SEMISYNTHESIS, CONFORMATION AND CYTOCHROME *c* OXIDASE ACTIVITY OF EIGHT CYTOCHROME *c* ANALOGUES

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1. Introduction

Chemical modification studies have been extensively employed in studies on the relationship between the structure of cytochrome *c* and its physical and biological properties [1]. Most of the methods employed lacked selectivity, introducing variations at several points along the polypeptide chain. Furthermore, modifications of certain internal residues can perturb the structural integrity of the protein. The changes in biological properties cannot then be ascribed wholly to the substituents. Currently, only modification of surface residues (mainly lysines) in cytochrome *c* has given easily interpretable results [2–6].

The semisynthetic procedure, recently developed for the assemblage of Hse^{65}-cytochrome *c* from three fragments [7,8] provides a new possibility for investigating the structure–function relationship of this protein. The effects of charge modification by acetylating the lysine residues 72, 73 and 79, and by the replacement of the almost invariant tyrosine residue at position 74 by a leucyl residue have been investigated and are now reported.

2. Materials and methods

Horse heart cytochrome *c* was isolated by a known method [9] or was purchased (type VI, Sigma). Beef heart cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) was prepared as described (procedure III in [10]).

Eight protected tetradekapeptides, selectively modified with respect to the sequence 66–79 of cytochrome *c* (scheme 1, compounds 1–8) were prepared in homogeneous solution in a similar manner to that described for the natural sequence [8]. Details of these syntheses will be published elsewhere. The synthetic peptides were coupled with the complementary fragments 80–104 and 1–65, obtained by CNBr-treatment of cytochrome *c*, as described for the semisynthesis of Hse^{65}-cytochrome *c* [7].

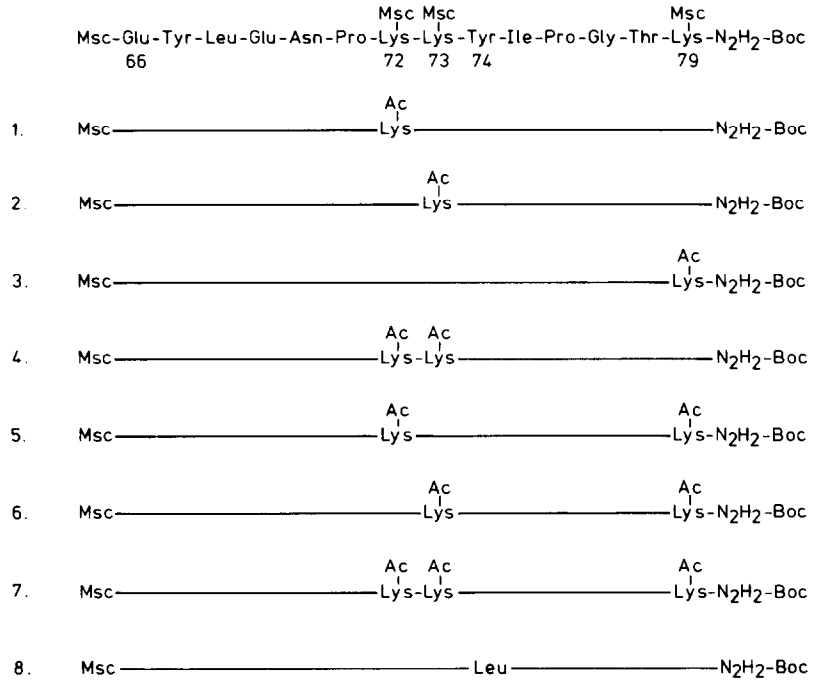
Products were purified by chromatography on CM-cellulose, using linear sodium phosphate buffer gradients (10–100 mM, pH 6.9); the Tris–acetyl derivative [7] was eluted with 5 mM sodium phosphate buffer (pH 6.9). All compounds were eluted as symmetrical peaks. Appropriate fractions were pooled and diluted with water.

The products were oxidized with the absolute minimum of ferricyanide and then absorbed onto small CM-cellulose columns, eluted as concentrated solutions with 0.12 M sodium phosphate buffer (pH 6.9) rapidly frozen at −78°C and then stored at −20°C. Prior to enzymatic assay, samples of each analogue were rechromatographed on Sephadex G-25 columns (1.0 × 30 cm) using 25 mM Tris–acetate buffer (pH 7.8) (25 mM in acetate).

Cytochrome *c* oxidase activity was determined, using the ascorbate–TMPD system [2,11] by polarographic measurement of the rate of oxygen uptake at 25°C with a Clark electrode, mounted on a Gilson oxygraph (see legends to fig.2,3).

