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The semisynthesis of analogues of cytochrome *c*

Modifications of arginine residues 38 and 91

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The arginine residues at positions 38 and 91 of horse cytochrome *c* are absolutely conserved throughout eukaryotic evolution. For studies of the functional roles of these residues, we have prepared, by semisynthetic techniques, analogues of cytochrome *c* in which one or the other of the arginine residues has been modified. The products of modification by adduct formation with pentane-2,4-dione were purified and extensively characterized. In biological tests, the arginine-91-modified cytochrome *c* showed little difference in behaviour from native horse cytochrome *c*. Modification of arginine-38, however, led to extensive changes in biological and chemical properties. We also prepared and tested adducts with cyclohexane-1,2-dione and camphorquinone-10-sulphonic acid. The same effects on biological properties were noted irrespective of the nature of the modifying group. We suggest reasons for the differences in sensitivity of the two sites.

A useful way to explore the role of specific amino acid residues of a protein is to study the biological behaviour of analogues of the protein that incorporate substitutions at one or more sites. Wallace & Offord (1979) developed a strategy for the semisynthesis of analogues of horse heart cytochrome *c* that involves the covalent coupling of fragments produced by cleavage with CNBr. The alternative technique for producing such analogues, modification of intact proteins by side-chain-directed reagents, has certain limitations. Whole-protein chemical modification is at once too specific, in that modification reagents are available for rather few types of side chain, and too general, in that, unless there is but a single representative of the amino acid residue to be modified, then more than one is liable to suffer change. Nevertheless, where it can be applied, whole-protein chemical modification is a particularly simple technique, especially when compared with a typical scheme (e.g. Wallace & Offord, 1979) for protein semisynthesis.

We have combined elements of both techniques: side-chain modification of a fragment, followed by the use of that fragment as an intermediate in the semisynthesis of the protein.

Inspection of the sequences of the CNBr-cleavage fragments of cytochrome *c* reveals many sites amenable to specific modification by this approach. Several of these are highly conserved residues, believed to be crucial to function.

We present an example of the simplest possible case, where a protein may be cleaved into two fragments, one of which contains only one example of the residue to be modified. Horse heart cytochrome *c* contains two arginine residues, at positions 38 and 91 (Margoliash *et al.*, 1961); both are absolutely conserved in eukaryotic cytochromes *c*.

By using conditions that limit CNBr cleavage to residue 65 (Corradin & Harbury, 1970), we obtained two fragments, both of which contain only one arginine residue. These residues can be independently modified and the molecule resynthesized. Resynthesis follows the spontaneous association, under reducing conditions, of the two CNBr-cleavage fragments and occurs by specific aminolysis of the *C*-terminal homoserine lactone by the α-amino group, so that side-chain protection is unnecessary.

Abbreviations used: DHCH, 1,2-dihydroxycyclohex-1,2-ylene; DMP, 4,6-dimethyl-2-pyrimidyl; CQS, camphorquinone-10-sulphonate; Hse, homoserine.

Materials and methods

Materials

Horse heart cytochrome *c* (type III) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

CNBr was from Kodak, Kirkby, Liverpool, U.K. Cyclohexane-1,2-dione was from Aldrich Chemical Co., Gillingham, Dorset, U.K. Triethylamine was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Camphorquinone-10-sulphonic acid was kindly given by Dr. John Glass, Mt. Sinai Medical School, New York, NY, U.S.A. All other reagents were obtained from BDH Chemicals, Poole, Dorset, U.K., Fluka, Buchs, Switzerland, or Merck, Darmstadt, West Germany, and were of analytical grade. Boronate affinity gels were made by the method of Rose *et al.* (1981) or purchased from Amicon Corp., Danvers, MA, U.S.A. (Matrex PBA 30).

Cleavage of cytochrome c

Cytochrome *c* was cleaved with CNBr, at a 3:1 excess over protein (Corradin & Harbury, 1970). Digests were gel-filtered on Sephadex G-50 (fine grade) (column dimensions 135 cm × 2 cm or 150 cm × 4.4 cm) in 7% (v/v) formic acid. Fractions corresponding to residues 1–65 and residues 66–104 were freeze-dried and rechromatographed separately on the same column.

