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Protein engineering of cytochrome *c* by semisynthesis: substitutions at Glutamic acid 66

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We have used protein semisynthesis to prepare four analogues of horse cytochrome *c*, in which the glutamic acid residue at position 66 has been removed and replaced by norvaline, glutamine, lysine and, as a methodological control, glutamic acid. This residue is quite strongly conserved in mitochondrial cytochrome *c*, and forms part of a cluster of acidic residues that occurs in all cytochromes *c* but whose function is obscure. Comparative studies of the physical and biochemical properties of the analogues have now disclosed two specific roles for Glu^{66} in the protein. It contributes significantly to the stabilization of the active conformation of the protein, probably by salt bridge formation, and it appears to participate in the redox-state-dependent ATP-binding site of cytochrome *c*. Our results also support two general views of the role of surface charged residues in cytochrome *c*, namely that their disposition influences both redox potential, through the electrostatic field felt at the redox centre, and the kinetics of electron transfer, through the dipole moment they generate.

Key words: ATP binding/conformational stabilization/cytochrome *c*/semisynthesis/structural analogues

Introduction

The function of mitochondrial cytochrome *c*, like many other proteins, is strongly dependent on the nature and distribution of the charged surface residues (Margoliash and Bosshard, 1983). The protein is unusual in its great predominance of basic residues (Dickerson and Timkovitch, 1975), and it has been shown that some of these residues participate directly in the binding of the cytochrome to its physiological partners. These residues tend to be evolutionarily invariant. Even those basic residues not thus implicated are quite well conserved, and the proposal has been made that the ensemble is important in setting the dipole moment of the protein, which in turn orients the cytochrome during its approach to partners and increases the productivity of collisions (Margoliash and Bosshard, 1983).

It also seems probable that surface charge plays a role in determining the redox potential of the haem iron (Rees, 1980) and that basic residues participate in the specific binding of certain physiologically important anions (Corthésy and Wallace, 1986) and in surface salt-bridges that confer structural stability (Osheroff *et al.*, 1980).

In contrast, little is known of the specific role of surface acidic residues, although the unusual distribution of carboxylate groups was remarked upon some time ago (Dickerson, 1972). In the horse protein, nine of the 12 acidic groups are clustered in the conventional upper left quadrant, and a glutamate is presented to the surface at each turn of the 62–70 helix. While it is possible that this arrangement has evolved to fulfil the demand for a particular dipole moment, the striking nature of the distribution suggests other, more specific roles for these residues. Such a view may be reinforced by the conservatism exhibited by the cluster and, frequently, by the individual residues composing it. The pattern of conservation of the acidic residues composing the left-side helix in 67 mitochondrial cytochromes *c* is shown in Table I

As a first step in the exploration of the role of these residues by protein engineering, we have prepared analogues of Glutamate 66, one of the highly conserved residues of the helix, by semisynthesis In protein semisynthesis the native protein is fragmented by chemical or enzymic means, the peptide that contains the residue of interest is manipulated to achieve the desired structure and the fragments are recombined to yield a specific analogue of the original structure (Offord, 1980).

Site-directed mutagenesis has recently been used for the preparation of yeast cytochrome *c* analogues (Pielak *et al.*, 1985), and chemical modification has yielded many useful derivatives of the horse protein for structure–function studies (Dickerson and Timkovitch, 1975); but the bulk of known analogues has been prepared by semisynthetic techniques.

The highly developed technology, combined with the versatility of the method and its capacity to cope with non-coded amino acids and prosthetic groups, means that semisynthesis plays a major role in studies of cytochrome *c* As of now, 15 (nine of them absolutely conserved) of 104 residues have been examined, involving the creation of 40 substitutions (for examples, see Wallace, 1979; Koul *et al*, 1979; Boon *et al.*, 1981; Wallace and Rose, 1983; Ten Kortenaar *et al.*, 1983). Additionally, semisynthetic techniques have been used to create many other analogues, including regiospecific modifications (Wallace, 1984a), interspecies chimerae (Wallace *et al.*, 1986) and two-fragment complexes (Proudfoot *et al.*, 1986).

