Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5729–5733, August 1990
Biochemistry

Amino acid sequence requirements for the association of apocytochrome *c* with mitochondria

(apocytochrome *c* constructs/truncated apocytochrome *c*/mutant apocytochrome *c*/targeting signals/high- and low-affinity associations)

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ABSTRACT **To examine the amino acid sequence requirements for the biphasic association of *Drosophila melanogaster* apocytochrome *c* with mouse liver mitochondria *in vitro*, recombinant constructs of the protein were prepared. Removal of the C-terminal sequence to residue 58 had little influence, but truncation to residue 50 decreased the association to low levels and removal to residue 36 was even more effective. However, a mutant missing the segment between residues 35 and 66 was fully functional, but, when the C-terminal segment from residue 36 was replaced with a noncytochrome *c* sequence, the high-affinity phase of the association was lost. A mutant in which residues 90, 91, 92, 96, and 100 were replaced by lysine, leucine, proline, proline, and proline, respectively, to prevent the possible formation of the C-terminal α-helix and another mutant in which the C-terminal segment from residue 90 to residue 120 was a noncytochrome *c* sequence had normal association. In contrast, replacing lysine-5, -7, and -8 by glutamine, glutamic acid, and asparagine, respectively, resulted in loss of the high-affinity phase. The same mutations in the apoprotein lacking the segment between residues 35 and 66 caused, in addition, a decrease of the low-affinity phase association. Thus, the N-terminal region is most critical for apocytochrome *c* association, but alternative segments of the central and/or C-terminal region can be utilized, where noncytochrome *c* sequences are ineffective. These results emphasize the wide disparity between the structural requirements for association with mitochondria and for the production of a functional holoprotein.**

Mitochondrial cytochrome *c* has been widely investigated (1–4), but little is known about the amino acid sequence requirements for the transfer of the apoprotein from its cytoplasmic site of biosynthesis (5–7) to the intermembrane space of mitochondria, its site of function as the holoprotein. *In vitro*, in the presence of a wheat germ extract factor (WGEF), a high-affinity association of apocytochrome *c* and mitochondria occurs. This leads to internalization of the apoprotein, even though up to 95% remains in equilibrium with the external medium with no covalent attachment of the heme (8).

Of the several hundred proteins imported into mitochondria, apocytochrome *c* is one of the few that do not have an N-terminal presequence (9–11), so that if any targeting sequence exists, it must be part of the apoprotein itself. Stuart *et al.* (12), using the total translation mixture of the isolated gene of *Neurospora crassa* cytochrome *c*, found that an apoprotein in which the 8 C-terminal residues were replaced by a 27-residue noncytochrome *c* sequence became associated with mitochondria to a small extent. This was taken to indicate that an "intact carboxyl-terminus was required for efficient import of apocytochrome *c* into mitochondria" (12).

By contrast, it is known that apocytochrome *c* can penetrate into artificial lipid vesicles (13–15) and N-terminal segments containing 38–65 residues are translocated nearly as well as the full-length apoprotein, whereas C-terminal fragments of 65–23 residues penetrate into, but not through, the lipid bilayer (16–18).

Although considerable evidence has accumulated that suggests a direct coupling between the import of apocytochrome *c* and the covalent attachment of the heme prosthetic group by the cytochrome *c* heme lyase system (19–24), we have found that replacing the two cysteines that normally bind the prosthetic group (residues 14 and 17) with either alanines or serines has no influence on the association with mitochondria (8). Nevertheless, the region of these two cysteines appears to be important for mitochondrial uptake, since mutants of yeast iso-1 cytochrome *c* with one or the other cysteine replaced by phenylalanine, tyrosine, or tryptophan do not make holoprotein *in vivo* and do not accumulate apoprotein *in vitro* (24). Similarly, the *N*-ethylmaleimide adduct of the apoprotein does not accumulate in mitochondria (24) and, as shown below, does not exhibit the typical high-affinity association with mitochondria in the presence of WGEF (8).

By using the WGEF-stimulated *in vitro* system (8) and various constructs of *Drosophila melanogaster* apocytochrome *c*, the amino acid sequence requirements for the import of the apoprotein into mouse liver mitochondria was examined. It was found that the C-terminal half of the sequence was not involved in the association phenomenon as long as the central and N-terminal portions were intact but that when the central portion was deleted, the C-terminal segment could replace it.