Modification of fragments

N^7N^8-(1,2-Dihydroxycyclohex-1,2-ylene)arginine-containing derivatives (DHCH derivatives) of fragment 1–65 were prepared by dissolving the peptide (at about 10 mg/ml) in deionized 8 M-urea, then adding an equal volume of 0.15 M-cyclohexane-1,2-dione in 0.25 M-sodium borate buffer, pH 9.0. After 2 h at 37°C an equal volume of 30% (v/v) acetic acid was added, and the product was dialysed at 4°C against several changes of 1% (v/v) acetic acid and freeze-dried. Urea is used to increase the solubility of the fragment and the accessibility of residues to be modified (Rose *et al.*, 1981).

Fragment 66–104 was dissolved directly (at about 20 mg/ml) in 8 M-urea made 0.15 M in cyclohexane-1,2-dione and 0.25 M in sodium borate buffer, pH 9.0. The solution was diluted, after 2 h at 37°C, by an equal volume of 30% (v/v) acetic acid and gel-filtered on Sephadex G-50 (fine grade) (column dimensions 92 cm × 2.6 cm) in 50% (v/v) acetic acid. Peptide-containing fractions were evaporated to a small volume on a rotary evaporator without heating, diluted with water and freeze-dried.

Two methods were used to prepare N^{δ}-(4,6-dimethyl-2-pyrimidyl)ornithine-containing derivatives (DMP derivatives).

(1) This method is an adaption of that of Morris *et al.* (1973). The peptide was dissolved in water (at about 60 mg/ml), and then to 1 vol. of the solution were added 1 vol. of triethylamine, 2 vol. of ethanol and 2 vol. of pentane-2,4-dione, in that order. The mixture was heated for 4 h at 95°C in a sealed tube, diluted with an equal volume of 50% (v/v) acetic acid and subsequently with half that volume of formic acid. The product was then gel-filtered on the Sephadex G-50 column equilibrated in 50% acetic acid. Peptide-containing fractions were evaporated to a small volume, diluted and freeze-dried.

(2) This method is an adaptation, with the use of urea, of the method of Gilbert & O'Leary (1975). The fragment was dissolved to about 10 mg/ml in a reagent solution made by adding pentane-2,4-dione (0.5 ml) to a saturated solution of $NaHCO_3$ in deionized 8 M-urea (4.5 ml) and adjusting the pH to 8.5 by dropwise addition of a solution (100 mg/ml) of Na_2CO_3 in deionized 8 M-urea. After 48 h at 37°C, the solution was either adjusted to pH 4 with careful addition of acetic acid or an equal volume of acetic acid was added to it, and gel-filtered on a column (150 cm × 4.4 cm) of Sephadex G-50 (fine grade) equilibrated in 7% formic acid. The peptide-containing fractions were freeze-dried directly.

Camphorquinone-10-sulphonate–arginine derivatives (CQS derivatives) were made by adding a 5-fold excess of camphorquinone-10-sulphonic acid to a solution of the fragment (1 mM) in 0.1 M-sodium borate, pH 8.8. The mixture was left at room temperature overnight and was then dialysed against three changes of distilled water. The non-diffusible residue was freeze-dried.

Purification and characterization of modified fragments

The extent of modification of the arginine residues in the DMP derivatives may be quantified by amino acid analysis. DMP-ornithine breaks down under the acid hydrolysis conditions employed (6 M-HCl containing 1% phenol, 108°C for 24 h) to release ornithine and no arginine (Gilbert & O'Leary, 1975). The degree of incorporation of the DMP moiety may also be calculated from the increase in absorption at 310 nm. Vetter-Diechtl *et al.* (1968) have shown the group to have a molar absorption coefficient of $5600\ M^{-1} \cdot cm^{-1}$ at pH 1.

The extent of modification of the arginine residues in the DHCH derivatives can be assessed by amino acid analysis. Although there is some regeneration of arginine under the standard hydrolysis conditions, inclusion of 10% mercaptoacetic acid in the 6 M-HCl yields an unidentified amino acid that is co-eluted with histidine, and no arginine regeneration is seen (Patthy & Smith, 1975).

CQS-arginine is not stable to the conditions of acid hydrolysis, and arginine is completely regenerated (Pande *et al.*, 1980).

Because the modification produces a charge change, modified fragments may be identified by their different elution properties on cation-exchange chromatography. Modified fragments are also retained by a boronate affinity gel, presumably because the diol forms a strong complex with borate

ion in mildly basic solutions, in analogy to the DHCH case (see Rose *et al.*, 1981).