^{1}H NMR spectra were determined using a Bruker 360 MHz spectrometer, operating in the Fourier transform mode, with 2,2-dimethyl-2-silapentane-5-sulphonate (δ = 0 ppm) as internal standard.

Scheme 1



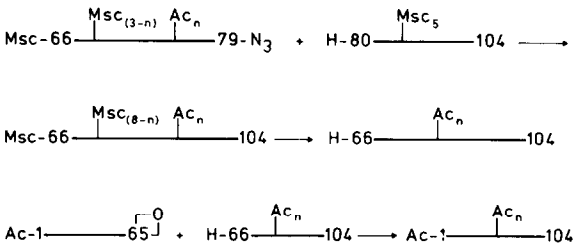
Eight tetradecapeptide derivatives for covalent insertion between isolated cytochrome *c* fragments 1–65 and 80–104.

3. Results and discussion

3.1. *Acetylated cytochromes* c

According to scheme 2, the azides of the synthetic tetradekapeptides 1–7 were condensed with the partially protected protein fragment 80–104, obtained from native cytochrome *c* as in [7]. After removal of the MSC (methylsulphonylethyloxycarbonyl-) groups by brief treatment with base and purification on CM-cellulose the semisynthetic 66–104 sequences were obtained in 34–43% yields.

Scheme 2



Semisynthesis of acetylated cytochrome *c* analogues from three fragments

The combination of modified fragments 66–104 with the lactone form of the fragment 1–65, obtained by CNBr-treatment of cytochrome *c*, proceeds successfully only when the reacting groups in the 1:1 complex of the complementing fragments are suitably aligned. It was anticipated that the acetylation of the lysyl residues 72, 73 and 79 would not interfere seriously with the coupling reaction, since the hydrophylic character of the sidechains was retained, irrespective of the loss of positive charge. The final couplings gave the desired analogues in yields of 21–33% (after purification).

The acylated cytochromes apparently retained the overall native conformation since:

(i) In the unaltered absorption spectrum the conformation-sensitive 695 nm band, indicative of ligation of Met^{80} to the heme iron, was of unreduced intensity.

(ii) Reduction by ascorbate was complete (95–98%) and proceeded with pseudo first-order rate con-

stants (22–30 × 10^{-3} s^{-1} at 22°C (pH 7.0), 2.5 mM ascorbate), comparable with that of cytochrome *c* (27 × 10^{-3} s^{-1}).

(iii) The reduced proteins did not bind carbon monoxide.

3.2. *Hse*^{65}, *Leu*^{74} *cytochrome* c

The substitution of tyrosine by a leucyl residue at position 74 in the 66–104 fragment also did not affect the 'conformation-directed' [7,8] coupling with the complementary part in the reduced form of the complex 1–65–66–104. The analogue was obtained in 53% yield after purification on CM-cellulose, thus suggesting that the conformation of the ferrous derivative was very similar to that of ferrocytochrome *c*. This conclusion was substantiated by the similarity of the visible spectra of ferrocytochrome *c* and the reduced analogue, and by the absence of binding of carbon monoxide by the latter. Moreover, its ^{1}H NMR spectrum showed unaltered resonance positions for Met^{80}-protons and for three heme methyl groups [13]. These data indicate that reduced Hse^{65}, Leu^{74}-cytochrome *c* has a native, heme-crevice structure.

The ferric form of the analogue exhibited decreased thermostability. The absorption band at 695 nm, normal shaped but of lower intensity (89%) at 25°C, was completely absent at 55°C (fig.1). The protein remains, however, in a low spin configuration [1].

Related changes were observed in the ^{1}H NMR spectrum. The CH_3-singlet of Met^{80} at –23.4 (25°C, pH 7.0; cytochrome *c*: –23.8) broadened and shifted downfield with increase in temperature; at 55°C the resonance became an almost unobservable signal at –18.6 ppm [cytochrome c: –20.4 (without appreciable broadening)]. The singlet resonances of the methyl groups of the pyrrole rings II and IV at 32.6 and 35.4 ppm, respectively, also broadened, concomitantly with the appearance of new resonances at 22.5 and 20.0 above 40°C [13].

These changes, which proved to be completely reversible, are similar to those observed for cytochrome *c* above pH 9 [13,14] or when the heme region is otherwise disrupted [15] and indicated displacement of Met^{80} as an iron ligand by another strong-field ligand.

The temperature dependence of the 695 nm band (fig.1) suggested that even at room temperature a small fraction of the analogue exists as the high temperature conformer. This was further indicated by the rate of diminuition in ascorbate reduction, which was initially equal to that of cytochrome *c*. The complete reducibility of the analogue could be ascribed to a shift in the equilibrium between the native and high temperature conformer.

