Semisynthesis of cytochrome *c* analogues

The effect of modifying the conserved residues 38 and 39

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We have used chemical and enzymic protein engineering techniques to create analogues of the semisynthetic two-fragment complex (1–37)·(38–104) of mitochondrial cytochrome *c*. This complex, unlike the natural product of specific tryptic cleavage, (1–38)·(39–104), from which it is prepared, quite closely resembles the parent protein in functional characteristics and is thus a suitable substrate for modifications designed to study structure–function relations. We have replaced the invariant Arg-38 and the conserved Lys-39 with a range of alternative amino acids and have studied the effects on the principal functional parameters. The hydrogen-bonding capacity of Arg-38 is crucial to the stabilization of the bottom Ω -loop, while the positive charge of Lys-39 helps maintain the high redox potential by electrostatic effects at the haem iron.

INTRODUCTION

A non-covalent complex of the fragments (1–38) and (39–104) is generated when tryptic cleavage of horse cytochrome *c* is limited to the peptide bond C-terminal to Arg-38 by acetimidylation of the lysine residues. Semisynthetic manipulations yield the modified fragments acetimidyl-(1–37) and acetimidyl-(38–104), which form a complex with functional properties much improved over those of (1–38)·(39–104) and close to those of the parent protein (Proudfoot *et al.*, 1986). We have therefore proposed that this complex could be a useful basis for protein engineering aimed at exploring the roles of individual amino acid residues around the cleavage site.

Studies of the complex (1–37)·(38–104) of normal sequence have already led us to propose a specific functional role in structural stabilization for the invariant residue Arg-38. An alternative hypothesis has been advanced for the conservation of this residue (Moore *et al.*, 1984), that it is an essential counterion to the buried inner haem propionate group, and so we have introduced substitutions at this position to test the validity of the two hypotheses. We have also used the same, and other, complex systems to examine the functional importance of Lys-39, which is invariant among animal species.

EXPERIMENTAL

Materials

Horse heart cytochrome *c* (type III) was from Sigma Chemical Co., St. Louis, MO, U.S.A. Amino acid derivatives were from Bachem, Bubendorf, Switzerland. Other chemicals are from Merck, Darmstadt, Germany.

Fragment preparation and modification

Preparation of semisynthetic complexes modified at residues 38 and 39 was achieved by using the scheme illustrated in Scheme 1.

Characterization of the analogues

The succinate oxidase activity of the analogues was assayed in cytochrome *c*-depleted mitochondria. This

tests the productivity of the interaction of cytochrome *c* with its reductase (Wallace & Proudfoot, 1987). The oxidation–reduction potential of analogues was determined by a ferrocyanide/ferricyanide redox titration using a method developed for small quantities of cytochromes (Wallace *et al.*, 1986; Wallace & Proudfoot, 1987).

RESULTS

The progress of fragment manipulation steps was monitored by cation-exchange chromatography. The elution positions on the ion-exchange column of the various intermediates can be predicted (Wallace & Proudfoot, 1987). Products purified by this method were checked by amino acid analysis.

The complexes prepared from these fragments were examined in a Cary 210 u.v.–visible spectrophotometer. The presence of a weak absorption band at 695 nm signals the interaction between the ferric haem and the sixth ligand methionine sulphur, and is critically dependent on the integrity of the haem crevice structure (Dickerson & Timkovitch, 1975). A well-developed band was observed in all cases. The complexes were all ascorbate-reducible.

The results of biological activity measurements and redox potential determinations are summarized in Table 1.

DISCUSSION

Replacement of Lys-39, whether it be in a complex of low [(1–39)·(40–104)] or high [(1–37)·(38–104)] initial potency, diminishes biological efficacy. The conservation of the residue in all animal species had signalled the probability that this residue has a significant functional role. It is not, however, one of the lysine residues implicated in binding to physiological partners (Margoliash & Bosshard, 1983). The members of the ring of lysine residues that surrounds the exposed edge of the haem group, through which electron transfer takes place, interact with groups of opposite polarity on the redox

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Scheme 1. Semisynthetic scheme for the preparation of cytochrome *c* analogues with substitutions at residues 38 and 39