Materials and methods

Materials

Cytochrome *c* (type III) and N^{α}-Boc-glutamine-N-hydroxysuccinimido ester were from Sigma Chemical Co., Munich, FRG N^{α},ϵ-di-Boc-lysine-N-hydroxysuccinimido ester was from Bachem, Bubendorf, Switzerland N^{α}-Boc-glutamic acid (O^{γ}-*t*-butyl ester) (Koch-Light, Colnbrook, UK) and N^{α}-Boc-norvaline (Sigma) were converted to the corresponding N-hydroxysuccinimido esters in this laboratory (Wallace and Offord, 1979) Phenylisothiocyanate and trifluoracetic acid were sequencer grade from Fluka, Buchs, Switzerland Other reagents and solvents were from Merck, Darmstadt, FRG, and were of the highest available purity

ϵ-Amino protection of cytochrome c

Side-chain functional groups of peptides that are capable of participating in the reactions employed for fragment modification or condensation must be temporarily blocked during the semisynthesis This is most conveniently performed prior to fragmentation, thus permitting, for example, the distinction between α- and ϵ-amino groups

Lysine side-chains were blocked by the acetimidyl group, using the method of Wallace and Harris (1984) Quantitative reaction, and lack of side-reactions, were checked by the methods of Corthésy and Wallace (1986) Since the functional consequences of complete acetimidylation are minor (Wallace, 1984a), the protecting group was not removed at the end of the semisynthesis

Table I. Distribution of acidic residues in the left-side helix of mitochondrial cytochromes *c*

Species	No	Acidic residues		
Man, chimp, rhesus, horse, donkey, zebra, cow, pig, sheep, dog, rabbit, kangaroo, chicken, turkey, emu, penguin, duck, pigeon, turtle, rattlesnake, bullfrog, carp, fruitfly, screwworm-fly, *Samia cynthia*, moth, *Humicola*, *Ustilago*, camel, whale seal	31	6162	66	69
Bat, bonito, *Debaromyces*, *Candida*, *Neurospora*	5	61	66	69
Tuna	1	62	66	69
Snail (Asn^{61}, Gln^{62}, Gln^{66})	1			69
Dogfish, lamprey	2	62		69
Crithida	1	62	65	69
Euglena	1	6162		69
Acer	1		66	
Nigella, mung, cauliflower, pumpkin, hemp, elder, abuliton, cotton, castor, tomato, maize, arum, sesame, leek, niger, sunflower, nasturtium, parsnip, wheat, buckwheat, spinach, ginkgo, rape, *Saccharomyces*	24	61	66	

Fragmentation of acetimidyl cytochrome c

In a semisynthesis it is desirable that the number of fragment condensations be kept to a minimum, so cleavage methods with narrow specificity are favoured CNBr cleavage is a suitable method since the protein contains only two methionine residues, and semisyntheses using the three fragments thus generated have been reported (Wallace and Offord, 1979, Wallace, 1979, Koul *et al*., 1979) However, under certain conditions reaction can be partially limited to the non-essential Methionine residue 65 (Corradin and Harbury, 1970) Since in the present work the object is modification of residue 66, the cleavage method of Corradin and Harbury (1970) for generation of fragments (1–65) and (66–104) (with methionine 80 intact) was adopted

Separation and purification of fragments

CNBr digests were gel-filtered on Sephadex G-50 in 7% formic acid (column dimensions 150 × 4 4 cm) Fractions corresponding to fragments (1–65) and (66–104) were pooled and lyophilized, then repurified by cation-exchange chromatography on trisacryl-SP (LKB) using the conditions described by Proudfoot *et al* (1986) for fragments (1–38) and (39–104)

Sequential degradation of fragment (66–104)

The Edman degradation was used to truncate the peptide (Wallace and Offord, 1979) Acetimidyl fragment (66–104) was dissolved in 50% pyridine at 0 5 mg/ml To 2 vol of this solution was added 1 vol of a 5% solution of phenyl-isothiocyanate in pyridine The mixture was degassed, sealed under N_2 and left at 30°C for 2 h, then dried under high vacuum The phenylthiocarbamyl peptide is stood in anhydrous TFA (10 mg/ml) at room temperature for 1 h, then dried

Truncated peptide is freed of phenylthiohydantoin by partition of the latter into *t*-butyl acetate from dilute aqueous HCl, and finally gel-filtered on Sephadex G25 in 0 01 M HCl and freeze-dried

Stepwise elongation of fragment (67–104)

