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TOTAL SYNTHESIS OF HORSE HEART CYTOCHROME C

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Summary: A strategy based on complexation-assisted condensation of large synthetic protein fragments and mitochondria-mediated stereospecific heme insertion has been utilized to assemble a functional molecule corresponding to native horse heart holocytochrome c. This original approach offers the unique opportunity of selective modifications both in the C-terminal and in the N-terminal regions of the apoprotein and may represent an useful alternative to *site-directed mutagenesis*[1,2], particularly when D-amino acids, chemically and/or isotopically modified or other unnatural amino acids have to be introduced in this important molecule. The present result is an example of how solid phase peptide synthesis of large protein fragments in conjunction with the availability of a specific recognition process may extend the potentiality of the chemical approach to the synthesis of an entire protein.

Cytochrome c is a well studied protein normally found on the outer surface of the inner mitochondrial membrane of all aerobic organisms[3]. The interest in the structure and properties of this molecule is due to its key role in electron-transfer and to the fact that it represents a unique tool to study molecular evolution[4], immunogenicity[5] and, in general, the intricate relationships between structure and function in proteins[6].

Studies on several two- and three-fragment non-covalently linked complexes have allowed the definition of two permissible regions for cleavage of the polypeptide chain, namely between residues 23-25 and residues 39-56 (Fig. 1a), without disrupting the overall fold of horse heart cytochrome c[6]. Interestingly, they correspond to two regions which are believed to be inserted during evolution. Some of these complexes, presumably because of interactions involving the heme moiety, are more stable than the similar ones given by other well characterized proteins such as ribonuclease A[7] or staphylococcal nuclease[8] and are therefore particularly suited to understand cooperative interactions involved in protein folding[6].

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Several years ago Corradin & Harbury[9] have reported on the formation of a native-like complex between the two CNBr fragments [Hse>^{65}](1-65)H heme fragment and (66-104) of horse heart cytochrome c and on the spontaneous re-synthesis between the reactive C-terminal Hse^{65}lactone and the α-amino function of the Glu^{66}, provided the heme iron is in the reduced ferrous state. This observation opened up the way to obtain semisynthetic cytochrome molecules by condensation of native [Hse>^{65}](1-65)H heme and synthetic (66-104) fragments, although the possibility of introducing substitutions was limited to the C-terminal region[10-12].

More recently, Gozzini et al.[13] have shown, using native fragments, that the peptide bond between the CNBr [Hse>^{65}](1-65) apofragment and (66-104), can be selectively restored when these non-heme segments form a three-fragment complex with the reduced (1-25)H heme-fragment (Fig. 1b). These findings have extended the possibility of selective substitutions to the entire apocytochrome c molecule. Restoration of the peptide bond was also observed with the apofragment [Hse>^{65}](23-65), confirming that in the (1-25)H:[Hse>^{65}](1-65):(66-104) complex residues 1 to 22 of the apofragment flexibly protrude from the ordered structure (Fig. 1b) and therefore do not cooperate in the formation and stabilization of the ferrous complex. Therefore, any modification in the 1-22 sequence, e.g. substitution with natural or unnatural amino acids, insertion or deletion of either amino acids or segments should not interfere with the restoration of the 65-66 bond and it seems like-

