Functional Consequences of the Excision of an Ω Loop, Residues 40–55, from Mitochondrial Cytochrome c*

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A novel technique for protein semisynthesis, enzymic activation, has been used to create a mitochondrial cytochrome c analogue in which the conventional bottom loop has been deleted. The resulting structure resembles that of cytochrome c₅₅₅ from a primitive photosynthetic sulfur bacteria. Comparisons of this analogue with natural cytochromes show which of the functional differences between cytochromes c and c₅₅₅ may be related directly to the incorporation of the loop. The structure is an example of an Ω loop, recently defined as a discrete category of protein secondary structure. The analogue maintains the overall structure of the parent protein, but a significant change in redox potential has been engineered. It provides support for the prediction that Ω loops act as independent modules in folding, function, and evolution. The rapidity of the synthesis and the high yield of product show that this technique for protein engineering is both competitive with, and complementary to, genetic methods.

Omega loops (Ω loops) have been proposed to be a discrete category of protein secondary structure (1). They are characterized by their length (6–16 residues), their compactness, and the necking in of the two termini, so that they adopt the Ω form. They appear relatively commonly in known protein structures; cytochrome c (Fig. 2) has four. They may contain α₉ bends (reversed turns) but not α- or β-structure and comprise the majority of what was formerly termed random coil. In line with the generality (1) cytochrome c has only 10% of residues not now included in defined secondary structure (Fig. 2).

Leszczynski and Rose (1) have further suggested that Ω loops will act as modules of folding, function, and evolutionary change and would be natural candidates for protein engineering studies. The present work sets out to test some of these ideas by examining the consequences of excising the 40–55 loop of horse heart cytochrome c. The protein analogue was prepared by using the techniques of protein semisynthesis (2, 3).

Semisynthesis takes, as its starting material, the natural protein which is fragmented, manipulated, and resynthesized.

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The number of chemical steps is reduced relative to totally synthetic methods for the production of protein analogues, although the variety of operations is increased, and in general the products are of higher quality.

One of the most problematic operations in semisynthesis is fragment condensation, but we have recently developed a novel approach that has greatly increased the efficacy of this step. Reverse proteolysis has been applied successfully to the activation, prior to fragment condensation, of peptides obtained from enzyme digests of natural proteins (2, 3). The method has advantages over conventional techniques of activation for the synthesis of peptides and proteins; side chain protection is minimal or unnecessary, and racemization and side reactions are absent. Moreover, in those cases where contiguous reacting peptides associate to form noncovalent complexes (4), very high coupling yields result even in aqueous buffers because of the proximity of the reacting termini achieved by the adoption of the native conformation. In these circumstances the protein catalyzes its own resynthesis. Since by definition the residues of the neck of an Ω loop are proximate (Fig. 4), similar behavior might be expected of noncontiguous complexes in which a peptide representing a loop sequence was missing.

The properties of the product of combining an activated 1–39 fragment with fragment 56–104 are compared with those of cytochrome c analogues in which the loop is nicked at various points (4) and with the cytochrome c₅₅₅ of the green sulfur bacterium Chlorobium thiosulfatophilum. This cytochrome differs in structure from the mitochondrial protein principally in lacking this same loop (6) and thus in itself provides an example of an Ω loop as a module of evolutionary change.

EXPERIMENTAL PROCEDURES

Materials—Horse cytochrome c (type III) and porcine trypsin were obtained from Sigma. Amino acid dichlorophenyl esters were prepared as previously described (3). Other reagents were purchased from Merck and were of the highest quality obtainable.

Semisynthesis—The reaction scheme is summarized in Fig. 1. Acetimidyl fragment (1–38) was obtained from the limited trypptic digest of the fully N-ε-acetimidyl protein, and fragment (56–104) from a 1-h trypptic digest of the contiguous complex ferro-(1–38)-(39–104) (4). The N-terminal fragment was activated to give acetimidyl fragment [Ala₈³]₁(1–39) dichlorophenyl ester (3). It was subsequently mixed with 1 eq of (56–104) in phosphate buffer, reduced with a crystal of sodium dithionite, and left to stand for 1 h. The coupled product was isolated by gel filtration on Sephadex G-50 in 7% HCOOH, and acetimidyl groups were removed by exposure to a crystal of sodium dithionite, and left to stand for 1 h. The coupled product was isolated by gel filtration on Sephadex G-50 in 7% HCOOH, and acetimidyl groups were removed by exposure to a concentrated ammonia-ammonium acetate buffer of pH 11.3 (7).

Purification and Characterization of the Product—The crude product obtained from gel filtration was purified by cation-exchange chromatography on SP-Triacryl (7). Amino acid compositions were determined by chromatography of 24-h 10⁸ °C 6 N HCl hydrolysates on HPLC (Waters) using a Radialpak C₄ cartridge, followed by o-phthalaldehyde derivatization.