The affinity gel may thus also be used for the purification of DHCH-arginine-containing fragments, as unmodified peptides are not retained by the gel when passed in 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 8.5. In either case the modified fragments were released from the gel by lowering the pH with 1% (v/v) acetic acid.

Ion-exchange chromatography is effective for the purification of DMP-ornithine-containing derivatives, for this modification, like the formation of the CQS adduct, results in a charge change. In both cases peptide was dissolved in 10mM-potassium phosphate buffer, pH 7.0, and chromatographed on SP-(sulphopropyl-)Sephadex C-25 cation-exchanger with a linear gradient from 10mM- to 200mM-potassium phosphate buffer, pH 7.0.

Resynthesis of modified cytochrome c

Freeze-dried protein fragments were dissolved, at concentrations of 0.2–0.4mM in 0.1 M-sodium borate buffer, pH 8.5 (DHCH derivatives), or 0.05 M potassium phosphate buffer, pH 7.0 (DMP or CQS derivatives), to make an equimolar mixture of modified 1–65 fragment and native 66–104 fragment, or of native 1–65 fragment with modified 66–104 fragment. Sufficient $Na_2S_2O_4$ was added to ensure complete reduction of the degassed mixture, which was then sealed in a glass-walled syringe so as to exclude all air. Under these conditions a strong complex is formed between the two fragments that leads to the spontaneous re-formation of the peptide bond between Hse-65 (which as the lactone form is mildly activated) and Glu-66 (Corradin & Harbury, 1971, 1974). After 24h at 20°C, mixtures were gel-filtered on Sephadex G-50 (fine grade) in 7% (v/v) formic acid (column dimensions 150cm × 4.4cm) and the coupled product was freeze-dried.

Purification and characterization of products

Freeze-dried coupled products were treated with 8 M-urea in 0.1 M-potassium phosphate buffer, pH 7.0, and then freed from urea on a gel-filtration column of Sephadex G-25 equilibrated in 0.1 M-potassium phosphate buffer. This treatment has the effect of renaturing molecules denatured by freeze-drying from 7% formic acid (Boon *et al.*, 1979).

Products were purified by either cation-exchange chromatography (DMP and CQS derivatives) or passage through a boronate affinity column (CQS and DHCH derivatives) as described above for fragment purification, to remove either unmodified cytochrome, or polymers of fragments, that are co-eluted with the desired product on gel filtration.

Purified cytochrome *c* analogues were checked for complete modification by amino acid analysis, and, for the DMP analogues, by the increase in absorption at 310nm. The absorption spectra of ferricytochrome and ferrocytochrome forms in the visible region were obtained on a Varian Superscan 3 spectrophotometer. Reduction to the ferrocytochrome form was effected with a single crystal of $Na_2S_2O_4$ added to the cell.

Redox potentials were determined by the method of mixtures (Davenport & Hill, 1952).

