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Biochemistry

Semisynthetic horse heart [65-homoserine]cytochrome *c* from three fragments

(peptide synthesis/reversible protection/heme protein/conformation direction)

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ABSTRACT **Horse heart cytochrome *c* was treated with methylsulfonylethyloxycarbonyl succinimide (Msc-ONSu) to give fully N^{ϵ}-protected cytochrome *c*. Treatment of this derivative with a hard base for 15 sec regenerated the native tetrahectapeptide chain. CNBr degradation of the protected compound produced three fragments bearing only protective Msc functions on ϵ-amino groups. The fragment comprising the sequence 81–104 was isolated from the mixture and acylated with *N*-hydroxysuccinimidyl-*t*-butyloxycarbonyl-L-methioninate. The resulting pentacosapeptide derivative was partially deprotected by treatment with acid and condensed in good yield (65%) with fully synthetic $N^{\alpha 66}$,$N^{\epsilon 72,73,79}$-tetra-Msc-cytochrome-*c*-(66–79)-tetradecapeptide azide. This pathway is preferred because the *penta*decapeptide azide derivative 66–80 acylated the N^{ϵ}-protected tetracosapeptide sequence 81–104 in an unpredictable manner. Subsequent treatment of the product with a base produced unprotected semisynthetic cytochrome-*c*-(66–104)-nonatriacontapeptide, which is known to undergo acylation by unprotected [Hse^{65}]cytochrome-*c*-(1–65)-pentahexacontapeptide lactone. The high specificity of this condensation is ascribed to "conformation direction." Semisynthetic [Hse^{65}]cytochrome *c* thus prepared reacts like native cytochrome *c* with a succinate cytochrome *c* reductase preparation and with cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1). This semisynthetic strategy may provide a rapid route for the production of cytochrome *c* analogs modified in the highly conservative sequence 66–80.**

The synthesis of large peptides is complicated by problems that tend to increase rapidly with increasing chain length. Most of these stem from the strongly decreasing solubility of the protected intermediates. The complete removal of protective groups likewise becomes increasingly troublesome when the sequences are longer. Furthermore, the decreasing probability of effective encounters between reactive sites implies an increase of the extent to which side reactions may occur during coupling reactions.

Solubility problems may be overcome by the development of more polar or even charged protective groups, which are more compatible with the highly polar solvents needed in the coupling reactions. Minimally protected, or in the ultimate case even unprotected, intermediates would then have to react specifically to obtain the pertinent peptide linkage. This would demand the proper guidance of the reactive sites, for instance under the direction of the preferred conformation of the fragments. It would also imply the use of weak carboxyl activations to prevent random acylations. This ideal situation has recently been achieved in the semisynthesis of basic pancreatic trypsin inhibitor (1). A similar synthesis has subsequently been described for a horse heart cytochrome *c* analog (2). In both cases the aminolysis of a homoserine lactone residue restored the uninterrupted peptide chain; a homoseryl residue thus connected the chains that were formerly linked by a methionyl residue. Both processes are to be considered as examples of reactions in which the correct orientation of the fragments is the cause of a highly selective, and, in view of the modest carboxyl activation, remarkably rapid coupling.

In this report we describe (*i*) the reversible protection of the 19 ϵ-amino functions in cytochrome *c* with the acid-stable methylsulfonylethyloxycarbonyl (Msc) group; (*ii*) the isolation of the selectively protected fragment 81–104 after CNBr degradation of N^{ϵ}-protected cytochrome *c*; (*iii*) the acylation of this fragment with fully synthetic peptide derivatives comprising the sequence 66–80 to give the reversibly protected nonatriacontapeptide 66–104; and (*iv*) the final condensation of this deprotected sequence with [Hse^{65}]cytochrome-*c*-(1–65)-pentahexacontapeptide lactone to give semisynthetic [Hse^{65}]cytochrome *c*.

Biological, chemical, and physical comparisons with native horse heart cytochrome *c* are included.

EXPERIMENTAL

Horse heart cytochrome *c* was either isolated according to the method of Margoliash and Walasek (3) or obtained from Sigma (type III). Sephadex gels were from Pharmacia; CM-cellulose (CM-52) and DEAE-cellulose (DE-52) were from Whatman.