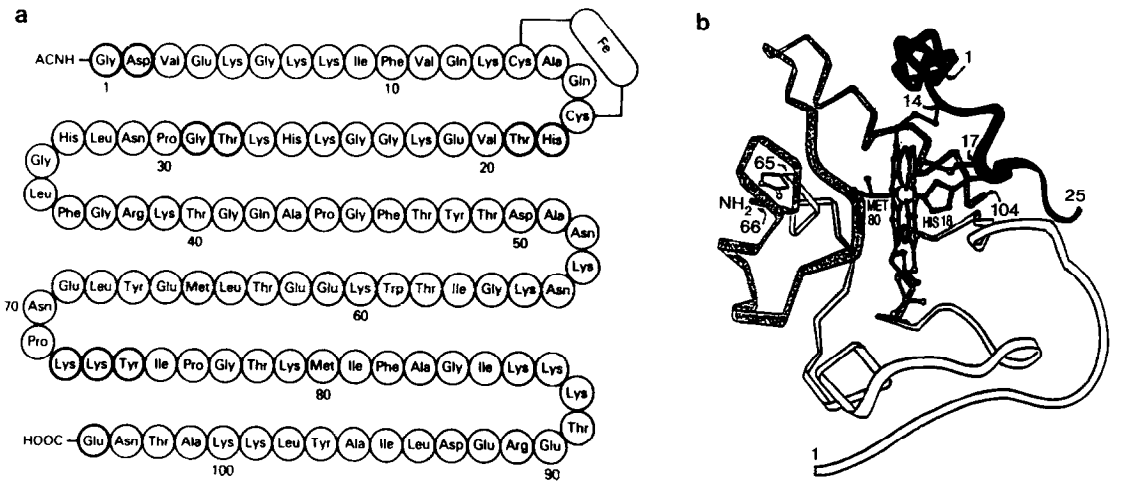

Fig. 1a. Primary structure of horse heart cytochrome c.

Fig. 1b. A schematic representation of the three-fragment complex. The drawing refers to tuna cytochrome c[22] and is taken from Gozzini et al.[13]. X-ray crystallographic investigations have shown a close structural homology among cytochromes c of different species[23-25]

