A Functioning Complex between Tryptic Fragments of Cytochrome *c*

A ROUTE TO THE PRODUCTION OF SEMISYNTHETIC ANALOGUES

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We report the discovery of a functioning non-covalent complex between two peptides obtained from a limited tryptic digest of horse heart cytochrome *c*. We have used the phenomenon to produce three modified versions of the complex. We have replaced lysine-39 semisynthetically with ornithine in the first analogue, with *p*-fluorophenylalanine in the second, and removed it entirely in the third.

We report the discovery of a functioning non-covalent complex between two peptides obtained from a limited tryptic digest of horse heart cytochrome *c*. The peptides correspond to residues 1–38 and residues 39–104 of the protein. We have used the phenomenon to produce three modified versions of the complex. We have replaced lysine-39 semisynthetically with ornithine in the first analogue, with *p*-fluorophenylalanine in the second, and removed it entirely in the third.

These experiments recall other instances in which fragments of proteins have been found to combine non-covalently to give a functioning complex. For example, the phenomenon has been observed for fragments of pancreatic ribonuclease (Richards, 1958), for *Staphylococcus aureus* nuclease (Taniuchi *et al.*, 1967) and for CNBr fragments of cytochrome *c* (Fisher *et al.*, 1973; Corradin & Harbury, 1974). (By analogy with the nomenclature used by previous workers, our complex could be called cytochrome *c*-T.) The smaller of the fragments of ribonuclease has been replaced by totally synthetic analogues (Scofield *et al.*, 1967) and similar experiments have been carried out on the *S. aureus* nuclease (e.g. Ontjes & Anfinsen, 1969).

We limited the tryptic cleavage of the protein to arginine by *N*^ε-acetimidylation (Hunter & Ludwig, 1962) of the lysine residues, a modification which did not greatly affect biological activity in the depleted mitochondrion assay (see below). The acetimidylation is reversible (Ludwig & Byrne, 1962) with retention of activity (C. J. A. Wallace & D. E. Harris, unpublished work). We found that, even after acetone denaturation of the cytochrome, cleavage at arginine-91 (which has an acidic residue on either side; Margoliash *et al.*, 1961) did not take place to any detectable extent. There is only one other arginine residue in the sequence, at position 38, and this constituted the sole site of tryptic cleavage. This residue

is the only site of tryptic attack in *N*^ε-trifluoroacetyl-cytochrome *c* (Fanger & Harbury, 1965).

Experimental

Materials

Cytochrome *c* (grade III) was obtained from Sigma (London) Chemical Co., Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH, U.K. Trypsin (Worthington type TRTPCK) was obtained from Cambrian Chemicals, 73 Cherry Orchard Road, Croydon CR9 6AG, U.K.

The *N*-hydroxysuccinimido ester of Boc^{*}-*p*-fluoro-L-phenylalanine was a gift from Dr. D. J. Saunders of this Laboratory. The corresponding ester of *N*^ε,^δ-di-Boc-ornithine was prepared as described by Schnabel (1967) and Anderson *et al.* (1964).

Methods

Acetimidylation of the cytochrome *c* was carried out by the method of Wallace (1976), which resembles that of Pettigrew *et al.* (1976). The *N*^ε-acetimidyl-cytochrome *c* was denatured by acetone. The protein was dissolved in distilled water (85 mg/ml), and sufficient acetone added to produce a slight precipitate. The solution was incubated at 37°C for 30 min, after which more acetone was added until the protein was completely precipitated. The precipitate was spun down and dried. Tryptic digestion was at an enzyme/substrate ratio of 1:100 (w/w) at a protein concentration of approx. 10 mg/ml in 0.1 M-NH₄HCO₃ at 37°C for 4 h, after which the digest was freeze-dried. The two fragments of acetimidylated cytochrome *c* were separated by gel filtration (50 mg of digest in 2 ml of eluent) on a column (135 cm × 2 cm diameter) of

*Abbreviation: Boc, *t*-butoxycarbonyl.

Sephadex G-50 in aq. formic acid (7%, v/v). The fragments were recovered by freeze-drying and gave the expected results when analysed for amino/acid composition and for free amino groups.