General procedures

Column eluates were monitored with an LKB type III instrument. Amino acid compositions were determined with a modified Jeol JLC-6AH analyzer after hydrolysis in 5.7 M hydrochloric acid (Merck, Suprapur) at 110–115°C for 24 hr in sealed, evacuated ampoules.

Spectroscopic measurements were made with a Cary 118 instrument. Concentrations of cytochrome *c* were calculated from ΔA_{550} (reduced − oxidized) by using a ΔA of 21.0 mM^{-1} cm^{-1} (4).

Synthetic procedures

A survey of the synthetic procedures leading to the semisynthesis of [Hse^{65}]cytochrome *c* is given in Fig. 1.

$N^{\epsilon 86-88,99,100}$-penta-Msc-cytochrome-*c*-(81–104)-tetracosapeptide (II). *N^{ϵ}-protected cytochrome* c (*I*). Cytochrome *c* (553 mg, 44.7 μmol) was dissolved in water (20 ml) and dimethylformamide (DMF) (15 ml). The apparent pH of the solution was adjusted to a value between 8.8 and 9.4 by addition of *N*-ethyldiisopropylamine (10% vol/vol solution in DMF). Methylsulfonylethyloxycarbonyl succinimide (5) (Msc-ONSu, 440 mg, 1.66 mmol, 2 equivalents per lysyl residue) in DMF (5 ml) was added in portions, while the apparent pH was maintained between 8.5 and 9.0 by manual injection of the base. The reaction mixture was transferred after about 10 min to a Sephadex G-25 column (3.5 × 50 cm) equilibrated and eluted with a sodium phosphate buffer (0.02 M, pH 6.9). The colored fraction was lyophilized (810 mg, containing buffer salts).

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Abbreviations: Msc, methylsulfonylethyloxycarbonyl; Boc, *t*-butyloxycarbonyl; ONSu, succinimido-oxy; DMF, dimethylformamide.

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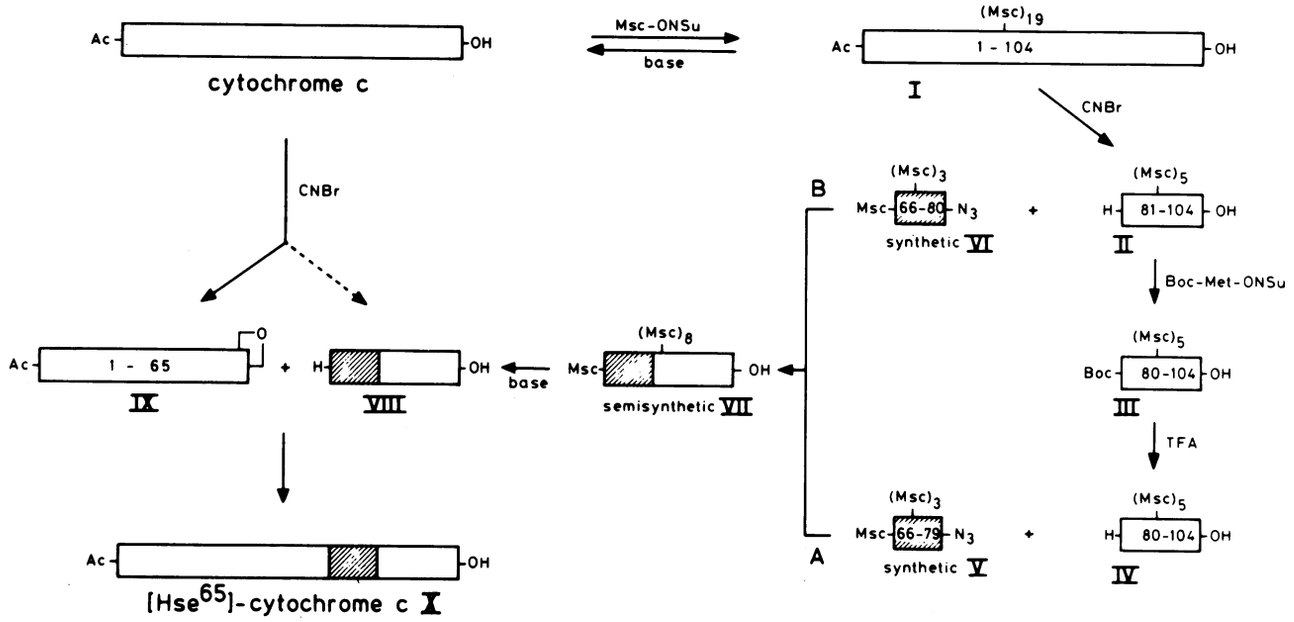