The N^{α}-Boc amino acid active esters listed in the Materials section were used for coupling to acetimidyl fragment (67–104) by methods based on those of Wallace and Offord (1979) To a solution of peptide HCl salt (1–5 mM) in anhydrous redistilled DMF or DMSO was added 10 equivalents of hydroxybenzotriazole, active ester and N-ethyl morpholine, in that order In one case (lysine) a three-fold excess was also employed The mixture was left at room temperature overnight At intervals aliquots corresponding to 10 nmol peptide were removed to Whatman no 4 paper for descending chromatography in butanol acetic acid water pyridine (30 6 24 20) Dried chromatograms were stained with ninhydrin

The bulk coupling mixture was diluted with dilute HCl (resulting pH 2.5–3 0), extracted twice with ethyl acetate and dried The residue was redissolved in anhydrous TFA (containing 1% w/v methionine) at about 5 mM and left at room temperature for 30 min, then precipitated with 5 vol of ice-cold ether The pellet was washed with ether, dried, redissolved in 8 M urea and chromatographed on an SP-trisacryl cation-exchanger, using the system described by Proudfoot *et al* (1986)

The purified product was desalted on Sephadex G25 in 1% acetic acid, lyophilized and redissolved (at 1 mg/ml) in 0.2 M Tris-HCl buffer, pH 8 5, made 10% in mercaptoethanol, before standing for 12 h at 30°C This treatment is intended to cause reversion to methionine of any methionine sulphoxide formed during coupling After drying and a final desalting, the product semisynthetic acetimidyl fragment (66–104) is ready for coupling to acetimidyl fragment (1–65)

Characterization of the semisynthetic fragment (66–104)

The successful replacement of residue 66 was controlled by amino acid analysis, and end-group determination by dansylation, of intermediates and final products, using standard procedures

Condensation of fragments (1–65) and (66–104)

In neutral aqueous solution these two fragments will form a stable complex that duplicates the conformation of the native protein (Corradin and Harbury, 1971) In the dithionite-reduced complex the missing peptide bond 65–66 is reformed by aminolysis of the C-terminal homoserine lactone of 1–65 by the α-amino group of 66–104 (Corradin and Harbury, 1974) For this reason all the semisynthetic analogues discussed in this paper have homoserine, not methionine, at position 65 This substitution does not affect the functional or physical properties of cytochrome *c* (Boswell *et al*., 1981)

The technique of Corradin and Harbury (1971, 1974), as simplified by Wallace and Rose (1983), was used to resynthesize the complete molecule from natural acetimidyl (1–65) and semisynthetic acetimidyl (66–104) fragments

Coupled product was separated from unreacted fragments by gel-filtration and purified by ion-exchange, by the methods used in fragment preparation or by the method of Wallace and Harris (1984)

Characterization of semisynthetic cytochromes c

The amino acid compositions of the purified products were determined, though the method cannot distinguish between [Gln^{66}] and [Glu^{66}] cytochrome *c*. In these cases paper electrophoresis at pH 6 5 (Offord, 1977) of chymotryptic digests of semisynthetic and native cytochromes was used to show whether peptide (60–67) contained glutamine or glutamic acid

Spectroscopy

U v -visible spectra were drawn over the range 250–750 nm using a Cary 210 spectrophotometer

Redox potentials

Oxidation–reduction potentials of semisynthetic cytochromes *c* were determined by the method of mixtures described by Wallace *et al* (1986)

Biological assays

All analogues were tested in the depleted-mitochondrial succinate oxidase assay system of Jacobs and Sanadi (1960) The method has been discussed by Wallace (1984b).

Equilibrium gel-filtration

Determinations of the anion-binding capacity of [Lys^{66}] cytochrome *c* were made using the method described by Corthésy and Wallace (1986)

Results

Ion-exchange chromatography has proved valuable not only for the separation of coupled products from unreacted materials or side-products, but also in an analytical role where modification involves a charge change. Table II shows the ionic strengths of elution of acetimidyl fragment (66–104) and its analogues, and the end groups determined by dansylation for these materials.