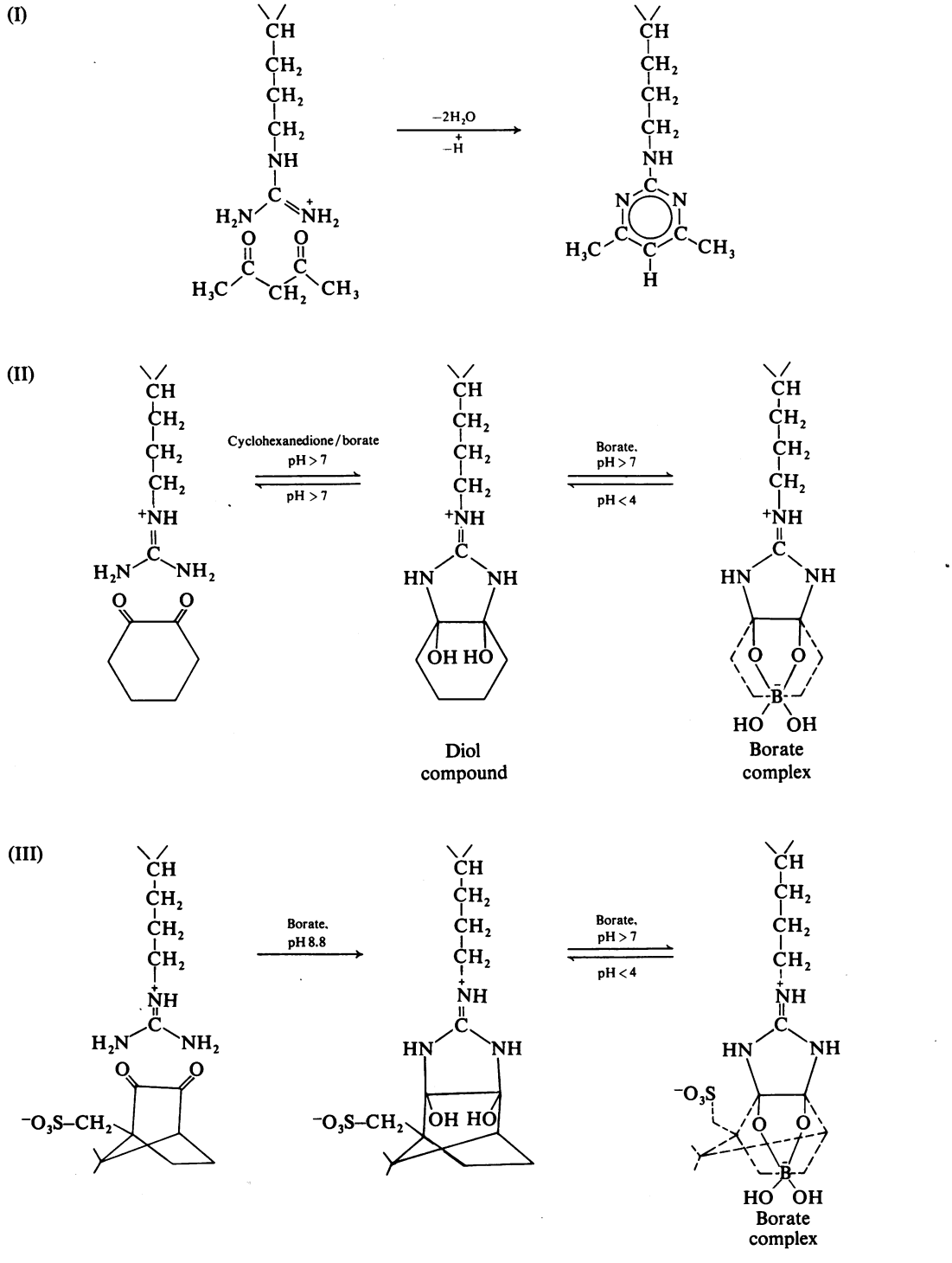
Biological activity was determined by the ability to stimulate succinate oxidation in the cytochrome *c*-depleted-mitochondria system of Jacobs & Sanadi (1960), as employed by Harris & Offord (1977).

Results and discussion

The structures of the adducts formed by reaction of arginine with the reagents employed are shown in Scheme 1. Modification of fragment 1–65 by a single treatment with pentane-2,4-dione or cyclohexane-1,2-dione resulted in the complete disappearance of arginine from amino acid analyses of the products, and the appearance of ornithine or an increase in the histidine value respectively (Table 1). The increase in 310nm absorbance of DMP-modified fragment 1–65 over unmodified fragment 1–65 is equivalent to the incorporation of 1.0DMP group/molecule. Treatment of fragment 1–65 with camphorquinone-10-sulphonic acid under conditions known to cause complete reaction with ribonuclease fragment 1–21 and soya-bean trypsin inhibitor resulted in an almost quantitative change in elution properties in the ion-exchange system described above.

Although DMP modification of fragment 1–65 was seen to have gone to completion, the product was purified by ion-exchange chromatography to remove an unwanted side product. This material was produced in greater quantities by method (1), for which reason method (2) is generally preferred for the formation of this fragment 1–65 derivative. The side reaction may involve attack on the haem group of this fragment, for the side product is discoloured. As it did not bind to the SP-Sephadex C-25 column under the ion-exchange conditions used, separation of this material from fragment with normal spectral characteristics was easy.

Complete modification of fragment 66–104 was more difficult to achieve. In some cases a second treatment with reagent was sufficient to decrease the arginine content to zero {[DMP-Orn^{91}]fragment 66–104 in Table 1, for example, received two treatments with method (1)}. With method (2) for the formation of the DMP derivative, spectrophotometric determination showed 24% modification after one cycle and 40% after two. Two cycles of cyclohexane-1,2-dione treatment gave a product with 0.15 residual arginine residue/molecule ([DHCH-Arg^{91}]fragment 66–104 in Table 1). Unmodified material was removed in these cases,



Scheme 1. *Chemical modification of arginine residues*
(I) Formation of DMP-ornithine; (II) formation of DHCH-arginine; (III) formation of CQS–arginine adduct. Reaction conditions are detailed in the Materials and methods section.