FIG. 1. Strategy for the semisynthesis of [Hse^{65}]cytochrome *c* (**X**). TFA, trifluoroacetic acid.

A sample (10 mg) of the fully protected derivative **I** could be deprotected in a mixture of DMF (0.75 ml) and methanol (0.25 ml) by a 15-sec treatment with 4 M sodium hydroxide (0.05 ml) under vigorous agitation (Vortex). After neutralization with excess acetic acid (0.1 ml), the cytochrome *c* was recovered by gel filtration on Sephadex G-25 in 0.02 M sodium phosphate buffer, pH 6.9.

Isolation of ***II.*** The crude protected cytochrome *c* (**I**) (800 mg, 44 μmol) was dissolved in 70% (vol/vol) aqueous formic acid (20 ml) and treated with CNBr (140 mg, 1.32 mmol, 30 molar equivalents) for 24 hr in the dark at room temperature. The resulting solution was diluted with water (8 ml) and applied to a Sephadex G-50 column (3.5 × 100 cm) and eluted with 50% (vol/vol) aqueous formic acid. Fractions containing the desired tetracosapeptide derivative were pooled, concentrated (30°C) under reduced pressure to about 2 ml, diluted with 50 ml of water, and lyophilized to give 115 mg of crude **II.** This material was dissolved in 10 ml of sodium phosphate buffer (0.01 M, pH 6.9, containing 4 M urea) and applied to a DE-52 column (1 × 26 cm) previously equilibrated with the same buffer. The column was developed with a linear gradient obtained by mixing 250 ml of the 0.01 M buffer with 250 ml of the same buffer 0.05 M in sodium phosphate. Material comprising the main peak (Fig. 2) was desalted on a Sephadex G-25 column (2.8 × 40 cm) with water as the eluent and recovered by lyophilization (yield 83 mg, 52%).

N^{α}-Boc,$N^{\epsilon 86\text{-}88,99,100}$-penta-Msc-cytochrome-*c*-(80–104)-pentacosapeptide (III). The partially protected, COOH-terminal cytochrome *c* fragment **II** (21 mg, 5.95 μmol) was suspended in DMF (10 ml). Upon addition of water (0.5 ml) a perfectly clear but somewhat viscous solution resulted, which was further diluted with water (1.0 ml). Boc-Met-ONSu (6) (20 mg, 58 μmol) was dissolved in DMF (0.2 ml) and added to the vigorously stirred solution. The apparent pH was kept between 8.0 and 8.5 by addition of ethyldiisopropylamine and within a few minutes the solution gelatinized. After 5 hr, 2-propanol (15 ml, peroxide free) was added to the sticky mass. The precipitate was collected by centrifugation, washed with 2-propanol and with ether, and dried (yield 18 mg, 80%).

Cytochrome-*c*-(66–104)-nonatriacontapeptide (VIII). *$N^{\epsilon 86\text{-}88,99,100}$-penta-Msc-cytochrome*-c-*(80–104)-pentacosapeptide IV.* Compound **III** (15 mg) was partially deprotected by dissolution in 90% (vol/vol) aqueous trifluoroacetic acid (1.0 ml) at −10°C. The solution was kept at room temperature for 40 min. The product was isolated by ether precipitation.

Amino components II and IV. The amino components, prior to the condensations to give **VII**, were dissolved in anhydrous trifluoroacetic acid, which was subsequently evaporated in a current of nitrogen. The compounds were dried under reduced pressure over moist potassium hydroxide.