MATERIALS AND METHODS

Apocytochrome *c* Constructs. Rat apocytochrome *c* was obtained by subcloning the *Bam*HI–*Eco*RI fragment of the RC9 pseudogene, which translates into the normal rat cytochrome *c* sequence (25), into pGEM-1. Deletion and site-directed mutants of the *D. melanogaster* cytochrome *c* gene, pDM*c*1.2 (8), were generated in M13 (26), subcloned into pGEM-4Z, and sequenced. These constructs and their abbreviations are listed in Fig. 1. The codons for residues 90 and 91 were altered to that of the *Hin*dIII restriction site yielding an apoprotein in which residues 90 and 91 were changed from Glu-Arg to Lys-Leu.

Mutants of Apocytochrome *c*. Two complementary 55-nucleotide oligomers were ligated into the new *Hin*dIII restriction site. After transformation, mutant plaques were screened using the oligomers to obtain clones containing the

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Abbreviation: WGEF, wheat germ extract factor.

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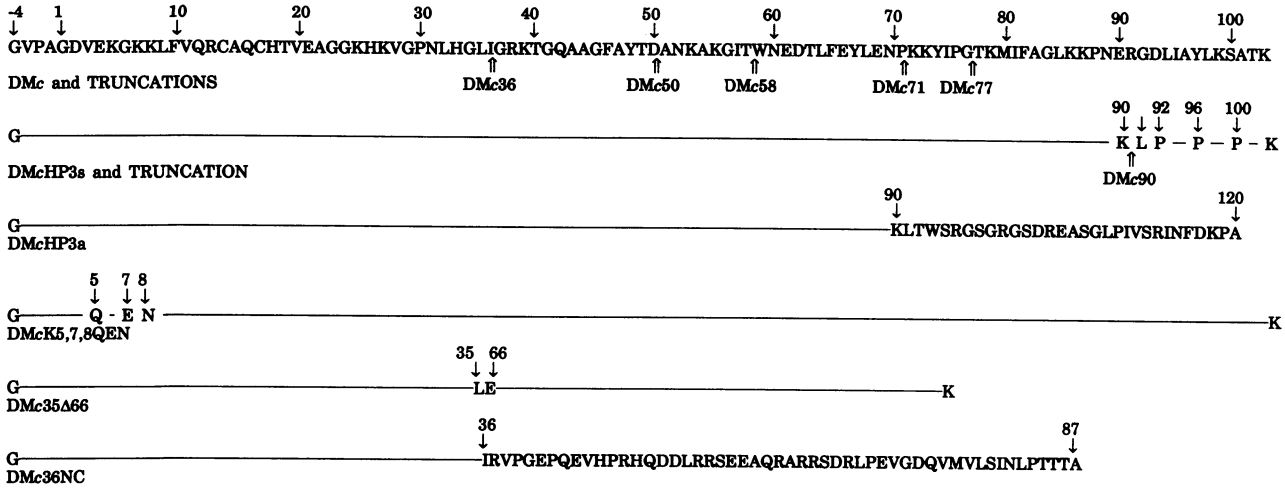

FIG. 1. Amino acid sequences of *D. melanogaster* apocytochrome *c* and its constructs. Truncated apocytochromes *c* are indicated (⇑) at the site at which the plasmid was linearized. *Mbo* I was used for DM*c*77; *Fok* I was used for DM*c*71; *Scr*FI was used for DM*c*58; *Aha* II was used for DM*c*50; and *Sau*3A was used for DM*c*36. *Hin*dIII was used to linearize pDM*c*HP3s to produce DM*c*90. DM*c*HP3s contains Lys-Leu at residues 90 and 91 and prolines at residues 92, 96, and 100. DM*c*HP3a carries a noncytochrome *c* sequence at residues 90–120. DM*c*35Δ66 contains a deletion of residues 36–65. DM*c*36NC has a noncytochrome *c* sequence after residue 36. DM*c*K5,7,8QEN contains the indicated replacements at lysine-5, -7, and -8.

oligonucleotides in both orientations. The sense direction coded for a full-length apocytochrome *c*, containing Lys-Leu at residues 90 and 91 and proline at residues 92, 96, and 100 (DM*c*HP3s); the antisense direction coded for an apocytochrome *c* with a noncytochrome *c* amino acid sequence from residue 90 to residue 120 (DM*c*HP3a; Fig. 1).

