The effect of complete or specific partial acetimidylation on the biological properties of cytochrome *c* and cytochrome *c*-T

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The biological consequences of acetimidylation of all 19 ϵ -amino groups of horse cytochrome *c* are a slight decrease in both the redox potential of the protein and its ability to stimulate oxygen uptake in the cytochrome *c*-depleted-mitochondria assay. Examination of a number of specific partially acetimidylated analogues and acetimidylated cytochromes *c* of other species has shown that the changes in biological properties, which are associated with a slight structural change as monitored by n.m.r. spectroscopy [Boswell, Moore, Williams, Harris, Wallace, Bocieck & Welti (1983) *Biochem. J.* 213, 679–686], appear to stem from modification of residues in a restricted region of the sequence. The failure of the redox potential of *Saccharomyces cerevisiae* cytochrome *c* to be affected by acetimidylation suggests that it is lysine-53, absent from that species, that is the sensitive residue.

The preparation of pure 19-*N*- ϵ -acetimidylated cytochrome *c* is described in an accompanying paper (Wallace & Harris, 1984). The biological properties of this derivative are presently compared with those of the native protein.

The present paper also describes the use of acetimidylation in the preparation of [Hse⁶⁵]cytochrome *c* with all the lysine residues in either the 1–65 sequence (11 residues) or the 66–104 sequence (8 residues) modified. [Hse⁶⁵]Cytochrome *c* is functionally identical with the native protein (Corradin & Harbury, 1974; Wilgus *et al.*, 1978; Boswell *et al.*, 1981), and thus it has been possible to compare directly the effects of the partial or complete acetimidylation on some of the biological properties of cytochrome *c*.

Cytochrome *c*-T is a non-covalent complex formed between fragments 1–38 and 39–104 (Harris & Offord, 1977) that exhibits approx. 20% of the specific activity of the native protein. By using fragments prepared from both native and acetimidylated proteins one can compare the biological activity of partially and fully acetimidylated cytochromes *c*-T with that of the unmodified complex.

The effect of acetimidylation on the biological properties of horse cytochrome *c* is a small but significant decrease in both the ability to stimulate

oxygen uptake in a succinate oxidase assay and the redox potential. Examination of the partially modified cytochromes shows that this effect is confined to derivatives where the lysine residues contained in the 39–65 sequence are modified.

The effect has been interpreted as a consequence of the modification of a single lysine residue, with reference to observations on the biological properties of acetimidylated *Candida krusei* and *Saccharomyces cerevisiae* cytochromes *c* and to structural studies (Bosshard & Zürrer, 1980; Takano & Dickerson, 1981; Boswell *et al.*, 1983; Robinson *et al.*, 1983) on these and other cytochrome *c* derivatives.

Experimental

Materials

Cytochromes *c* [horse heart (type III), *Saccharomyces cerevisiae* (type VIII) and *Candida krusei* (type VII)] were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Methyl acetimidate hydrochloride was prepared by the method of Hunter & Ludwig (1962). Other chemicals were of analytical grade.

Acetimidylation of cytochromes *c*

This was performed by method (iv) of Wallace & Harris (1984). The purity of the products was checked by ion-exchange chromatography on SP-

Abbreviations used: Hse, homoserine; Acim, acetimidyl.

(sulphopropyl)-Sephadex C-25 as described in that paper.

Preparation of partially acetimidylated [Hse⁶⁵]cytochromes c

This was achieved by the formation of chimerae from fragments of acetimidylated and native horse cytochrome *c*, by using the CNBr-cleavage and resynthesis methods described by Wallace & Rose (1983).

Preparation of cytochromes c-T

The two tryptic fragments, 1–38 and 39–104, of 19-*N*- ϵ -citraconylated horse cytochrome *c* were prepared and deprotected [A. E. I. Proudfoot & C. J. A. Wallace, unpublished work, based on Juillerat & Homandberg (1981)]. Acetimidylated fragments 1–38 and 39–104 were prepared by tryptic digestion of 19-*N*- ϵ -acetimidylated cytochrome *c* (Harris & Offord, 1977). Cytochrome *c*-T, acetimidylated cytochrome *c*-T and the two chimeric complexes were prepared by purifying equimolar mixtures of the fragments on Sephadex G-50 (fine grade) equilibrated with 0.1 M-Tris/HCl buffer, pH 9.4. The products were freed of Tris by passage through a column of Sephadex G-25 (fine grade) in 0.1 M-NH₄HCO₃.