Msc-Glu-Tyr-Leu-Glu-Asn-Pro-Lys(Msc)-Lys(Msc)-Tyr-Ile-Pro-Gly-Thr-Lys(Msc)-N_3 (***V***). The parent hydrazide was synthesized in homogeneous solution (7) as the *tert*-butyl carbazate and liberated by treatment with trifluoroacetic acid. The resulting trifluoroacetate (16.8 mg, 7.0 μmol) was dissolved in DMF (250 μl) and treated with dry HCl in ethyl acetate (14 μl of a 3.9 M solution, 55 μmol) with cooling at −20°C and stirring. *tert*-Butyl nitrite [25 μl of a 4% (vol/vol) solution in DMF, 8.75 μmol] was then added, and the solution was kept at −15°C for 15 min, then cooled to −30°C and neutralized by the addition of ethyldiisopropylamine [75 μl of a 10% (vol/vol) solution in DMF, 43.5 μmol]. The presence of the azide function was established by infrared spectroscopy.

Msc-Glu-Tyr-Leu-Glu-Asn-Pro-Lys(Msc)-Lys(Msc)-Tyr-Ile-Pro-Gly-Thr-Lys(Msc)-Met-N_3 (***VI***). This compound was obtained from the parent hydrazide trifluoroacetate (21.0 mg, 8.2 μmol) in a manner analogous to that for **V.** The presence of the azide function was again established by infrared spectroscopy.

Ubiquitously N-*Msc-protected cytochrome*-c-*(66–104)-nonatriacontapeptide* (***VII***). Route A: The activated peptide derivative **V** (6.62 μmol) was added to a precooled solution of **IV** (15 mg, 4.16 μmol) in a mixture of dimethyl sulfoxide (120

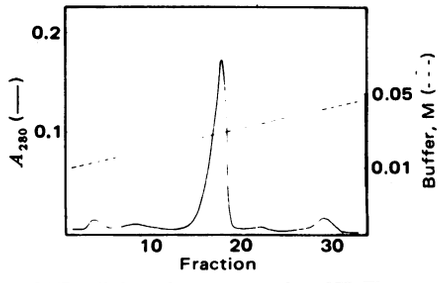

FIG. 2. DEAE-cellulose chromatography of **II.** Flow rate, 50 ml/hr; fraction volume, 12.5 ml.

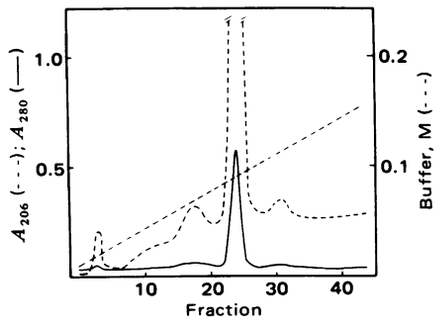

FIG. 3. CM-cellulose chromatography of **VIII.** Flow rate, 25 ml/hr; fraction volume, 4.2 ml.

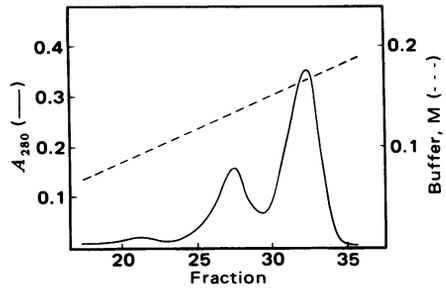

FIG. 5. Purification of **IX** (35 mg) on CM-cellulose (1.4 × 28 cm). Linear sodium phosphate gradient at pH 6.8, 0.01–0.20 M; flow rate, 35 ml/hr; fraction volume, 10.5 ml.

μl) and hexamethylphosphoric triamide (100 μl). The apparent pH of the reaction mixture, as indicated by moist indicator paper, was adjusted to 7.0–7.5 with ethyldiisopropylamine [92 μl of a 10% (vol/vol) solution in DMF, 53.4 μmol]. The pH had to be readjusted after about 38 hr at 0°C with the base. Crude **VII** was precipitated by addition of dry, precooled ether after a reaction period of 4 days. Route B: Compound **II** (17.4 mg, 4.83 μmol) was acylated with **VI** (8.2 μmol) as described for route A.