Fragments (1-38) and (39-104) are obtained by tryptic digestion of *N*^ε-acetimidyl-cytochrome *c*. The fragments are not subsequently deprotected and thus they and the complexes derived therefrom, shown above, are all acetimidylated. Arg-38 is specifically removed from (1-38) by carboxypeptidase B (Harris, 1978); (39-104) is truncated by a single Edman degradation cycle by the method of Wallace & Corthésy (1986) for (66-104), but with the use of diethyl ether precipitation, instead of ethyl acetate extraction, to free the peptide of small molecule contaminants. Elongation of (1-38) is performed by reverse proteolysis with trypsin: the equilibrium is shifted by high concentrations of organic solvent and the nucleophile, an amino acid ester (Rose *et al.*, 1983). Stepwise elongation at the *N*-terminus of (38-104) or (39-104) requires amino acid active esters; for residues other than arginine, we employed *N*-hydroxysuccinimide esters (Wallace & Corthésy, 1986). With arginine, the hydroxybenztriazole ester was formed just before couplings (Proudfoot *et al.*, 1986). α -Amino-group and α -carboxy-group protections were removed before complex-formation. Fragment purification and characterization were performed, and complexes were formed from pairs of fragments, and purified, as previously described (Proudfoot *et al.*, 1986; Wallace & Proudfoot, 1987).

partner. They thus participate directly in the electron-transfer process and their modification has pronounced effects on electron-transfer rates, though the changes in redox potential are small (Rees, 1980; Margoliash & Bosshard, 1983). Analogues containing such modifications should not therefore obey the relationship between biological activity and redox potential adduced by Wallace & Proudfoot (1987) for those in which the modification is distant from the active site. The fact that

the [Glu³⁹] analogue does obey the relationship (Fig. 1) confirms that this residue does not have that sort of binding function.

The substitution might influence the redox potential and/or electron transfer with reductase by the following. 1. Both lysine (animals) and glutamine (plants) could act as hydrogen donors in a stabilizing hydrogen bond within the bottom loop, whereas glutamate cannot. Although such a bond, between the ϵ -nitrogen atom of Gln-39 and the main-chain carbonyl group of Ser-40, has been reported in the rice cytochrome *c* crystal structure (Ochi *et al.*, 1983), it is not seen in the tuna protein (Takano & Dickerson, 1981). 2. A charge change could alter the dipole moment of the protein, and hence its orientation relative to, and efficiency of electron transfer with, oxidase and reductase (Margoliash & Bosshard, 1983). However, such a change, if it were significant, should also lead to divergence from the relationship shown in Fig. 1. 3. There might be an electrostatic effect: surface charge changes can modify the redox potential by altering the field felt at the haem iron, which changes the stability of one of the redox states relative to the other (Rees, 1980). The work of Moore *et al.* (1984) suggests that this may be the case for this residue. In *Saccharomyces cerevisiae* cytochrome *c* the redox potential titrates with a pK of 6.9, corresponding closely to a value of 6.8 determined for the ionization of His-39 (Robinson *et al.*, 1983). A net change of approx. 25 mV is observed

Table 1. Biological activities and redox potentials of cytochrome *c* and analogues

Cytochrome or analogue	Biological activity (%)	Redox potential (mV)
Horse cytochrome <i>c</i>	100	260
Acetimidyl-cytochrome <i>c</i>	85	245
Acetimidyl-(1-37)·(38-104)	55	215
Acetimidyl-(1-37)·([Glu ³⁹]38-104)	34	190
Acetimidyl-(1-37)·([Lys ³⁸]38-104)	26	166
Acetimidyl-(1-37)·([Gln ³⁸]38-104)	19	148
Acetimidyl-(1-38)·(39-104)	25	150
Acetimidyl-(1-39)·(40-104)	25	—
Acetimidyl-([Tyr ³⁹]1-39)·(40-104)	18	—
Acetimidyl-([Thr ³⁹]1-39)·(40-104)	19	—

Fig. 1. Plot of redox potentials versus biological activities

A series of analogues modified at positions distant from the active site (dark bars) show a direct relationship between potential and the logarithm of electron-transfer rate (Wallace & Proudfoot, 1987). The analogues presently discussed (asterisks) conform to this relationship, which demonstrates that the residues modified do not participate directly in electron transfer.

in this case, as in the [Glu³⁹] analogue presently under consideration.

The cytochromes *c* of all higher plants have glutamine at position 39, yet show redox potentials comparable with those of vertebrate examples (Wallace & Boulter, 1987). However, plant cytochromes differ from the horse protein by an average of more than 45 residues (out of 112), including an eight-residue *N*-terminal extension. We suggest that this degree of variation gives ample scope for changes that would compensate for the decrease in potential seen on modification of this residue alone in horse cytochrome *c*.