Spectroscopy—UV-visible spectra were drawn from 750–250 nm in 0.1 M potassium phosphate buffer, pH 7.0, at room temperature using a Cary 210 spectrophotometer. The disappearance of the weak charge-transfer band at 695 nm with increasing pH was followed as described by Wallace (9). Midpoint oxidation-reduction potentials were determined by changes in the absorption at 550 nm due to the ferrous form of the cytochrome in ferri-/ferrocyanide redox buffers of known

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**RESULTS AND DISCUSSION**

The yield of coupled product from the stoichiometric complex is 60%; this is high by the general standards of fragment condensation in protein synthesis but is comparable with the few known natural cases of conformationally assisted coupling (13). This efficiency, greater even than that seen in couplings of (1-39) and (40-104) (3), implies that the complex (1-39)-(56-104) must adopt the conformation of the native protein, wherein residues 39 and 56 are brought together at the neck of the \( \Omega \) loop (Fig. 4). It further demonstrates the capacity of this method to rapidly produce informative protein analogues in high yield from relatively few manipulations (Fig. 1).

A sample of the principal peak of ion-exchange purification was subjected to amino acid analysis. The composition was that expected of native cytochrome \( c \) from which residues 40–55 have been subtracted and in which a lysine to alanine substitution has occurred.

The \( \lambda_{\text{max}} \) of the principal bands of both des-(40–55)-ferro- and ferricyanocobyrinic acid are compared in Table I with those of related cytochromes. Also included are values for the ratios of heights of the \( \gamma \) (Soret) and \( \alpha \) bands for the ferrocyanocobyrinic acid and the \( pK_a \) for the loss with rising pH of the ferricyanocobyrinic acid absorbance at 695 nm, diagnostic of methionine 80 ligation at the 6th coordination position (9).

One of the characteristics that distinguishes Chlorobium \( c_{553} \) from other cytochromes is the marked and universal shift in \( \lambda_{\text{max}} \). Des-(40–55)-cytochrome \( c \) does not share this attribute, which cannot thus be a consequence of the lack of a bottom loop. It is not obvious from the structure (6) what other factor might be responsible. Shifts in \( \lambda_{\text{max}} \) for protoheme are generally related to the nature of the substituents attached to the heme ring (10); it may be that the heme of \( c_{553} \) differs in some as yet undetected manner from that of the mitochondrial protein.

Another distinguishing feature of the spectra, the ratio of \( A_{408}/A_{550} \), clearly does depend on the polypeptide conformation around the heme. Fragment (1-65) of horse cytochrome \( c \) has similar \( \lambda_{\text{max}} \) to the parent protein, but the ratio is like that of the analogue and Chlorobium \( c_{553} \) differs greatly from that of cytochrome \( c \).

The weak charge-transfer band observed around 695 nm is a sensitive indicator of the structural integrity of the heme crevice (10). It is absent in denatured nonfunctional molecules that may continue to exhibit an otherwise normal visible spectrum, and so the height of the band is diagnostic of the quality of the preparation. Ion-exchange purified des-(40–55)-cytochrome \( c \) shows a fully developed 695-nm band at pH 6.

The values for \( pK_a \)s of the transition at 695 nm show that the removal of the loop greatly destabilizes the protein against alkaline denaturation. The observations emphasize the role of the polypeptide chain in maintaining the crucial Fe–S bond at the sixth coordination position. In the oxidized state, this ligation is weak, and the sulfur atom may be easily displaced by a strong field ligand upon loss of the normal protein conformation (10). It is not known what the value is in the Chlorobium protein, but complexes with nicks in the loop (4) also exhibit low \( pK_a \)s. We may conclude, then, that for these contiguous complexes it is not the chain break, but the lack of an intact bottom loop, that is responsible for this characteristic.

A redox potential of 130 mV for des-(40–55)-cytochrome \( c \) (Fig. 3) was determined by the method of mixtures (13). This is close to the potential of Chlorobium \( c_{553} \), 140 mV (11), but differs greatly from that of the parent mitochondrial cyto-

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**Table I**

| Ferrocyanocobyrinic acid | \( \alpha \) | \( \beta \) | \( \gamma \) | \( A_{\alpha}/A_{\gamma} \) |
|-------------------------|------|------|------|------------------|
| Horse cytochrome \( c \) | 550  | 520  | 418  | 4.7              |
| Complex (1-50) (51-104) | 550  | 520  | 414  | 5.6              |
| des-(40–55)-cytochrome \( c \) | 550  | 520  | 416.5 | 6.2             |
| Fragment (1-65) | 550  | 520  | 415  | 6.7              |
| Chlorobium \( c_{553} \) | 555  | 523  | 418.5 | 7.1              |
| Ferricyanocobyrinic acid | \( \alpha \) | \( \gamma \) | \( \delta \) | \( pK_{695} \) loss |
| Horse cytochrome \( c \) | 528.5 | 409.5 | 360  | 9.30             |
| Complex (1-50) (51-104) | 529  | 408.5 | 358  | 7.35             |
| des-(40–55)-cytochrome \( c \) | 529  | 406.5 | 356  | 7.40             |
| Chlorobium \( c_{553} \) | 535  | 412.5 | 363  | NA*              |