Deprotection of VII: cytochrome-c-(66–104)-nonatriacontapeptide (VIII). The crude material (30 mg) from route A was dissolved in a mixture of dimethyl sulfoxide (0.3 ml) and hexamethylphosphoric triamide (1.2 ml). The mixture was diluted with methanol (0.5 ml), and 4 M sodium hydroxide (0.1 ml) was injected at once into the clear solution with vigorous agitation (Vortex mixing). The initial base concentration was thus 0.2 M. After exactly 30 sec the base was neutralized by the addition of excess acetic acid. The product was precipitated by the addition of pre-cooled ether, then dried. The sediment was redissolved in 7% (vol/vol) aqueous formic acid and applied to a Sephadex G-50 column (1.3 × 125 cm) which was equilibrated and eluted with the same solvent. Fractions comprising the first peak were pooled and evaporated under reduced pressure (<25°C). The residue was lyophilized twice from water (yield, 12.3 mg, 65%). The fluffy material was dissolved in 5.0 ml of sodium phosphate buffer (0.01 M, pH 6.9) and applied to a CM-52 column (0.8 × 27 cm) equilibrated in the same buffer. The column was eluted with a gradient obtained by mixing 100 ml of the 0.01 M sodium phosphate buffer with 100 ml of the same buffer 0.2 M in sodium phosphate. Fractions comprising the main peak (Fig. 3) were pooled and desalted on Sephadex G-25 (1.2 × 35 cm) with 0.1 M acetic acid as the eluent. The product was isolated by lyophilization.

[Hse^{65}]Cytochrome-*c*-(1–65)-pentahexacontapeptide Lactone (IX). Cytochrome *c* was treated with a 3-fold molar amount of CNBr in 70% (vol/vol) aqueous formic acid for 24 hrs (8). Fragmentation products were separated on Sephadex G-50 (3.5 × 125 cm) in 7% (vol/vol) aqueous formic acid (Fig. 4). Fragment 1–65 was rechromatographed on the same column and finally purified on a CM-52 column, with a linear sodium phosphate gradient (Fig. 5). This step removed the unreactive form of the fragment, in which the COOH-terminal homoserine lactone had been hydrolyzed.

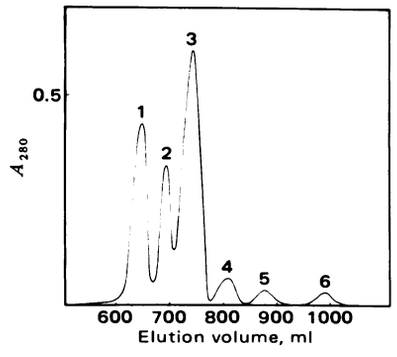

FIG. 4. Gel filtration of the fragments from a limited CNBr degradation of cytochrome *c* (130 mg). Flow rate 29 ml/hr; peak 1, 1–104; peak 2, 1–80; peak 3, 1–65; peak 4, 66–104; peak 5, 81–104; peak 6, 66–80.

Semisynthetic [Hse^{65}]Cytochrome *c* (X). The cytochrome *c* chain was reconstituted by a procedure similar to that described by Corradin and Harbury (9). In a typical experiment the (1–65)-lactone **IX** (7.0 mg, 0.91 μmol) and semisynthetic fragment 66–104, **VIII,** (3.0 mg, 0.65 μmol) were dissolved in 0.1 M sodium acetate buffer pH 5.6 (2.0 ml). The mixture was freed from oxygen by flushing with highly purified nitrogen in a rotating, modified Thunberg assembly with a 2-mm cuvette attached. The heme-containing component **IX** was reduced by addition of a minimal amount of sodium dithionite (16 μl of a 22 mM solution in deaerated sodium acetate buffer, pH 5.6:0.35 μmol). The solution was kept in the reduced form for 48 hrs (a 70% conversion of **VIII** was estimated from the absorbance change at 550 nm upon oxidation with oxygen). The reaction mixture was then subjected to gel filtration on a Sephadex G-50 column in 7% (vol/vol) aqueous formic acid. Fractions corresponding to the peak that was eluted at the position of native cytochrome *c* were pooled and lyophilized. This material was dissolved in 0.1 M sodium phosphate buffer, pH 6.8, containing 8 M urea (2.0 ml), applied to a Sephadex G-25 column (1.0 × 35 cm), and eluted with 0.1 M sodium phosphate buffer, pH 6.8. The colored fraction was oxidized with potassium ferricyanide and chromatographed on a CM-52 column (0.6 × 18 cm) in 0.1 M sodium phosphate buffer, p*H* 6.8 (Fig. 6). [Hse^{65}]Cytochrome *c* was eluted as a single, symmetrical peak (yield, 0.28 μmol, 43%).