Amino acid analyses and end-group determinations were carried out by standard methods, e.g. as described by Gonzalez & Offord (1971). All the materials described in this paper gave analyses with ratios within 10% of the expected values and substantially no other end groups than those expected. N.m.r. spectra were taken at 37°C at a concentration of between 1 and 4 mM in $^2\text{H}_2\text{O}$, adjusted to pD 6 with NaO^2H . The instrument was a Bruker 270 MHz with an Oxford Instrument Co. magnet. Visible and u.v. spectra were taken in a Unicam SP.800 spectrophotometer with a cell of 1 cm path length. Spectra in the range 600–750 nm were recorded by using a Zeiss PMQ II spectrophotometer. The biological assay of the materials was carried out with an O_2 electrode in the depleted mitochondrion system of Jacobs & Sanadi (1960). The electrode chamber contained, in a total volume of 5.7 ml, 10 mM-potassium phosphate, pH 7.0, 15 mM-potassium succinate, 60 mM-glucose, 7.5 mM- MgCl_2 , 1.5 mM-ATP (disodium salt) and 0.3 M-sucrose. It also contained 10 units of hexokinase (Sigma) and mitochondria, depleted in cytochrome *c*, obtained from 1 g of rat liver (Schneider, 1948). The assays were performed at 25°C. The test solutions were added in no more than 30 μl of 50 mM-sodium phosphate, pH 7.4.

Results

The absorption spectrum of *N*^ε-acetimidyl-peptide T1 (the haem peptide, representing residues 1–38) in 0.1 M-sodium phosphate buffer, pH 7.4, is given in Fig. 1. The spectrum of the oxidized peptide closely resembles that of ferricytochrome *c* with respect to the position and intensity of the main absorption bands. The major difference occurs at 280 nm and results from the absence of tryptophan and tyrosine residues from the peptide. The spectrum of the reduced peptide differs from that of ferrocytochrome *c* in several respects. All absorption bands are diminished in intensity and the Soret maximum (at 414 nm) is decreased in width and has side bands at 404 nm and 435 nm. The spectrum of *N*^ε-acetimidyl-peptide T23 (representing residues 39–104) is, as expected, flat in the Soret and visible regions and has the u.v. absorption to be expected from its content of aromatic amino acids.

The formation of the complex was observed by mixing unbuffered aqueous solutions of the two fragments. For example, *N*^ε-acetimidyl-peptide T1 was dissolved in water (250 nmol/200 μl), and 10 μl portions of a solution of *N*^ε-acetimidyl-peptide T23 (500 nmol/50 μl) were added. After each addition 10 μl was removed and added to 3.0 ml of 0.1 M-

sodium phosphate buffer, pH 7.4, and oxidized and reduced spectra were recorded in the range 370–420 nm. Immediate changes in the reduced Soret band were observed for the first three additions of *N*^ε-acetimidyl-peptide T23 solution, at which point the oxidized and reduced Soret bands were indistinguishable from those of cytochrome *c* (Fig. 1). Further additions of *N*^ε-acetimidyl-peptide T23 solution caused no further changes in the spectra.

Solutions of the complex were purified after the addition of solid NH_4HCO_3 to approx. pH 8 on a Sephadex G-50 column (135 cm $\times$ 1.6 cm diameter) equilibrated with 50 mM- NH_4HCO_3 , pH 8. In contrast with what was observed with the 7% formic acid column, the complex remained intact under these conditions. A haem-containing fraction was eluted in the position characteristics of cytochrome *c*, well clear of the positions at which the free peptides emerged. The haem-containing fraction had an absorption spectrum identical with that of cytochrome *c* in the range 240–750 nm and was thus judged to be a 1:1 complex between *N*^ε-acetimidyl-peptide T1 and *N*^ε-acetimidyl-peptide T23, a conclusion confirmed by amino acid analysis. The complex was further characterized spectroscopically. When oxidized, it exhibits the 695 nm absorption characteristically seen when the sulphur atom of methionine-80 is the sixth ligand of the iron (Schechter & Saludjian, 1967). Studies on the proton magnetic resonance of the reduced form also indicated that the juxtaposition between the methionine residue and the haem ring is close to that found in the intact protein (Table 1), even though they belong to different tryptic fragments. Such differences as there are between the chemical shifts seen in the complex and those in the native protein may be due to slight structural differences rather than to the different conditions at which the measurements were made, since the spectrum of the protein is almost invariant over a wide range of temperature and pD (McDonald & Phillips, 1973).

The results of assay in the depleted mitochondrion system are shown in Fig. 2. *N*^ε-Acetimidyl-cytochrome *c* had nearly the full activity of the unmodified protein. The formation of the acetimidyl complex enhanced the residual activity of the isolated haem peptide to a very significant extent.