Biological activities of cytochromes c

These were determined in the succinate oxidase assay system of Jacobs & Sanadi (1960). The assay conditions were as described by Harris & Offord (1977).

The assay system employs cytochrome *c*-depleted mitochondria, and is thus the nearest equivalent to an assay of cytochrome *c* *in vivo* available to the investigator. It is in consequence a complex system and does not distinguish between effects on activity with oxidase or reductase. In practice, because the rate of reaction of cytochrome *c* with isolated oxidase is 3-fold that with reductase (Dickerson & Timkovitch, 1976; Antalis & Palmer, 1982), it is probable that small effects on overall activity reflect changed activity in the reductase complex.

The assay as employed is widely used and gives consistent results, and, as a holistic system, is potentially sensitive to functional effects that would not be picked up by the study of reactions with isolated (and, as these are membrane systems, rather artificial yet still quite complex) subsets of the electron-transport system.

Assay results should be interpreted with caution and in the light of the knowledge of redox potentials [see Dickerson & Timkovitch (1976), p. 473, for a discussion].

Oxidation-reduction potentials

These were determined by the method of mixtures described by Davenport & Hill (1952).

All determinations were performed at pH 7.0, with 50 mM-phosphate buffer, and at room temperature. The solutions used to prepare the mixtures were 10 mM in either ferricyanide or ferrocyanide, and cytochrome or analogue concentration was in the range 10–40 μ M.

Results and discussion

The biological activities of cytochrome *c* and its derivatives are shown as plots of rates of oxygen uptake versus quantity of haemochrome added. Fig. 1 gives the results from multiple determinations for fully acetimidylated cytochrome *c* and the two chimeric forms compared with those for native horse cytochrome.

Oxidation-reduction potentials are determined from the plots shown on Fig. 2. In both Figures it can be seen that curves for cytochrome *c* and the (1–65)-Acim-(66–104)-[Hse⁶⁵]cytochrome *c* chimera are superimposable, as are those for acetimidyl-cytochrome *c* and Acim-(1–65)-(66–104)-[Hse⁶⁵]cytochrome *c*. These latter two derivatives have biological activities of about 85% of the native protein [Harris & Offord (1977) reported

Fig. 1. *Biological activity in the cytochrome c-depleted-mitochondria assay of cytochrome c, acetimidylated cytochrome c and chimerae*

For full experimental details see the text. ●, Horse cytochrome *c*; ▲, acetimidyl-cytochrome *c*; ■, (1–65)-Acim-(66–104)-[Hse⁶⁵]cytochrome *c*; ○, Acim-(1–65)-(66–104)-[Hse⁶⁵]cytochrome *c*; the last three assays were performed in triplicate.

about 80%), and redox potentials of 242 mV, compared with 254 mV for cytochrome *c* and (1-65)-Acim-(66-104)-[Hse⁶⁵]cytochrome *c*.

The two chimeric derivatives are [Hse⁶⁵] forms, but this substitution does not significantly affect the chemical or physical properties of cytochrome *c* (Corradin & Harbury, 1971, 1974; Wilgus *et al.*, 1978; Boswell *et al.*, 1981). It may thus be fairly assumed that the results obtained in each of the four cases are comparable, and that differences in behaviour are a result of the effects of acetimidylation of lysine residues.

The difference observed between native and fully modified horse cytochrome *c* is significant, and agrees with the observations made in another lysine-modified cytochrome *c*, amidino-cytochrome *c*. In this case, too, the positively charged lysine ϵ -amino group (pK about 10.5) is replaced by the similarly charged guanidino group (pK about 12.6). The pK of the acetimidino group is 12.7-12.8 (G. R. Moore & C. J. A. Wallace, unpublished work). 19-*N*- ϵ -Amidino-cytochrome *c* shows quantitatively similar changes in redox potential and biological activity to those seen in the acetimidylated protein.