Ascorbate/Tetramethylphenylenediamine Cytochrome *c* Oxidase Activity. Beef heart cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) was purified

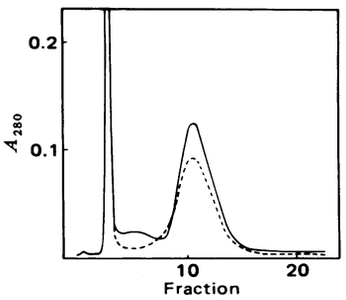

FIG. 6. Elution patterns of **X** (—) and native cytochrome *c* (- - -) on a CM-cellulose column. Flow rate, 10.7 ml/hr; fraction volume, 2.7 ml. The profiles were matched on the potassium ferricyanide peak.

Table 1. Amino acid compositions of intermediate peptide fragments in the semisynthesis of X*

Residue	II	II†	III	V	VI	VIII‡	VIII§	VIII†§
Asp	2.02	2.08	2.04	1.03	1.00	2.93	2.94	3.07
Thr	1.88	1.87	1.92	0.90	0.96	2.91	2.84	2.86
Glu	2.88	2.91	2.89	2.15	2.08	5.02	5.16	4.30
Pro				1.98	1.99	2.08	2.13	2.28
Gly	1.03	1.05	1.04	1.05	1.03	2.04	2.01	2.03
Ala	2.98	2.97	3.03			2.99	2.89	2.83
Met			0.84		0.92	0.82	0.79	0.78
Ile	2.99	2.03	2.85	0.96	0.95	3.90	3.87	3.77
Leu	2.06	2.04	1.99	0.99	1.02	2.97	3.01	3.19
Tyr	1.00	0.10	1.00	1.96	1.94	2.91	2.78	0.65
Phe	1.01	0.96	1.03			0.98	0.96	0.90
Lys	4.95	4.93	4.94	2.87	3.02	7.67	8.07	0.46
Arg	1.03	1.07	1.03			1.05	0.97	0.97

* Theoretical values correspond to the nearest integer.
† After dinitrophenylation. Dinitrophenylation of **III** yielded: 2.74 Ile, 4.91 Lys.
‡ Obtained through route A (Fig. 1).
§ Obtained through route B (Fig. 1).

as described by Van Buuren (10). The activity was measured polarographically with a Clark electrode mounted on a Gilson oxygraph. The reaction mixture contained 50 nmol of cytochrome aa_3, 50 mM potassium phosphate at pH 6.8, 0.5% Tween, 250 mM sucrose, 6.0 mM ascorbate, and 1.0 mM *N,N,N′,N′*-tetramethylphenylenediamine. Cytochrome *c* concentrations ranged from 0.2 to 1.2 μM.

Succinate Cytochrome *c* Reductase Activity. A succinate cytochrome *c* reductase preparation as described by Yu *et al.* (11) was used. The activity was measured spectrophotometrically by recording the absorbance change at 415 or 550 nm. The reaction mixture (1 ml) contained 7.5 μg of the reductase, 50 mM Tris·HCl at pH 8.0, 250 mM sucrose, 10 mM succinate, and 0.4 mM KCN. Cytochrome *c* concentrations ranged from 1 to 4 μM.

RESULTS AND DISCUSSION

The spontaneous reformation of a broken peptide chain in a rigid molecule (basic pancreatic trypsin inhibitor), as described by Dyckes *et al.* (1), exemplified a potential new tool in peptide and protein chemistry. This was followed by the genuine intermolecular condensation of two separated fragments of horse heart cytochrome *c* to give the complete enzyme (2). In this case the reaction was preceded and apparently stimulated by the formation of a stoichi6ietric molecular complex (9). This recombination from the heme-containing (1–65)-fragment lactone and the 66–104 fragment is the basis of the semisynthesis of [Hse^{65}]cytochrome *c* from three fragments that is outlined in this paper (Fig. 1).