N^ε-Acetimidyl-peptide T23 has only one free amino group, the α -amino group of residue 39 (*N*^ε-acetimidyl-lysine). This fact has been exploited to prepare semisynthetic analogues of the peptide [the techniques of protein semisynthesis have been briefly reviewed (Offord, 1973) and two other papers (Garner & Gurd, 1975; Halban & Offord, 1975) give more recent references]. Residue 39 was removed by the Edman reaction and replaced by coupling the trifluoroacetyl salt of the truncated peptide in dimethylformamide solution with a 20-fold excess of 1-hydroxybenzotriazole together with the *N*-hydroxy-

Fig. 1. Absorption spectra of (a) *N*²-acetimidyl-cytochrome *c*, (b) *N*²-acetimidyl-peptide T1 and (c) *N*²-acetimidyl-cytochrome *c*-T

----, Reduced forms; —, oxidized forms. The spectra of unmodified cytochrome *c* are indistinguishable from those in (a). Spectra were recorded at a concentration of $10\mu\text{M}$ in 0.1 M-sodium phosphate, pH 7.4. Reduction was effected by the addition of solid $\text{Na}_2\text{S}_2\text{O}_4$. The spectra in (c) were taken immediately on mixing the components. The spectra of *N*²-acetimidyl-des-Lys³⁹-cytochrome *c*-T and of *N*²-acetimidyl-[Orn³⁹]cytochrome *c*-T were very similar to those of the unmodified complex. The insets show the continuation of the spectra of the oxidized forms.

Table 1. Comparison between selected ring-shifted proton resonances in the reduced acetimidylated complex and those in the reduced native cytochrome *c*

Observations were made at 37°C at a concentration of 1–4 mM in $^2\text{H}_2\text{O}$ adjusted to pD 6 with NaO^2H . The instrument was a Bruker 270 MHz with an Oxford Instrument Co. magnet. A chemical shift was observed 1 p.p.m. downfield from 2,2-dimethyl-2-silapentane-5-sulphonate = +1.0.

Observed chemical shifts		Number of protons	Assignment	Published values		
Acetimidylated cytochrome <i>c</i> -T	Cytochrome <i>c</i>			pD 7.0, 35°C*	pD 6.4, 22°C†	pD 6.9, 24°C‡
–1.90	–1.89	1	$\gamma\text{-CH}_2\text{-}$ of Met-80	–1.9	–1.87	—
–2.70	–2.66	1	$\beta\text{-CH}_2\text{-}$ of Met-80	–2.7	–2.59	—
–3.30	–3.30	3	-S-CH_3 of Met-80	–3.3	–3.30	–3.2
–3.76	–3.74	1	$\gamma\text{-CH}_2\text{-}$ of Met-80	–3.7	–3.77	—

* Wuthrich (1969).

† McDonald & Phillips (1973).

‡ Stellwagen & Shulman (1973).

Fig. 2. Oxygen-electrode assay of the preparations in the depleted mitochondrion system

For details, see the text. $\circ$, Cytochrome *c*; $\square$, N^α -acetimidyl-cytochrome *c*; $\triangle$, deprotected N^α -acetimidylcytochrome *c*; $\bullet$, N^α -acetimidyl-cytochrome *c*-T; $\blacksquare$, N^α -acetimidyl-[Orn³⁹]cytochrome *c*-T; $\blacktriangle$, N^α -acetimidyl-des-Lys³⁹-cytochrome *c*-T; ∇ , N^α -acetimidyl-peptide T1.

succinimide ester of $N^{\alpha,\delta}$ -di-Boc-ornithine or (in a separate experiment) of N^α -Boc-*p*-fluorophenyl-alanine. The basicity of the reaction mixture was controlled by the method of Rees & Offord (1976). After removal of the Boc groups by exposure to anhydrous trifluoroacetic acid (30 min at 20°C, removed at the end of the reaction with a stream of N_2) the semisynthetic analogues of N^α -acetimidyl-peptide T23 were characterized by end group and composition, and run on the 7% formic acid column, freeze-dried and mixed with samples of N^α -acetimidyl-peptide T1 as described above. Some of the truncated product of the Edman reaction that had not

been coupled was used to form a complex in the same way. All three mixtures produced a 1:1 complex, as judged by the position of elution of the haem peak on Sephadex G-50 at pH 8, and by amino acid analysis. Two of the complexes have so far been subjected to further characterization. Both of these (the N^α -acetimidyl-des-Lys³⁹-cytochrome *c*-T and the N^α -acetimidyl-[Orn³⁹]cytochrome *c*-T) had spectra closely similar (including a band at 695 nm) to that of cytochrome *c* and both have the biological activity of N^α -acetimidyl-cytochrome *c*-T.

It would be worth while to attempt to re-form the bond between residues 38 and 39, but even without this possibility the discovery of the complex should be of great assistance in the production of functioning semisynthetic analogues of cytochrome *c*. The fact that the complex has 50% biological activity (at high protein concentrations) may make it possible to interpret the results of such studies with at least as much confidence as with *S. aureus* nuclease T, where the activity is only 8% of that of the native form.

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