The diminished ability of the acetimidylated protein to stimulate oxygen uptake may be a simple consequence of the altered redox potential, for the replacement of one positive charge by another should not be expected to affect the binding to either oxidase or reductase (Capaldi *et al.*, 1982). If, indeed, it is the reductase activity that is rate-limiting in the cytochrome *c*-depleted-mitochondria assay, then shifting the redox potential of cytochrome *c* (normally about 255 mV) towards that of the reductase [c_1 , 220 mV; *a*, 290 mV (Loach, 1968)] would displace the equilibrium:

with a consequent decrease in overall rate.

The observation that the redox titration and biological assay curves for (1-65)-Acim-(66-104)-

[Hse⁶⁵]cytochrome *c* and Acim-(1-65)-(66-104)-[Hse⁶⁵]cytochrome *c* are different from one another and superimposable on those of cytochrome *c* and the acetimidylated protein respectively (Figs. 3 and 4) shows that the residue or residues responsible for this change on acetimidylation are located in the 1-65 fragment of horse cytochrome *c*.

The specific activity in the succinate oxidase assay system of cytochrome *c*-T is considerably lower than that of the native protein (Fig. 3). Nevertheless, acetimidylation is effective in decreasing it further, by a similar proportion to that observed on acetimidylation of the whole protein.

As with the cytochrome *c* chimerae, those of cytochrome *c*-T match one or other of the 'parent' proteins precisely (Fig. 3). In this case it is the Acim-(1-38)-(39-104)-[Hse⁶⁵]cytochrome *c* hybrid that corresponds to the unprotected protein, and thus in cytochrome *c*-T it is the 39-104 fragment that contains the sensitive residue(s).

If we assume that the effects of acetimidylation are the same or very similar in both cytochrome *c* and cytochrome *c*-T, then the two sets of data combine to localize the residue or residues responsible to the 39-65 region, in which are found four lysine residues: 39, 53, 55 and 60.

N.m.r.-spectroscopic comparisons of native and modified horse cytochrome *c* have shown that the most significant conformational change on acetimidylation of the protein occurs in the region of Ile-57 (Boswell *et al.*, 1983): the CH₃ resonance of this residue experiences an upfield shift of 0.4 p.p.m. In the native protein this resonance shifts with temperature or pH; in the modified protein pH-sensitivity is lost and the resonance is found at the position that it occupies in native cyto-

chrome *c* only at pH 13. In this respect amidino-cytochrome *c* and the Acim-(1-65)-(66-104)-[Hse⁶⁵]cytochrome *c* chimera behave like the

Table 1. Oxidation-reduction potentials determined by the method of mixtures for cytochromes *c* and derivatives

For full experimental details see the text. Values for cytochromes *c*-T are approximate, being at the limit of determination by the system employed.

Cytochrome or derivative	Redox potential (mV)
Horse cytochrome <i>c</i>	258 $\pm$ 2
Acetimidylated horse cytochrome <i>c</i>	242 $\pm$ 3
Acim-(1-65)-(66-104)-[Hse ⁶⁵]cytochrome <i>c</i>	242
(1-65)-Acim-(66-104)-[Hse ⁶⁵]cytochrome <i>c</i>	254
Cytochrome <i>c</i> -T	~100
Acetimidylated cytochrome <i>c</i> -T	
<i>S. cerevisiae</i> cytochrome <i>c</i>	
Acetimidylated <i>S. cerevisiae</i> cytochrome <i>c</i>	270
<i>C. krusei</i> cytochrome <i>c</i>	271
Acetimidylated <i>C. krusei</i> cytochrome <i>c</i>	261

Fig. 2. Redox titration plots of cytochrome *c* and derivatives. For full experimental details see the text. Redox potentials calculated by extrapolation are: horse cytochrome *c* (○) and (1-65)-Acim-(66-104)-[Hse⁶⁵]cytochrome *c* (■), 254 mV; acetimidylcytochrome *c* (●) and Acim-(1-65)-(66-104)-[Hse⁶⁵]cytochrome *c* (▲), 242 mV.