Table 1. *Amino acid compositions of native and modified CNBr-cleavage fragments 1–65 and 66–104 of cytochrome c*
Fragment 1–65 and its derivatives are [Hse^{65}]. Values given in parentheses refer to the known amino acid compositions of fragments 1–65 and 66–104 of horse heart cytochrome *c* (Corradin & Hanbury, 1970). —, Not determined.

	Amino acid composition (mol of residue/mol)					
Amino acid	Fragment 1–65	[DHCH-Arg^{38}]-fragment 1–65	[DMP-Orn^{38}]-fragment 1–65	Fragment 66–104	[DHCH-Arg^{91}]-fragment 66–104	[DMP-Orn^{91}]-fragment 66–104
Asp	5.0 (5)	5.2	5.1	3.0 (3)	3.0	3.3
Thr	7.3 (7)	5.9	6.3	2.5 (3)	2.8	3.1
Ser	0.0	0.0	0.0	0.0	0.0	0.0
Glu	7.2 (7)	6.6	6.5	4.7 (5)	4.7	5.0
Pro	2.1 (2)	—	—	— (2)	—	—
Gly	10.0 (10)	10.0	10.3	2.8 (2)	2.1	2.4
Ala	3.2 (3)	3.0	3.1	3.4 (3)	3.4	3.5
Cys	— (2)	—	—	—	—	—
Val	2.9 (3)	2.1	2.2	0.0	0.0	0.0
Met	0.0	0.0	0.0	0.4 (1)	0.7	0.9
Ile	1.9 (2)	1.4	1.5	3.4 (4)	3.3	2.4
Leu	3.0 (3)	3.0	3.0	2.5 (3)	2.9	2.6
Tyr	1.0 (1)	0.9	1.0	1.9 (3)	2.7	2.6
Phe	2.8 (3)	2.3	2.5	1.0 (1)	0.9	0.7
His	3.3 (3)	4.3	3.3	0.0	1.1	0.0
Lys	11.4 (11)	11.5	11.1	7.6 (8)	7.4	7.2
Arg	1.0 (1)	0.0	0.0	1.1 (1)	0.1	0.0
Orn	0.0	0.0	0.9	0.0	0.0	0.6

and in that of [CQS-Arg^{91}] fragment 66–104, where the extent of modification was not quantified, by one of the purification methods described in the Materials and methods section, or, more conveniently, by purification of the final product.

When coupling mixtures were gel-filtered, good separation of product cytochrome *c* from non-coupled fragments was noted (Fig. 1). In those mixtures where fragment 66–104 contained the modification, uniformly good yields were observed; by comparison, the yields with modified fragments 1–65 were low. This difference must reflect a diminished capacity of the latter modified fragments to form a productive complex, and implies a structural derangement induced in the complex by a change in functional group at position 38, but not by change at position 91.

Table 2 gives the amino acid compositions determined for the other cytochrome *c* analogues, with the exception of the putative [CQS–Arg^{38}]- and [CQS–Arg^{91}]-cytochromes *c*. In each case loss of one arginine residue is accompanied by the appearance of ornithine or an increased histidine content. The comparison of spectra of [DMP-Orn^{38}]cytochrome *c* and native cytochrome *c* shows an increase in 310nm absorption corresponding to incorporation of 1 DMP group/molecule.

By virtue of the resynthesis method described above, all the cytochrome *c* analogues prepared contain homoserine at residue 65. This substitution

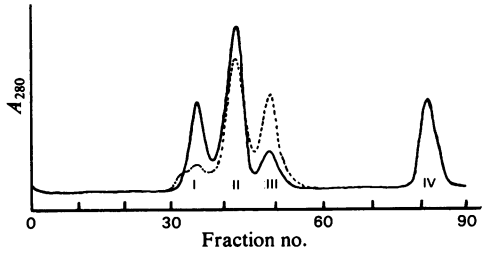


Fig. 1. *Superimposition of elution profiles of Sephadex G-50 gel filtration of coupling mixtures for synthesis of [DHCH-Arg^{38}]cytochrome c* (------) *and [DMP-Orn^{91}]-cytochrome c* (——)
Peak I is product cytochrome, peak II is fragment 1–65, peak III is fragment 66–104, and peak IV is salt. For details of the separation system see the Materials and methods section.

does not significantly affect the chemical or physical properties of cytochrome *c* (Corradin & Harbury, 1971, 1974; Boswell *et al.*, 1981), and so, for convenience, no further mention of this fact is made.