The product of Edman degradation of (66–104) gave two

Table II. Ionic strengths (expressed as conductivities) of phosphate–urea buffers eluting derivatives of fragment (66–104), and the end-groups of those fragments

Fragment	Conductivity (ms)	End-group
66–104	5 1	Glu
Glu.67–104	4 5	None
67–104	7 2	Tyr
[bis-Boc Lys[66]] 66–104	4 6	None
[Lys[66]] 66–104	9 7	Lys
[Glu[66]] 66–104 (semisynthetic)	5 0	Glu
[Nva[66]] 66–104	7 5	Nva
[Gln[66]] 66–104	7 4	Glu

The fragment or its derivatives were loaded on trisacryl-SP cation-exchanger in a 0 02 M potassium phosphate buffer, pH 7 0, in 7 M urea, and eluted from that column by a gradient between the loading buffer and a 0 2 M potassium phosphate–7 M urea buffer, pH 7 0 Conductivities of peak tubes were measured on a Radiometer CDM 3

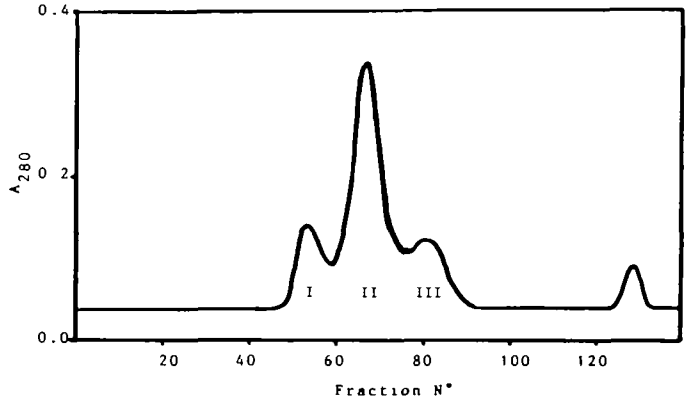

Fig. 1. Elution profile of gel-filtration on Sephadex G50 of a coupling mixture containing acetimidyl fragment (1–65) (**peak II**) and acetimidyl([Lys[66]] 66–104) (**peak III**) **Peak I** is product acetimidyl[Lys[66]] cytochrome *c* Conditions as in text

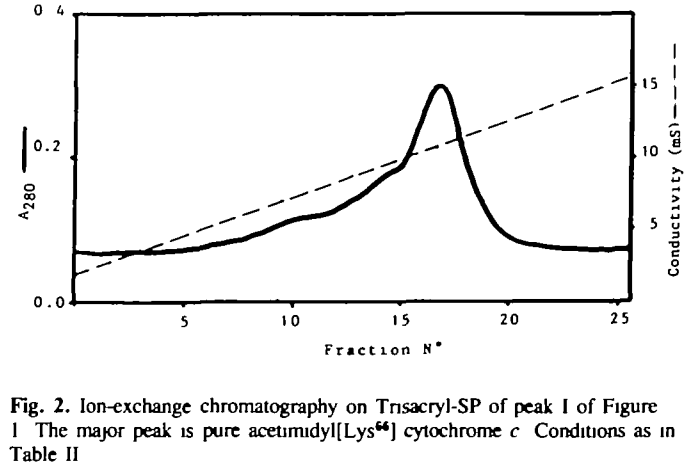

Fig. 2. Ion-exchange chromatography on Trisacryl-SP of peak I of Figure 1 The major peak is pure acetimidyl[Lys[66]] cytochrome *c* Conditions as in Table II

peaks on ion-exchange chromatography. The major peak eluted later in this system than the starting material, as would be expected after removal of an acidic residue. The minor peak eluted at the (66–104) position. Since no end-group was determined this is likely to be the pyroglutamic acid form of the peptide. Peptide (66–104) is known to be prone to such cyclisation (Wallace and Offord, 1979).

Table III. Oxidation–reduction potentials of cytochrome *c* analogues

Cytochrome or analogue	Redox potential (mV)
Native horse cytochrome *c*	258
Acetimidyl cytochrome *c*	245
Acetimidyl[Gln[66]] cytochrome *c*	261
Acetimidyl[Lys[66]] cytochrome *c*	269

Potentials were determined by the method of mixtures in 0 05 M phosphate buffer, pH 7 0, using the ferricyanide/ferrocyanide couple to set the redox equilibrium (Wallace *et al* , 1986)

Ninhydrin staining of paper chromatograms showed that, at excesses of protected amino acid active esters over acetimidyl peptide (67–104) of 10-fold, the α-amino group was completely concealed after overnight reaction. Three-fold excesses did not produce quantitative reaction, as shown by the presence of a substantial residual peak, eluting at 7.2 mS, after coupling of lysine. However, with a lower excess, substantially less material eluting at other positions was seen, with a consequently greater total recovery of desired product. Final yields of up to 29% of ([Lys[66]] 66–104) relative to the native (66–104) employed were obtained

Untreated semisynthetic peptides did not couple well with (1–65), but after mercaptoethanol treatment yields of 30–40% of resynthesized protein were the norm (Figure 1), except in the case of [Nva[66]] cytochrome *c*, where the recovery was much lower. Ion-exchange purification of the high mol wt peak from gel-filtration rid the products of small amounts of contaminants (Figure 2) and they eluted in the conditions of Wallace and Harris (1984) at values of ionic strength consistent with the changes in net charge induced: [Glu[66]] 25 mS, [Nva[66]] and [Gln[66]] 33 mS, [Lys[66]] 41 mS.