* Not available.
The reaction of cytochrome c is only about 15% of that of horse cytochrome c. This value can be seen from Fig. 3 to be in accord with the prediction of Leszczynski and Rose (1) that the role of the loop is but marginally more detrimental to function than the effect of stabilizing the oxidized form (charge +1) relative to the reduced heme center (no net charge) and lowering accessibility at the bottom of the heme crevice (4). This has the effect of stabilizing the oxidized form (charge +1) relative to the reduced heme center (no net charge) and lowering potential (8). Clearly the loss of the loop altogether will permit increased solvent exposure, but it is of interest that the resulting redox potential is little different from that of one of these complexes, (1-38)-(39-104), at 150 mV. Since loss of the loop is but marginally more detrimental to function than the loosening of its structure, we may conclude that the role of the loop is exclusively that of shielding the bottom heme edge and that this Ω loop is, therefore, also a module of function.

The analogue was tested in a succinate oxidase assay system employing cytochrome c-depleted mitochondria. In this system the reaction of cytochrome c with its reductase is limiting (4). The specific activity determined for des-(40-55) cytochrome c is only about 15% of that of horse cytochrome c. This value can be seen from Fig. 5 to be in accord with the relationship established between electron transfer rate and redox potential for cytochrome c analogues that do not involve modification of residues directly involved in the active site (4). Therefore, no part of the (40–55) Ω loop participates in the electron transfer process nor does the lack of the bottom loop induce any distortion in the conformation of the active site.

The ability of Chlorobium c555 to react with a related reductase assay system has been tested by Davies et al. (5). A specific activity of 2% was found. Fig. 3 shows that this value deviates very greatly from that expected for a redox potential of 140 mV, should the active sites be homologous.

Fig. 4 sketches the binding domain of mitochondrial cytochrome c. The sequence of Chlorobium c555 shows considerable homology with respect to the lysine residues comprised in it, but with some spatial displacement. Obviously, this arrangement is not a sufficiently close approximation to that of the mitochondrial protein. The result provides an indication of the degree of discrimination exercised by the cytochrome c binding site of the mammalian reductase. The high activity with mammalian oxidase (5) noted for c555 suggests that this enzyme is not as particular.

The normal reactivity of des-(40–55)-cytochrome c implies that the active site is not distorted, and the 695-nm band shows that the heme crevice structure is not disturbed. These considerations, together with the evidence from coupling yields that the two fragments adopt a normal configuration in the interaction region.

FIG. 3. A comparison of the relationships between electron transfer rates in reductase assays and redox potential for Chlorobium cytochrome c555 and des-(40–55) cytochrome c. The latter falls on the line established for a set of analogues in which active site residues are not modified (4). The numbered bars represent cytochrome c (1), 19-N-c-acetimidyl cytochrome c (2), acetimidyl complex (1-37)-(38-104) (3), (1-55)-(56-104) (4), (1-65)-(56-104) (5), (1-50)-(51-104) (6), (1-50)-(66-104) (7), and acetimidyl (1-38)-(39-104) (8). c555 does not obey this relationship, implying a substantial difference of conformation in the interaction region.

FIG. 4. A sketch, looking into the active site, based on the published three-dimensional structure of mitochondrial cytochrome c (8) emphasizing (a) the positions of the reactive termini of the complex, residues 39 and 56, shown arrowed, and (b) the relative positions of the ε-amino groups (solid circles) of lysine residues known to mediate binding of cytochrome c to reductase (complex III), and those (dashed circles) of the lysine residues conserved among the three known sequences of the c555 group. The N- and C-termini, and the heme and its 5th and 6th coordinating ligands, His46 and Met46, are also shown.
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has often been felt that loops of random coil serve to link together units of more ordered structure, this is clearly not the role of the 40–55 loop.

It has also been suggested (1) that Ω loops may be independent folding, as well as functional, units in view of their generally globular structures which depend more on the packing of side chains within the loop than on interactions with the rest of the protein for their compactness. The insensitivity of the cytochrome c structure to the removal of the 40–55 loop supports this idea. The fact that the two termini, formerly the neck of the loop, are able to maintain their relative proximity must be in some measure due to the stability afforded by the packing of the other structural elements of the molecule, in particular the Ω loops, against the heme faces.

In summary, the loop-deleted analogue of cytochrome c behaves like the parent protein in its interaction with physiological partners and its spectral properties. It is unlike it, and more like bacterial c555, in structural stability and hence redox potential, implicating the loop in the control of this parameter. These properties are consistent with the idea that Ω loops are employed in protein structure as independent functional modules.

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