In the semisynthesis of the 66–104 fragment, the previously demonstrated (12, 13) suitability of the acid-stable Msc group for the reversible protection of amino functions was further substantiated. All 19 ε-amino functions of cytochrome *c* could be protected within 2 min by using only a slight excess of the mixed succinimidyl carbonate. The product was readily soluble in aqueous media at pH >5.5. After reaction with fluorodinitrobenzene, amino acid analyses showed unaltered values for lysine, 18.4–18.8 residues per mol of protein, compared with 0.4–0.9 residue per mol for the unprotected protein.

Tryptic digestion of the derivatized protein gave only two fragments, due to cleavage at the Arg^{38}-Lys(Msc)^{39} bond. **I** behaved as a single entity upon ion-exchange chromatography on DE-52 in sodium phosphate buffer at pH 6.9 containing 4 M urea. No differences in the visible absorbance spectrum were detected, compared with the native unmodified protein. The full intensity of the conformation-sensitive 695-nm band was present, which was indicative of the unaltered methionine-80 ligation to the heme iron. The product was only very slowly reduced by ascorbate.

Msc protective groups could be split off by a 15-sec treatment with base. The ensuing product was indistinguishable from the native cytochrome *c* by its elution position on a CM-52 column, its lysine content of 0.6 residue per mol of protein after dinitrophenylation, and its UV-visible absorbance spectrum. The deprotected product was completely and rapidly reduced by ascorbate.

Treatment of the N^{ϵ}-protected cytochrome *c* (**I**) with CNBr produced the selectively N^{ϵ}-protected 81–104 fragment (**II**). Chromatography on DE-52 (Fig. 2) gave one major product. Dinitrophenylation and hydrolysis of this tetracosapeptide derivative proved Ile-81 to contain the only free amino group present (Table 1).

III was obtained by acylating **II** with Boc-Met-ONSu. The extent of acylation calculated from amino acid analyses (Table 1) appeared to be 85%, evidently due to the significantly reduced solubility of **III**. Io attempts were made to remove the remaining starting compound **II**, because the absence of methionine-80, one of the heme ligands, in the final "conformation-directed" coupling reaction would preclude effective complex formation. Deprotection of the product in 90% (vol/vol) aqueous trifluoroacetic acid afforded the crude N^{ϵ}-protected sequence 80–104 (**IV**).

The syntheses of the N^{α},N^{ϵ}-Msc-protected peptide hydrazides **V** and **VI**, comprising the sequences 66–79 and 66–80, respectively, were done by conventional procedures (7). *tert*-Butyl-derived protecting groups were used for temporary protection of the other side-chain functional groups. Peptides **V** and **VI** were found to be homogeneous by thin-layer chromatography, prior to and after deprotection, and by amino acid analyses (Table 1). A detailed description of the syntheses will subsequently appear elsewhere.

Fig. 1 shows two routes for the production of the semisynthetic (66–104)-peptide (**VIII**). The direct approach, route B, in which **VI** should acylate **II**, proved to have a rather unpredictable course. In one series of experiments the azide failed to acylate **II** and simple model compounds. Therefore route A, via **III**, **IV**, and **V**, was devised, giving **VIII** in a very satisfactory yield of 65%. In later experiments route B afforded yields of 20–37%. The model reactions between Msc-Gly-Thr(Bu^t)-Lys(Msc)-Met-N_3 and a series of amino components gave nearly quantitative yields, whereas in the absence of the *tert*-butyl ether function the same reluctance to react was noted (30–50%

