26–44, 48–inner haem propionate, 49–outer haem propionate (twice), 55–74, 59–inner haem propionate, 63–58 and 79–47. It is proposed that the conformations of the complexes are in equilibrium between forms where the bottom loop is more or less open, and that in the (1–37)–(38–104) complex the presence of arginine-38 in the loop shifts that equilibrium in favour of the closed form.

group, and its main-chain carbonyl is hydrogen-bonded to the main-chain amide of tryptophan-59 (Takano & Dickerson, 1981). We propose that the role of residue arginine-38 is to participate in the shielding from solvent of the redox centre by providing a significant proportion of the binding energy pinning the loop formed by residues 36–59, which encloses the conventional bottom edge of the haem (Fig. 2), to the rest of the molecule.

Of the 11 hydrogen bonds that stabilize the interaction between the loop formed by residues 36–59 and the rest of the cytochrome *c* molecule, three are provided by the side chain of residue arginine-38 (Takano & Dickerson, 1981). In addition to these hydrogen bonds, it is suggested that there is an ionic bond between arginine-38 and the propionate carboxy group of the haem (Moore *et al.*, 1984). If the continuity of the peptide chain between residues 38 and 39 is maintained, these interactions will still have a stabilizing effect on the relationship between the loop and the rest of the molecule whether or not bond 37–38 is intact. If, on the other hand, bond 38–39 is broken, the interactions of arginine-38 will no longer influence the relationship between the loop and the rest of the molecule.

We have emphasized above that, in the former case [complex (1–37)–(38–104), with bond 38–39 intact], the properties of the derivative suggest a greater degree of stabilization of the loop enclosing the bottom edge of the

haem than in the latter case [complex (1–38)–(39–104), with bond 38–39 broken]. Thus our experimental results support the role that has been proposed for arginine-38.

Why is the complex (1–37)–(38–104) not fully functional? Although most of the loop 36–59 should still in principle be well stabilized in this analogue, a small part of the loop (namely residues 36–37) will not be, and may be more free to move than in the native molecule. Although it is difficult to imagine that access of solvent to the haem pocket would be as easy in this case as when virtually the whole loop has increased freedom to move, some adverse effect on function is to be expected. In addition, even in complex (1–37)–(38–104), one of the covalent bonds of the native structure is missing, and a new pair of ions has been created by the liberation of α -amino and α -carboxy groups.

Are the different specific activities of electron transfer observed for cytochrome *c* and the various complexes discussed here a consequence of differences in conformation affecting ability to bind to physiological partners, or of the differing redox potentials? N.m.r. spectroscopy shows that differences between the structures of complex (1–38)–(39–104) and cytochrome *c* are confined to the conformation of the loop formed by residues 36–59 itself (D. E. Harris & G. R. Moore, unpublished work). This region is diametrically opposite to the binding site of the cytochrome for oxidase and reductase (Takano & Dickerson, 1981). It therefore seems likely that changes in redox potential, and not changes to binding sites, are the governing factor. Differences between the observed redox potentials of the various analogues (Table 1) correlate with the differences in the biological activities as shown in Fig. 1, and Moore *et al.* (1984) have reported a potential for complex (1–37)–(39–104) 12 mV lower than that of complex (1–38)–(39–104).

The much greater similarity of complex (1–37)–(38–104) than of complex (1–38)–(39–104) to the native protein means that semisynthetic analogues of the former are likely to produce informative results in structure–function studies of cytochrome *c*.

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