The expected amino acid compositions were obtained in each case, and the paper electrophoretic analysis demonstrated that in acetimidyl[Gln[66]] cytochrome *c*, peptide (60–67) contained glutamine and not glutamic acid. In the chymotryptic digest of cytochrome *c*, this is the sole peptide to migrate towards the anode at pH 6.5 (mobility relative to aspartic acid: observed, 0.45; calculated for mol. wt 1011, charge −2, 0.48). This peptide is missing from the digest of acetimidyl[Gln[66]] cytochrome *c*, but a new acidic peptide of mobility 0.24 appears (calculated for mol. wt 1011, charge −1, 0.26).

Typical cytochrome *c*-like spectra, including the diagnostic 695 nm band, were recorded for all the analogues but one. In acetimidyl[Nva[66]] cytochrome *c*, this absorption band, which signals co-ordination of the haem iron by methionine sulphur in the ferric state, and which is lost upon even minor perturbation of the protein conformation (Dickerson and Timkovitch, 1976), is missing.

Normally, the 695 band disappears with rising pH, with a p*K* for the transition in acetimidyl cytochrome *c* of 9.95 (Wallace, 1984b). However, the p*K* observed for the Lys[66] analogue is 9.25, and that of the Gln[66] analogue 9.20, in 0.1 M potassium phosphate buffer. At low ionic strength the difference is yet more pronounced (Lys[66] analogue, 7.95; acetimidyl cytochrome *c*, 9.50).

Redox potentials were determined for both Glu[66] and Lys[66] analogues (Table III), giving values close to, but somewhat higher than, the parent acetimidyl cytochrome *c*. All analogues were assayed for biological activity (Figure 3). Most gave values of >90% of the parent protein, but that of the Nva[66] analogue was only about 20%.