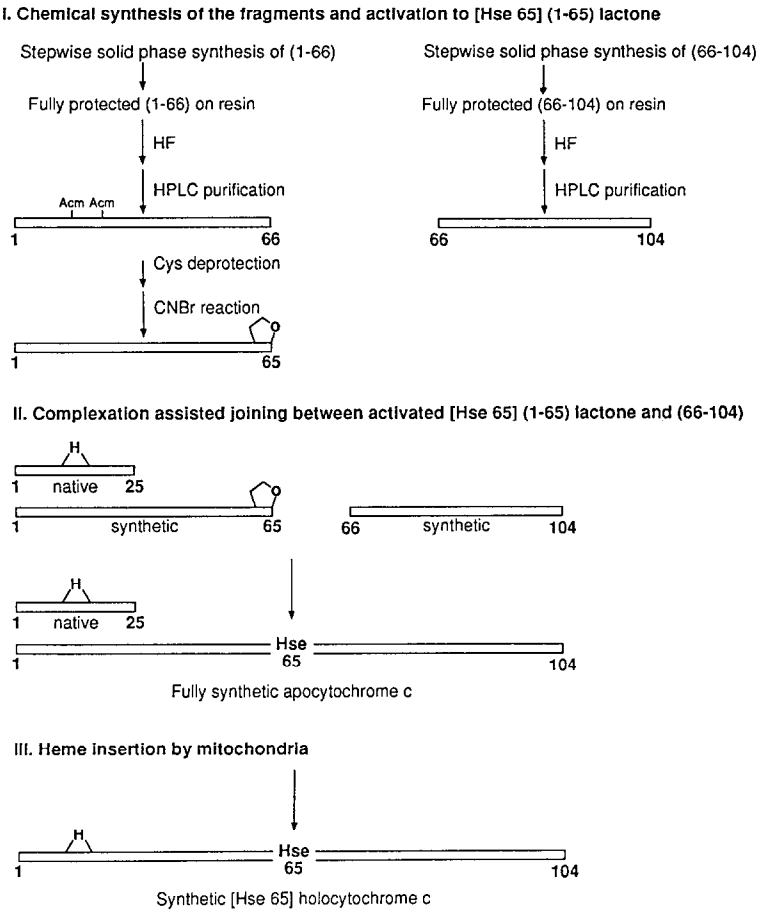

Fig. 2. Strategy followed for the total synthesis of [Hse^{65}]holocytochrome c. **I.** Peptides corresponding to (1-66) and (66-104) sequences were synthesized by stepwise solid-phase methods on Pam resin[26-28]. After HF cleavage, purification was achieved by semipreparative HPLC. Homogeneity was determined by analytical HPLC, amino-acid composition, Edman degradation, electrophoresis, tryptic and chymotryptic peptide mapping. Details are reported elsewhere[26,28]. Cys Acm-protection was removed[29] by Hg^{2+}, then CNBr treatment resulted in cleavage of the 65-66 peptide bond with simultaneous formation of activated [Hse^{65}](1-65)-lactone. The native (1-25)H heme-fragment was obtained by tryptic cleavage of reduced (1-38)H:apocythocrome complex according to Taniuchi et al.[6]. **II.** "Optimized" rejoining conditions involve incubation of synthetic [Hse>^{65}](1-65) and (66-104) with native (1-25)H in HEPES buffer, pH 7, in the absence of oxygen. (1-25)H and (66-104) were in equimolar amounts, while 50% molar excess [Hse>^{65}](1-65) was used to take into account for lactone hydrolysis. Dithionite and radical scavengers were added to keep the heme-iron reduced and to prevent damages to the heme. The reaction was monitored by SDS-PAGE (Fig. 3). Rejoining yields up to 80% were obtained after 48 h, as determined by HPLC (data not shown). The synthetic [Hse^{65}]apocytochrome c was purified by ion-exchange and RP-HPLC and characterised by amino-acid composition and, after non-covalent binding to ferri [1-25]H, by UV-visible and UV-CD spectroscopy, and biological activity[21]. **III.** Mitochondria incubation was performed essentially as described in ref. 14. Briefly, 0.5 mg synthetic [Hse^{65}]apocytochrome c was radioactively labelled at Lys residues by reductive methylation[30] (^{14}C specific activity = 15 x 10^6 dpm/mg). The radioactive material was incubated for 40 min at 25 °C with a fresh beef heart mitochondria suspension (100 mg protein in 2.5 mL total volume) and hemin. The reaction was monitored by RP-HPLC (Fig. 4). Then, the newly formed radioactive [Hse^{65}]holocytochrome c was extracted with 0.5 M NaCl, submitted to ion-exchange chromatography, precipitated by 10% TCA, redissolved in 6 M guanidinium chloride and purified by RP-HPLC. The purified synthetic material gave

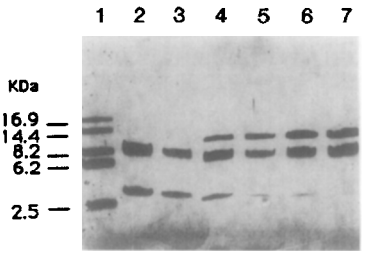

Fig. 3. SDS-PAGE time course analysis of synthetic [Hse^{65}]apocytochrome c formation by complexation-assisted joining. Synthetic [Hse^{65}](1-65) was incubated with synthetic (66-104) and native (1-25)H under the experimental conditions described in the legend to Fig. 2 (point II). The joining reaction was monitored by electrophoresis on a Pharmacia-LKB Phast System using High Density Phast Gel and silver staining. Samples taken at 0, 1, 6, 11, 32, 48 h (lanes 2 to 7) reveal that [Hse^{65}]apocytochrome c (12 KDa) becomes detectable only after 6 h, while (66-104) (4 KDa) has reacted almost quantitatively after 32-48 h. Fragment (1-25) is not detectable in the present experimental conditions. Lane 1 contains low MW standards.

ly that this concept could be extended also to the 39-56 region. Moreover these results, combined with the known availability of a mitochondrial enzymatic system specific for heme attachment to the apoprotein [14-17], have also suggested a possible strategy for the total synthesis *in vitro* of horse heart cytochrome c[13].

According to the procedure illustrated in Fig. 2, the total assembly of a functional holoprotein has now been achieved by mitochondria-mediated insertion of the heme to the side-chains of Cys^{14} and Cys^{17} of fully synthetic [Hse^{65}]apocytochrome c which, in turn, has been obtained with good yield by complexation-assisted covalent ligation of two chemically synthesized fragments spanning the entire 104-residues sequence of the horse heart apoprotein. As shown in Fig. 3, the formation of [Hse^{65}]apocytochrome c was followed by the disappearance of the electrophoretic band corresponding to the (66-104) fragment. Yields as high as 80% were determined after 48 h by HPLC analysis. Then, the synthetic [Hse^{65}]apocytochrome was radioactively labelled at Lys residues by reductive methylation and converted into [Hse^{65}]holocytochrome c by incubation with beef heart mitochondria and hemin. The overall yield was calculated to be > 8% with respect to the synthetic [Hse^{65}]apocytochrome initially treated (Fig. 4). The only difference between the native and the newly synthesized protein is the replacement of Met^{65} by Hse^{65}, a modification which has no effect on the structure and properties of cytochrome c[18]. The synthetic holoprotein was purified by a combination of semipreparative ion-

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the expected amino-acid composition and resulted indistinguishable from authentic horse heart holocytochrome c by different criteria, including intensity and position of the maxima of the principal absorption bands, biological activity[21] and monoclonal antibody binding[20].