To obtain the deletion mutant, in which residues 36–65 were missing, DM*c*35Δ66, and the truncated construct with a noncytochrome *c* extension, DM*c*36NC, samples of pDM*c*1.2 were digested with either *Sau*3A or *Taq* I. A sample from each digestion was blunt-ended; another was filled to produce a 1-base overhang. Each of the two sets of *Sau*3A fragments was cut with *Eco*RI and the required *Eco*RI–*Sau*3A fragments were isolated on an agarose gel. Likewise, the two sets of *Taq* I fragments were digested with *Hin*dIII and the *Taq* I–*Hin*dIII fragments were isolated. The *Eco*RI–*Sau*3A fragment with the 1-base overhang was ligated into M13mp18 (*Eco*RI/*Hin*dIII cut) in the presence of the 1-base overhang *Taq* I–*Hin*dIII fragment. Transformation and sequence analysis of selected colonies identified the deletion mutant DM*c*35Δ66. This procedure yielded a deletion of residues 36–65. Similarly, the *Eco*RI–*Sau*3A blunt-ended fragment was ligated into M13mp18 (*Eco*RI/*Hin*dIII cut) in the presence of *Taq* I–*Hin*dIII blunt-ended fragment, transformed, and sequenced, and a construct was identified that contained a 51-residue out-of-frame noncytochrome *c* segment from residue 36, DM*c*36NC. The apocytochrome *c* constructs were subcloned into pGEM-4Z for transcription and translation.

The N-terminal mutants, in which lysine-5, -7, and -8 were replaced by glutamine, glutamic acid, and asparagine, respectively (DM*c*K5,7,8QEN), and in which cysteine-14 or -17 was replaced by serine, were obtained by site-directed mutagenesis (27). The same N-terminal mutations of lysine-5, -7, and -8 were produced in pDM*c*35Δ66.

After mutagenesis and transformation into *Escherichia coli* JM101, all M13 recombinant DNA constructs were identified by restriction mapping and sequencing.

Truncated apocytochrome *c* molecules were obtained by digesting pDM*c*1.2 with various restriction enzymes chosen to cut at selected unique sites. The DM*c*90 apoprotein was produced by linearizing pDM*c*HP3s with *Hin*dIII.

Association of Apocytochrome *c* and Mitochondria. Linearized apocytochrome *c* plasmids were transcribed and translated, and the radiolabeled apoprotein was routinely purified to the monomeric fully reduced state by reverse-phase HPLC (8). Since, in many of the constructs employed, the single methionine at position 80 had been eliminated, it became necessary to radiolabel positions 14 and 17 with [^{35}S]cysteine. The mutants that had one or the other cysteine replaced by serine were radiolabeled with [^{35}S]methionine. The [^{35}S]-cysteine and [^{35}S]methionine (both ≥40 TBq/mmol) were obtained from Amersham. PAGE of apocytochrome *c* and its constructs was performed by the method of Swank and Munkres (28).

Mouse liver mitochondria were isolated and the association of apocytochrome *c* constructs with the mitochondria were monitored, as described (8). In the current experiments, 300 μg of mitochondrial protein was employed in 200 μl of mitochondrial medium (10 mM Hepes, pH 7.4/220 mM mannitol/20 mM sucrose/10 mM sodium succinate/2.5 mM K_2HPO_4) with 2.5% (vol/vol) WGEF and 10–1000 pM apoprotein.

N-Ethylmaleimide-modified apocytochrome *c* was prepared by incubation with a 30-fold molar excess of the reagent (Pierce) (mitochondrial medium, 4°C, 60 min), followed by the addition of 2-mercaptoethanol [2% (vol/vol)] and guanidine hydrochloride (50 mM) at 4°C for 10 min and subsequent purification of the protein by reverse-phase HPLC (8).