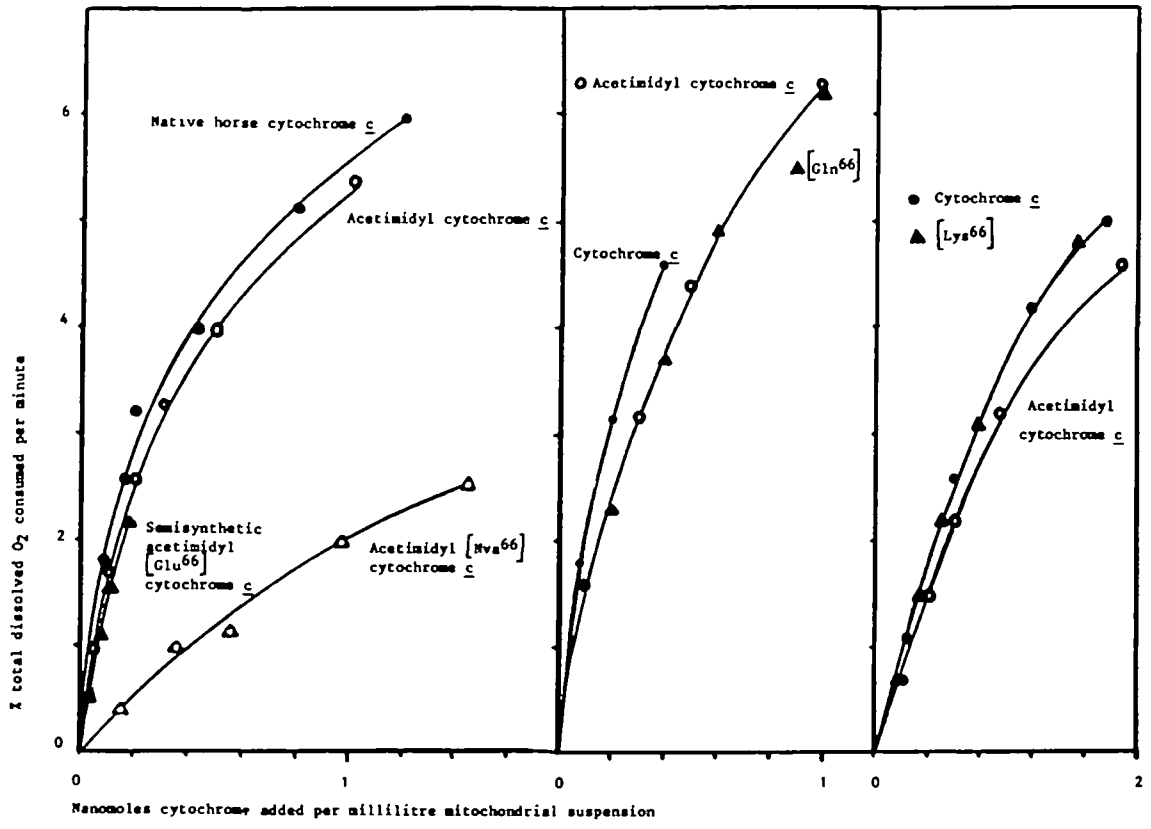

Fig. 3. Biological activities of cytochrome *c* and its analogues in the depleted mitochondria succinate oxidase system Cytochrome *c*-depleted mitochondria are suspended in a buffer containing succinate, O_2, glucose, ATP and hexokinase in the chamber of Rank Brothers O_2 electrode Cytochrome *c* or its analogues are added incrementally and the O_2 consumption measured polarographically

Table IV. ATP-binding ability of oxidized and reduced forms of cytochrome *c* and analogues, measured by the equilibrium gel-filtration method

	ATP bound (mol/mol cytochrome)	
	Ferric state	Ferrous state
Native cytochrome *c*	2 47	1 64
Acetimidyl cytochrome *c*	1 97	1 60
Acetimidyl[Lys^{66}] cytochrome *c*	1 80	1 70
[DMP-Orn^{91}] cytochrome *c*	1 59	1 51

Only one analogue was used for tests of ATP-binding ability, because of the large amounts of protein necessary for these studies. The Lys^{66} analogue was chosen, since it represents the most extreme substitution of the original residue that does not derange the native conformation of the protein. It was tested in both oxidized and reduced states (Table IV). In native cytochrome *c* there is a significant difference between the ability of the two redox states to bind this ligand (Corthésy and Wallace, 1986). Although in acetimidyl cytochrome *c*, as with other charge-retaining lysine-modified cytochromes, ATP binding by the oxidized form of the protein is diminished, it remains significantly greater than that of the reduced form. In the analogue this differentiation is virtually lost.

Discussion

Semisynthetic acetimidyl[Glu^{66}] cytochrome *c* is highly active, indicating that the semisynthetic scheme adopted does not cause structural damage to the protein or its component peptides. The non-coded amino acid norvaline was chosen to replace glutamate,

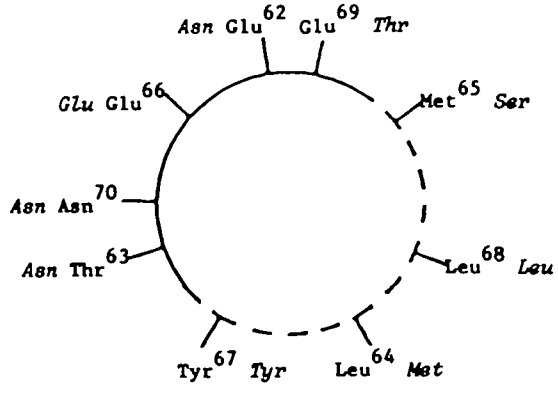

Fig. 4. Residues 62–70 of horse and yeast (italics) cytochrome *c* plotted as a helical wheel The hydrophilic and hydrophilic arcs are shown as solid and dotted lines, respectively

since, whilst conferring hydrophobicity at this position, the basic carbon skeleton of the residue is retained. Thus the very low biological activity of acetimidyl[Nva^{66}] cytochrome *c*, and the absence of the diagnostic 695 nm band, imply that in this analogue the active cytochrome conformation has been lost as a consequence of the substitution and that, since the Lys^{66} and isosteric Gln^{66} analogues are viable, hydrophobicity cannot be tolerated at this position.