We have previously proposed that the role of the essential Arg-38 is in structural stabilization (Proudfoot *et al.*, 1986). Comparisons of the complexes (1-37)·(38-104) and (1-38)·(39-104), where in the former Arg-38 is part of the bottom loop, whereas in the latter it is not, show that in (1-37)·(38-104) the loop is far more resistant to proteolysis, and the redox potential is much closer to the value for the intact protein. It was concluded that the three hydrogen bonds that Arg-38 makes to other elements of the protein structure (Takano & Dickerson, 1981) are crucial to the close contact between the loop and the bottom edge of the haem.

Moore *et al.* (1984) have suggested that the positive charge on the guanidino group effectively neutralizes the negatively charged inner haem propionate, thus diminishing its potential electrostatic effect at the haem iron and thereby raising the observed mid-point potential.

They proposed the low redox potential of the complex (1-38)·(39-104) to be a consequence of the inability of Arg-38 to interact with the propionate. Yet there seems no reason to suppose that such an interaction would be any more difficult to achieve than in the other nicked complex (1-37)·(38-104), where the potential is relatively high.

Replacement of arginine in the more active (1-37)·(38-104) complex by a polar glutamine residue

has the effect of lowering the redox potential to the value seen in (1-38)·(39-104), thus confirming our supposition that it is the position of the arginine residue in relation to the nick, and not the position of the nick itself, that is the major determinant of the differences between the two complexes of native sequence. Unlike glutamine, lysine should be capable of providing a counterion to the propionate group. Nevertheless, replacement of Arg-38 by lysine, although an improvement over glutamine, also results in a markedly lower redox potential (Table 1).

Both analogues obey the relationship illustrated in Fig. 1, so that Arg-38 is not directly involved in the electron-transfer process, although this possibility has been suggested by Tanaka *et al.* (1975).

The combined weight of these observations leads us to conclude that the predominant factor in the conservation and importance of an arginine residue at position 38 in mitochondrial cytochrome *c* is its capacity for extensive hydrogen-bonding and the consequent stabilization of the haem-enclosing bottom loop. This conclusion is supported by the observation that a decrease in potential to 130 mV occurs in an analogue where Arg-38 is conserved, but in which this loop, comprising residues 40-55, is deleted (Wallace, 1987).

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REFERENCES

- Dickerson, R. E. & Timkovitch, R. (1975) *Enzymes* 3rd Ed. **11**, 397-547
- Harris, D. E. (1978) in *Semisynthetic Peptides and Proteins* (Offord, R. E. & Di Bello, C., eds.), pp. 127-137, Academic Press, London
- Margoliash, E. & Bosshard, H. R. (1983) *Trends Biochem. Sci.* **8**, 316-320
- Moore, G. R., Harris, D. E., Leitch, F. A. & Pettigrew, G. W. (1984) *Biochim. Biophys. Acta* **769**, 331-342
- Ochi, H., Hata, Y., Tanaka, N., Kakudo, M., Sakurai, T., Aihara, S. & Morita, Y. (1983) *J. Mol. Biol.* **166**, 407-418
- Proudfoot, A. E. I., Wallace, C. J. A., Harris, D. E. & Offord, R. E. (1986) *Biochem. J.* **239**, 333-337
- Rees, D. C. (1980) *J. Mol. Biol.* **141**, 323-326
- Robinson, M. N., Boswell, A. P., Huang, Z.-X., Eley, C. G. S. & Moore, G. R. (1983) *Biochem. J.* **213**, 687-700
- Rose, K., De Pury, H. & Offord, R. E. (1983) *Biochem. J.* **211**, 671-676
- Takano, R. & Dickerson, R. E. (1981) *J. Mol. Biol.* **153**, 79-115
- Tanaka, N., Yamone, T., Tsukihara, T., Ashida, T. & Kakudo, M. (1975) *J. Biochem. (Tokyo)* **77**, 147-162
- Wallace, C. J. A. (1987) *J. Biol. Chem.*, in the press
- Wallace, C. J. A. & Boulter, D. (1987) *Phytochemistry*, in the press
- Wallace, C. J. A. & Corthésy, B. E. (1986) *Protein Eng.* **1**, 21-27
- Wallace, C. J. A. & Proudfoot, A. E. I. (1987) *Biochem. J.* **245**, 773-779
- Wallace, C. J. A., Corradin, G., Marchiori, F. & Borin, G. (1986) *Biopolymers* **25**, 2121-2132