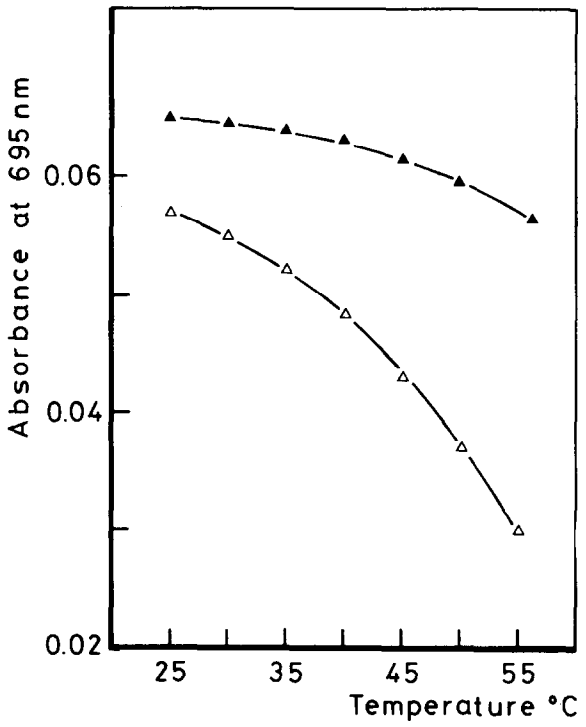


Fig.1. Changes in 695 nm absorption with temperature of cytochrome *c* (▲) and Hse^{65}, Leu^{74}-cytochrome *c* (△) in 20 mM sodium phosphate buffer (pH 6.9).

3.3. *Cytochrome* c *oxidase activities*

The steady-state kinetic analyses of the reactions of the cytochrome *c* analogues with cytochrome oxidase were carried out under conditions, which yield primarily information about the binding of the analogues to the oxidase [2,11].

Native cytochrome *c* reacts with the oxidase according to biphasic kinetics. The app. K_m values of the two phases correlate with the K_d values of two molecules of cytochrome *c* on the oxidase. Only the high affinity phase of the reaction was studied at the cytochrome *c* concentrations used (0.01–1.0 μM). The K_m-value, which characterizes the corresponding

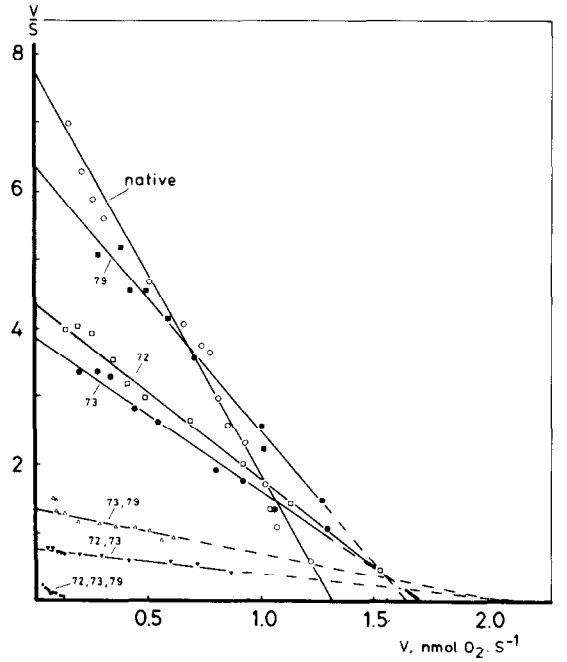


Fig.2. Steady-state cytochrome *c* oxidase activities of acetylated cytochrome *c* derivatives at low cytochrome *c* concentrations (0.01–1.0 μM). The sites of acetylation are marked next to the plots. *V* is in nmol O_2 reduced/s and *S* is in μM cytochrome *c*. The assay medium, 25 mM Tris–acetate (pH 7.8) (1.25 ml), contained in addition to cytochrome *c*: 250 mM sucrose, 0.1% Tween-20, 20 mM ascorbate, 2 mM TMPD and 0.114 μM of a purified cytochrome *c* oxidase preparation.

binding-site, is sensitive to changes in the ionic strength of the medium and the charge distribution on cytochrome *c*.

V_{max}-values of the monoacetylated cytochrome *c* (fig.2) appeared to be nearly equal, but ~25% higher than that of cytochrome *c*. The influence of monoacetylation on the app. K_m-values was more varied. Removal of charge at Lys^{72} or Lys^{73} resulted in a 2–2.5-fold increase. It also exerted a larger decrease in binding affinity than acetylation at Lys^{79}, which produced a much smaller increase of K_m.