The reason for this sensitivity may well lie in local secondary structural requirements. The 62–70 helix is a common feature of all cytochrome *c* crystal structures so far determined. When plotted in a helical wheel diagram (Schiffer and Edmundson, 1967), the characteristic pattern, of opposed hydrophobic and

hydrophilic arcs, of a surface helix is revealed (Figure 4). This distribution of polarity in the region is highly conserved, and it is to be assumed that such a helix is a vital structural feature of the protein.

The substitution of a hydrophobic for a hydrophilic residue in the centre of the hydrophilic arc without compensating adjustment is likely to be disruptive. Alternatively, norvaline might destabilize through a reduced α-helical potential: glutamic acid has a very pronounced α-helical propensity (Chou and Fasman, 1974) The potential of norvaline has not been determined, but it is to be noted that the propensities of Gln and Lys are lower than those of Leu or Val.

Since the hydrophobic face of the helix is in contact with the coordinating methionine side-chain (Takano and Dickerson, 1981), disruption of the 62–70 region would lead to displacement of the methionine sulphur, causing both loss of the 695 nm band and electron transfer ability, as is in fact observed. None the less, in terms of electron transfer capacity there does not seem to be a specific requirement for glutamic acid at position 66, since other hydrophilic residues are effective in producing normal spectra and differences in functional parameters are minor.

Rees (1980), citing the experimental work of Smith *et al.* (1977), proposed that the redox potential was in part determined by the electrostatic field at the redox centre, and that the more positive the field the higher the resulting potential. Analogues in which lysine amino groups within 11–14 Å of the haem iron were blocked showed redox potentials lower than the native protein by about 15 mV. Our results support this postulate, since in blocking or reversing the charge of a carboxylate group at a comparable distance from the iron atom a significant increase in redox potential is achieved.

At limiting cytochrome *c* concentrations, however, the biological electron transfer rate supported by the Gln[66] analogue is equal to, and by the Lys[66] analogue is slightly greater than, that for the parent protein. Such an observation is consistent with the idea (Margoliash and Bosshard, 1983) that the dipole moment (which will necessarily be altered in these analogues) is important to binding to physiological partners [since in other analogues in which binding ability is unaffected a higher redox potential is accompanied by a higher electron transfer capacity in the assay system employed (C.J.A.Wallace and A.E.I.Proudfoot, unpublished results)], but the effect is scarcely detectable. These two observations go some way towards explaining the preservation of the acidic stripe in cytochrome *c*, though neither function should require a fixed distribution pattern.

The altered p*K*s of the alkaline transition imply a reduced stability of the haem crevice (Osheroff *et al.*, 1980; Wallace, 1984b), and show that the Glu→Lys substitution is no more destabilizing than the Glu→Gln transition. Such results point to the involvement of Glu[66] in a surface salt bridge. Potential partners in such an arrangement are Lys[73] or, possibly, Lys[88] or Lys[55]. Glu[66] clearly has a specific and significant role in the maintenance of the physiological active conformation of cytochrome *c*.

Our measurements of the ATP-binding capacity of the Lys[66] analogue suggest a second specific function for this carboxyl group. At the redox state-dependent ATP binding site of cytochrome *c* the essential Arg[91] is implicated in the binding of the terminal phosphate (Corthésy and Wallace, 1986). The specificity for ATP implies that a recognition site for the nuleoside moiety must also exist. Since the 62–70 helix lies close to this arginine residue, and since dispositions of acidic residues such as found thereupon are generally employed in hydrogen bonding to nucleotide ribosyls in dehydrogenases (Rossman *et al.*, 1975), we tentatively attribute such a role to this feature

Strong binding at this site is only seen in the oxidized form: the ferricytochrome binds 0.83 mol more ATP per mol than the ferrocytochrome When the site is blocked, as in [DMP-Orn[91]] cytochrome *c*, the amount of ATP bound by the two oxidation states is similar, and close to the amount bound by the reduced form of the native protein (Table IV). Replacement of Glu[66] by lysine also gives values for the oxidized and reduced forms that are much closer to one another than are found in unmodified acetimidyl cytochrome *c* It would thus appear that in this case, too, modification has resulted in loss of the higher affinity of ferricytochrome *c* for ATP. Thus replacement of a glutamate by a residue that cannot H-bond to a ribosyl –OH group (glutamine could, and does, in other nucleotide-binding sites) reduces the ATP affinity of the redox state-dependent site. This finding lends support to our proposal that the conservation of some acidic residues in the left-side helix is a consequence of their role in nucleoside ribosyl binding, in analogy with other nucleotide-binding proteins

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