The effects of these modifications on the physical and biological properties of the product cytochromes *c* depends much more on the site of the modification than on the nature of the modifying group. All three modifications have the effect of

Table 2. *Amino acid compositions of cytochrome c analogues compared with those for the native and [Hse^{65}]proteins*
All analogues are [Hse^{65}]. Hydrolysis of [DMP-Orn^{38}]cytochrome *c* was performed at 110–120°C, which accounts for the low recovery of threonine; the analysis of [Hse^{65}]cytochrome *c* hydrolysed under the same conditions is included for comparison. Values given in parentheses refer to the known composition of horse heart cytochrome *c* (Corradin & Hanbury, 1970). Abbreviation: Cyt.*c*, cytochrome *c*. —, Not determined.

	Amino acid composition (mol of residue/mol)					
Amino acid	Cyt.*c*	[DHCH-Arg^{91}]-Cyt.*c*	[DMP-Orn^{91}]-Cyt.*c*	[DHCH-Arg^{91}]-Cyt.*c*	[DMP-Orn^{38}]-Cyt.*c*	[Hse^{65}]-Cyt.*c*
Asp	7.9 (8)	8.0	8.2	8.5	8.1	8.5
Thr	9.5 (10)	9.6	10.0	9.6	4.6	5.1
Ser	0.0	0.0	0.0	0.0	0.0	0.0
Glu	11.7 (12)	11.8	12.1	12.3	12.8	12.0
Pro	4.4 (4)	—	4.6	5.1	2.3	4.5
Gly	12.0 (12)	13.4	13.1	12.7	12.0	13.4
Ala	5.9 (6)	6.4	6.0	5.9	6.7	6.2
Cys	— (2)	—	—	—	—	—
Val	3.0 (3)	3.5	3.8	3.0	3.9	4.0
Met	1.4 (2)	0.5	—	1.0	0.9	1.0
Ile	5.8 (6)	4.4	5.4	4.9	4.7	5.2
Leu	6.0 (6)	5.8	6.0	6.0	5.5	6.0
Tyr	3.8 (4)	2.8	3.4	3.5	2.4	3.2
Phe	4.0 (4)	3.7	4.1	3.5	4.3	3.3
His	2.7 (3)	4.4	3.5	4.7	3.3	2.9
Lys	17.5 (19)	17.8	21.0	17.7	16.5	16.4
Arg	1.9 (2)	1.0	0.9	1.2	1.2	2.2
Orn	0.0	0.0	0.8	0.0	0.8	0.0

significantly increasing the bulk of the side chain; but, whereas the DHCH-arginine retains its positive charge at physiological pH, DMP-ornithine is uncharged and CQS–arginine has no net charge (Scheme 1).

All analogues containing modifications at residue 38 show very low biological activity (Fig. 2). [CQS–Arg^{38}] and [DMP-Orn^{38}] analogues were obtained in sufficient quantity for spectral and electrochemical studies. The spectra exhibit characteristics typical of loss of Met-80 co-ordination to the haem iron. The weak 695nm absorption is absent and there are shifts in the maxima of other bands in the oxidized forms (Fig. 3).

Reducibility of the analogues by ferrocyanide was slight, so that the determinations of redox potential were made at the limits of detectability by the system employed. Both analogues, though, gave almost identical values, nearly 100mV lower than that of native cytochrome *c* (Table 3).

All these effects are characteristic of a gross perturbation of the structure of the haem crevice (Dickerson & Timkovitch, 1976) and indicate an important structural role for Arg-38. In their description of the three-dimensional structure of tuna cytochrome *c* at 0.15nm (1.5Å) resolution, Takano & Dickerson (1981) show the side chain of Arg-38 to be lying half-buried on the molecular surface at the bottom of the haem crevice. The guanidino group makes hydrogen bonds with the inner haem propionate group and the main-chain carbonyl group of Trp-33 (His-33 in horse cytochrome *c*). The primary function of the residue, they postulate, is to 'fill space'.