These differences between the Lys residues were also reflected in the activities of the di-acetylated analogues: the K_m-values increased ~8- and 16-fold for the $Ac^{73,79}$ and $Ac^{72,73}$ derivatives, respectively. The elimination of a second charge lead, however, to a larger decrease of activity than mono-acetylation. The tri-acetyl derivative had only a very low activity.

The results agreed with those of other studies on analogues, in which a number of lysyl residues had been modified [2–4]. The lysines at positions 8, 13, 25, 27, 72, 79, 86 and 87 probably form a binding domain in cytochrome *c* which is situated in the upper left of the front surface of the molecule, which is marked by the exposed heme edge [17]. Virtually the same residues appear to be shielded against methylation and acetylation in the cytochrome *c* oxidase complex [18].

The current observation that removal of two and three charges causes increased reduction of affinity to the enzyme compared with a single charge elimination is in agreement with the fact that the lysine residues at 72, 73 and 79 are all situated near the positive end of the dipolar charge distribution of cytochrome *c* [19].

The measurements with Hse^{65}, Leu^{74}-cytochrome *c* gave reasonably similar results to those with Ac-Lys^{72}-cytochrome *c* (fig.3). The Tyr^{74} in cytochrome *c* is

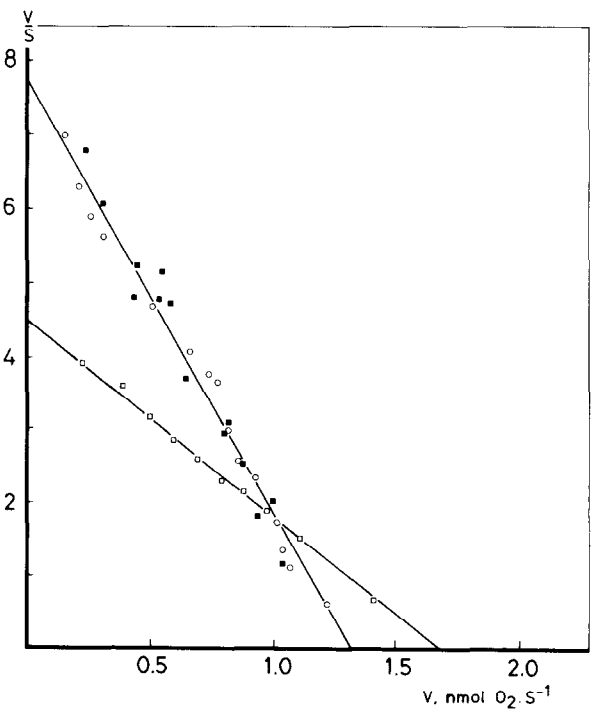


Fig.3. Steady-state cytochrome *c* oxidase activities of cytochrome *c* (○), Hse^{65}-cytochrome *c* (■) and Hse^{65}, Leu^{74}-cytochrome *c* (□). Conditions are as indicated in legend to fig.2.

an almost constant residue in eukaryotic cytochrome *c*. It has been rarely replaced by other aromatic amino acids. The aromatic ring is near to the surface of the molecule and forms part of the 'left channel', a hydrophobic region, also containing Tyr^{67} and Trp^{59} and extending from the surface to the heme moiety [1]. This particular region was thought once to form the path for the incoming of the electron. The presence of leucine at the homologous position in the bacterium *Paracoccus denitrificans* was the main reason for abandoning this hypothesis. Iodination of Tyr^{74} does not affect the electron transfer properties of cytochrome *c* [16], in agreement with this conclusion.

The decreased activity of the Hse^{65}, Leu^{74}-cytochrome *c* could be due to the increased thermolability as compared with cytochrome *c*. Preliminary stop–flow kinetic analyses [20] of the reduction of ferri-Hse^{65}, Leu^{74}-cytochrome *c* and the Ac^{73} analogue by purified cytochrome c_1 (10°C, pH 7.0, 250 mM phosphate buffer) gave a second order rate constant ($1.0 \pm 0.2 \times 10^6\ M^{-1} \cdot s^{-1}$) for both proteins. The same value was found for the unmodified protein. These observations supported the view that the aromaticity of the Tyr^{74} does not play a crucial role in the electron transport function of the protein.

We are tempted to conclude that an aromatic residue at position 74 is needed to provide the 'left channel' of the protein with the necessary rigidity, what could also explain its invariancy in eukaryotic cytochromes *c*.

Finally, it is noteworthy that the single substitution of Met^{65} by a homoseryl residue, present in all the analogues investigated, does not influence the activity; the behaviour of Hse^{65}-cytochrome *c* is indistinguishable from that of cytochrome *c* ([7] and fig.3).

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