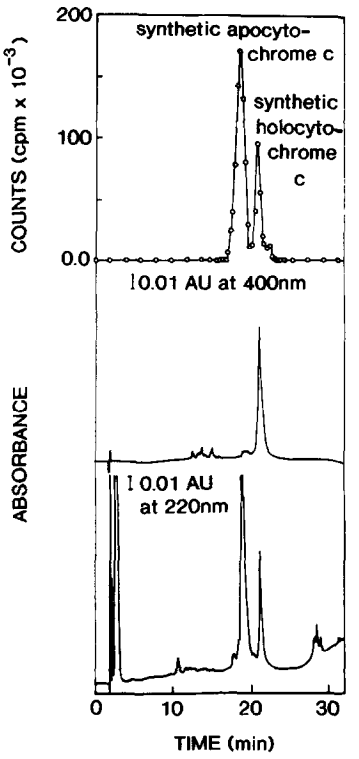

Fig. 4. RP-HPLC analysis of mitochondria-mediated stereo-specific heme-insertion on synthetic [Hse^{65}]apocytochrome c. The stereospecific attachment of the heme to $Cys^{14, 17}$ residues by beef mitochondria (see legend to Fig. 2, point III) was determined by RP-HPLC analysis on a Vydac C_4 column (0.46 x 15 cm) eluted with an acetonitrile gradient in 0.1% TFA (5 to 28% in 5 min, 28 to 38% in 20 min, 39 to 90% in 3 min). The eluate was analysed at 220 nm for total protein detection, at 400 nm for heme-containing proteins and by ^{14}C scintillation counting for [Hse^{65}]apocytochrome c conversion. Synthetic [Hse^{65}]apocytochrome c elutes at 19 min, while the newly produced ^{14}C radioactive heme-containing [Hse^{65}]cytochrome c is eluted at 21 min, as expected for native cytochrome c. The total radioactivity recovery for both peaks was 40%, due to losses during work up of the reaction mixture. Accordingly, the final yield of the newly formed [Hse^{65}]holocytochrome c corresponds to about 8% with respect to the [Hse^{65}]apocytochrome c initially treated.

exchange and RP-HPLC and was found to be indistinguishable from native horse heart holocytochrome c on the basis of spectroscopic and biological characterization. Proofs of identity included: UV-visible spectra in the oxidized and ascorbate-reduced state; UV-CD spectra with the characteristic negative ellipticity band at 222 nm[19]; recognition by monoclonal antibodies[20] and reduction by a yeast lactate dehydrogenase in the presence of lactate[21].

Our results demonstrate the possibility to achieve, in a complete *in vitro* system, the total synthesis of a functional horse heart holocytochrome c molecule by a combination of synthetic, conformation-assisted and enzyme-mediated procedures. The method, which in principle can be used to obtain cy-

tochromes carrying either minute or extended modifications in the N- and C-terminal parts of the apocytochrome sequence, reduces the synthetic effort to the preparation of relatively short peptides that can be produced easily by modern automated synthesizers and purified by current HPLC techniques. The advantages of the present approach for the study of protein structure and function may be summarized as follows: (i) relatively large amounts of the desired peptide can be produced in a matter of days in yield and purity that allow direct functional analysis; (ii) multiple variants of a given sequence can be made in short time by combination of different sequences of the N- and C-terminal fragments; (iii) variants containing non naturally occurring amino acids can be synthesized.

Although the general applicability of the conformation-assisted coupling method may be limited by the requirement of tight complex formation and by the permissibility of the Hse → Met substitution, the present strategy is by no means restricted to the case of cytochrome c and it may be extended to other systems containing potential resynthesis sites.

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