Substitution of the guanidino groups could cause a loosening of the crevice by driving in a 'wedge' of increased bulk at this point, or by disrupting a structurally important hydrogen bond, or both.

By contrast, analogues modified at Arg-91 show little change in biological properties. Their spectra are identical with that of cytochrome *c*, including the presence of the diagnostic 695nm band (Fig. 3), and they have biological activities (Fig. 2) and redox potentials (Table 3) close to or identical with those of the native protein. Thus this conserved residue is inessential to the basic electron-transport function of the cytochrome *c*.

Takano & Dickerson (1981), in their summary of the roles of the invariant residues in the cytochrome *c* sequence, suggest that the primary function of this residue, which forms a hydrogen bond to the main-chain carbonyl group of Ile-85, is to 'stabilize 80's chain'. However, if such a function is not essential, or if the hydrogen bond can be provided by side chains with quite widely differing chemical properties (Scheme 1) it is noteworthy that residues of no other amino acid have ever appeared at this position throughout the evolution of the eukaryotes.

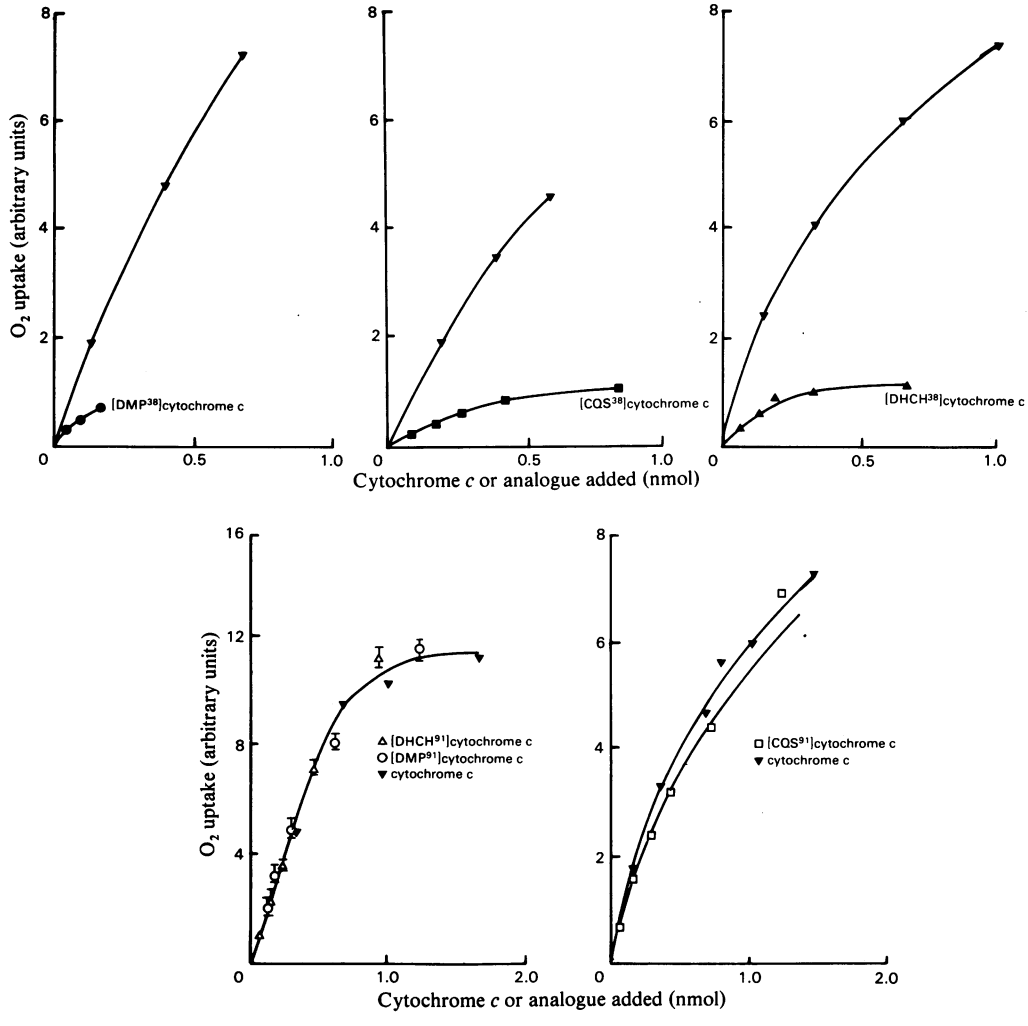


Fig. 2. *Biological assays of arginine-modified cytochromes c*

Stimulation of oxygen uptake plotted versus quantity of added cytochrome *c* or analogue in the cytochrome *c*-depleted-mitochondria assay method of Jacobs & Sanadi (1960). ▼, Cytochrome *c*; ●, [DMP-Orn^{38}]cytochrome *c*; ■, [CQS–Arg^{38}]cytochrome *c*; ▲, [DHCH-Arg^{38}]cytochrome *c*; ○, [DMP-Orn^{91}]cytochrome *c*; □, [CQS–Arg^{91}]cytochrome *c*; △, [DHCH-Arg^{91}]cytochrome *c*. All analogues are [Hse^{65}].

Table 3. *Redox potentials determined by the method of mixtures for cytochrome c and analogues*