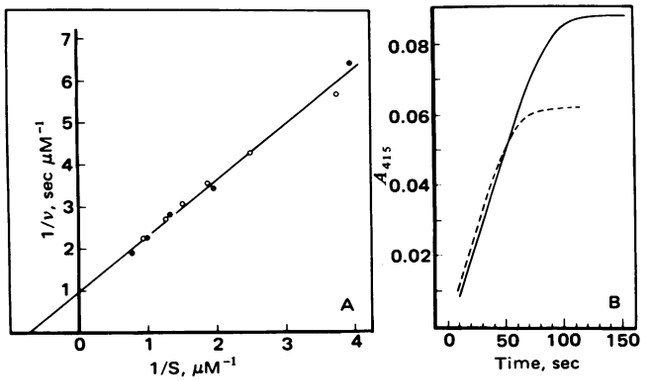

FIG. 7. (*A*) Comparison of the activity of **X** (○) and native cytochrome *c* (●) with cytochrome *c* oxidase. Velocity *v* is expressed in μmol of O_2 per sec and substrate concentration S is expressed in μM cytochrome *c*. (*B*) Changes in absorbance at 415 nm as 1.8 μM **X** (- - -) and 2.5 μM native ferricytochrome *c* (—) are reduced by a succinate cyrochrome *c* reductase preparation.

yields). The low and variable yields of acylations by **VI** may be ascribed to the presence of the unblocked side chain of Thr-78.

The selectively protected peptide **IV**, and to a lesser extent **II**, exhibited a troublesome tendency to associate in mixed aqueous/organic solvents, as already indicated above for compound **III**. Pretreatment of **II** and **IV** with trifluoroacetic acid effectively precluded gelatination during the subsequent acylations, if carried out under anhydrous conditions. Semisynthetic **VIII** (66–104) was isolated, after removal of the Msc protective groups with base, by gel filtration. Amino acid analyses (Table 1) of **VIII** obtained through route A as well as through route B gave the expected ratios. The semisynthetic (66–104)-peptide was eluted from a CM-52 column (Fig. 3) at the same position as the "native" fragment obtained from a limited CNBr degradation of cytochrome *c*.

The final product, [Hse^{65}]cytochrome *c* (**X**), was synthesized by reformation of the Hse^{65}-Glu^{66} bond between reduced "native" (1–65)-lactone (**IX**) and semisynthetic (66–104)-peptide (**VIII**). **X** was isolated by gel filtration. When condensation was performed with two purified "native" fragments, the efficiency was found to be over 90%; after thorough purification a 65% yield of **X** was obtained, and **X**'s properties showed no significant deviations from those of the native cytochrome *c* (14). In a recently described semisynthesis of **X** (15), a yield of only 6% was recorded for the final recombination reaction; the 66–104 fragment was obtained by a solid-phase synthesis, a possibly inadequate procedure.

It is noteworthy that the crude product **X**, isolated by lyophilization from 7% (vol/vol) aqueous formic acid, exhibited only a very modest activity towards cytochrome *c* oxidase at this stage. CM-cellulose chromatography revealed that only 15% of the material was eluted at the same position as native cytochrome *c*, while approximately 50% appeared to be irreversibly bound. However, native cytochrome *c* gave the same elution pattern when treated similarly. This indicated that the combined effects of formic acid and lyophilization had a profound influence on the chromatographic behavior of the protein.

Lyophilized semisynthetic [Hse^{65}]cytochrome *c* could be renatured by a short exposure to 8 M urea at neutral pH. Subsequent chromatography on CM-52 gave the elution profile of native cytochrome *c* (Fig. 6). The semisynthetic product (43%) had the same spectrum as the native protein and showed the normal reactivity towards ascorbate, and the reduced form did not bind any carbon monoxide. A treatment with CNBr produced the fragments 1–80 and 81–104 as the sole products.

The cytochrome *c* oxidase activity was determined under conditions that gave rise to monophasic kinetics. From Fig. 7*A* a K_m value of 1.3 μM can be calculated for the semisynthetic analog. The same value is found for native cytochrome *c*, in agreement with the value for the second polarographic phase (16). Fig. 7*B* shows the change in 415-nm absorbance during enzymatic reduction. In the concentration range of 1–4 μM cytochrome *c* no differences in the zero- and first-order parts of the kinetic curves (17) were observed. It is significant that [Hse^{65}]cytochrome *c* was completely (98%) reduced.

The semisynthetic approach from two natural fragments and a synthetic middle fragment appears to present an efficient route towards the construction of [Hse^{65}]cytochrome *c*, and possibly of analogs with completely defined sequences. The modification of the small but highly conservative middle portion 66–80, made possible by the proper location of the methionine residues, provides interesting possibilities for further studies into the mechanism of the electron transport to and from cytochrome *c*.

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