Fig. 4. Redox titrations of acetimidylated and native *C. krusei* cytochrome *c* and of acetimidylated and native *S. cerevisiae* cytochrome *c*.

For full experimental details see the text. □, *C. krusei* cytochrome *c*; ○, acetimidylated *C. krusei* cytochrome *c*; ●, *S. cerevisiae* cytochrome *c*; ■, acetimidylated *S. cerevisiae* cytochrome *c*.

Fig. 3. Biological assays in the cytochrome *c*-depleted-mitochondria assay of cytochrome *c*-T and derivatives, compared with native horse cytochrome *c*.

For full experimental details see the text. ■, Horse cytochrome *c*; □, cytochrome *c*-T; ○, acetimidylcytochrome *c*-T; ▲, Acim-(1-38)-(39-104)-cytochrome *c*-T; ●, (1-38)-Acim-(39-104)-cytochrome *c*-T.

acetimidylated protein; the other chimera is like native cytochrome *c*. Such behaviour precisely parallels the observations made on the biological and redox properties of the analogues and described above.

Furthermore, the region identified above as that containing the residue or residues responsible for the observed effects is precisely that which undergoes the conformational change. In particular, the side chains of Lys-55 and Ile-57 are juxtaposed.

Robinson *et al.* (1983) have concluded that the pH-dependent conformational change around Ile-57 is due to the deprotonation of Lys-55; thus it was thought possible that the conformational change occurring in the Ile-57 region on acetimidylation or amidination is due to modification at this residue alone.

Candida krusei and *Saccharomyces cerevisiae* cytochromes *c* contain a region structurally and functionally analogous (Dickerson & Timkovitch, 1976; Robinson *et al.*, 1983) to that around Ile-57 in the horse protein. *C. krusei* cytochrome *c* lacks, however, the lysine residue at position 55, and that of *S. cerevisiae* lacks that at position 53. Both species lack the lysine residues at positions 39 and 60. Comparisons of the redox potentials of acetimidylated and native *C. krusei* and *S. cerevisiae* cytochromes *c* with the horse protein are drawn in Fig. 4 and Table 1. These results show conclusively that acetimidylation of *Saccharomyces* cytochrome *c* does not decrease its redox potential whereas modification of the *Candida* protein does, and give strong support to the inference that the sensitive residue is in fact Lys-53.

Lys-53 is not part of the ring of positive charge that mediates the interaction of cytochrome *c* with other members of the electron-transport system, and hence the change in biological activity is unlikely to be a result of altered binding characteristics. It is probable that the conformational change around Ile-57, as the only detectable structural consequence of the modification, is a signal of an internal re-adjustment that is manifested as a decrease in redox potential.

It is unlikely that the altered pK of the amino group on acetimidylation could be responsible for such a re-adjustment, as both groups are essentially fully charged at neutral pH. However, the acetimidino group has also increased bulk and is adjacent to Asp-52 in the 49–55 helix. This residue is a member of a hydrogen-bond network that defines the structure of the 'lower left' region of the molecule and connects surface residues to the central iron atom of the haem group (Takano & Dickerson, 1981). During oxidation and reduction, movement of this side chain is co-ordinated with movement of Ile-57, and any shift in its orientation is likely to be accompanied by conformational change in this region. It is believed that it is the conformation assumed by the residues packed around the haem group that produces the fine-tuning of the redox potential (Schlauder & Kassner, 1979).

Conclusions

(1) Acetimidylation of horse cytochrome *c* causes small, but significant, changes to the functional properties of the molecule. These are a diminution of the redox potential and electron-transfer ability of the protein.

(2) The study of partially acetimidylated cytochromes *c* has located the residue or residues responsible to the region between residues 39 and 65. *Candida krusei* cytochrome *c*, which lacks the lysine residue at position 55, is affected in the same way as the horse protein by acetimidylation, but *Saccharomyces cerevisiae* cytochrome *c*, lacking the lysine residue at position 53, is not.

(3) The observed changes in biological properties on acetimidylation of horse cytochrome *c* seem likely to be a consequence of a change of the

structure in the conventional 'lower left' region induced by the increased bulk of the ε -acetimidyl-lysine residue at position 53.

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