Experimental details are indicated in the text. Values for the Arg-38 analogues are approximate, being at the limits of the range of detection of the system used.

Cytochrome	Redox potential (mV)
Cytochrome *c*	258
[Hse^{65}]cytochrome *c*	258
[CQS–Arg^{91}]cytochrome *c*	258
[DMP-Orn^{91}]cytochrome *c*	248
[CQS–Arg^{38}]cytochrome *c*	~170
[DMP-Orn^{38}]cytochrome *c*	~170

Conclusions

(1) Analogues of cytochrome *c* incorporating specific modifications to arginine residues can be prepared by a combination of semisynthesis and chemical-modification techniques. The methodology could be extended to yield analogues modified at other functionally important residues in this and other proteins.

(2) Modification of the evolutionary invariant Arg-38 residue changes the spectral and electrochemical properties of the analogue and renders it

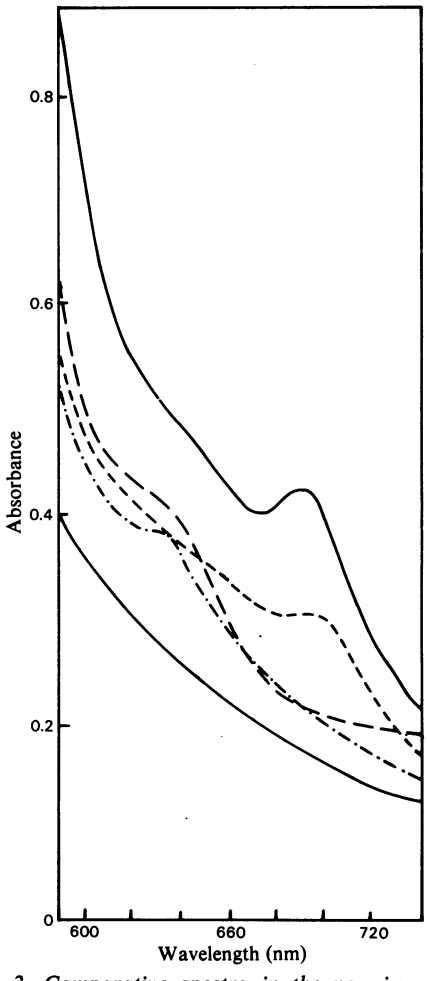


Fig. 3. *Comparative spectra in the near-i.r. region of cytochrome c* (——, *at top oxidized, at bottom ascorbate-reduced*) *with the analogous* [*DHCH-Arg*^{91}]*cytochrome c* (– – – –), [*CQS-Arg*^{38}]*cytochrome c* (— — —) *and* [*DMP-Orn*^{38}]*cytochrome c* (—·—·—)

The concentrations of the cytochrome *c* solutions were about 50mM, and of the analogues about 30mM. The cytochromes modified at residue 38 lack the characteristic band at 695nm, said to be indicative of correct co-ordination of Met-80 at the sixth ligand position.

inactive. This effect supports the hypothesis for the role of this residue developed from crystallographic studies.

(3) Modification of the Arg-91 residue, also invariant throughout eukaryote evolution, does not change the physicochemical properties of cytochrome *c*. Full activity is retained. We therefore believe that the importance of this residue is in a role peripheral to electron transport between phosphorylation sites II and III or the mitochondrial electron-transport system.

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