RESULTS

Preparation of Apocytochrome *c* Constructs. Truncated *D. melanogaster* apocytochromes *c* (Fig. 1) were produced from plasmid linearized at restriction sites that would produce fragments containing the SP6 promoter and the desired lengths of the apoprotein from the N terminus. The exact lengths of the truncated apoproteins made from the run-off transcript of the truncated genes were not determined, but, based on $NaDodSO_4$/urea/PAGE of the purified apoproteins, these lengths were proportional to the length of the gene fragment, indicating that the polypeptides were identical or close to their predicted sizes (data not shown). Reverse-phase HPLC elution times of the truncated constructs varied by only a few minutes from that of the full-length apocytochrome *c*. These, as well as the other apoprotein constructs, were as stable as the full-length material when prepared and stored as described (8).

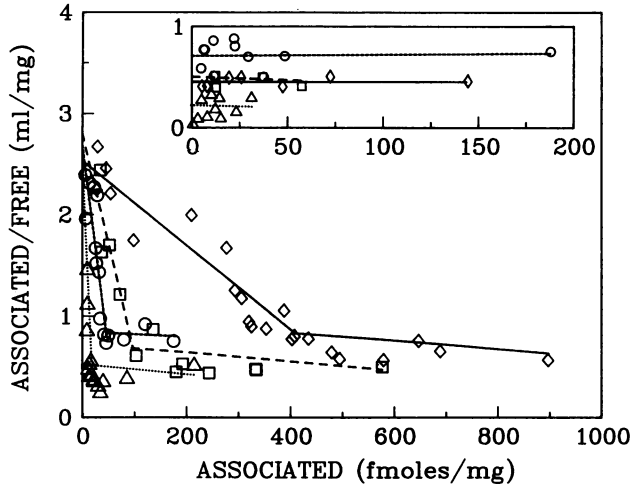

FIG. 2. Association of *D. melanogaster* apocytochrome *c* with mouse liver mitochondria, measured according to Hakvoort *et al.* (8), in the presence or absence (*Inset*) of WGEF. Three preparations (△···△, ○---○, □—□) were labeled with different batches of [^{35}S]-cysteine, and one preparation (◇—◇) was labeled with [^{35}S]methionine.

Association of Apocytochrome *c* Constructs with Mitochondria. When the association curves for the [^{35}S]methionine-labeled native apocytochrome *c* were compared to those for the [^{35}S]cysteine-labeled material (Fig. 2), it was found that the results with the former yielded apparent K_d values of 0.7–3.0 × 10^{-10} M, and 200–500 fmol of binding sites per mg of mitochondrial protein (8). By contrast, the results with the cysteine-labeled apoproteins were more variable, apparent K_d values ranging from 0.8 to 4.7 × 10^{-11} M, and binding sites from 20 to 130 fmol/mg of mitochondrial protein. However, with any single batch of radiolabeled amino acid, the association parameters tended to be more reproducible. These effects may be due to chemical changes in the ^{35}S-labeled amino acid, particularly with the cysteine (29, 30), which result in the loss of the sulfur, or to the presence of unlabeled amino acids in the translation system, again predominantly with the cysteine (30). In either case, the apoprotein preparation would contain a variable proportion of unlabeled protein competing for mitochondrial association with the labeled protein, so that measuring the ^{35}S label would yield the lower apparent K_d values and the smaller number of binding sites, characteristic of the cysteine-labeled apoproteins, as compared to the methionine-labeled material (Fig. 2). Because of these phenomena, the association curves with the various constructs were determined always with those for the native apoprotein prepared with the same batch of ^{35}S-labeled amino acid or with batches yielding the same curves and always in the presence, as well as the absence, of WGEF, which permitted an accurate assessment of whether the high-affinity or the low-affinity phase of the binding curve was principally affected.

To resolve the role of the C-terminal segment (12, 16, 31), mutations were introduced into that region by site-directed mutagenesis. Surprisingly, the disruption of the C-terminal α-helix by the introduction of three prolines (residues 92, 96, and 100), in addition to the changes at residues 90 and 91 (Glu-Arg to Lys-Leu), had no effect on the association of the mutant apoprotein (DM*c*HP3s) in the presence or absence of WGEF (Fig. 3*A*). The apoprotein with the noncytochrome *c*

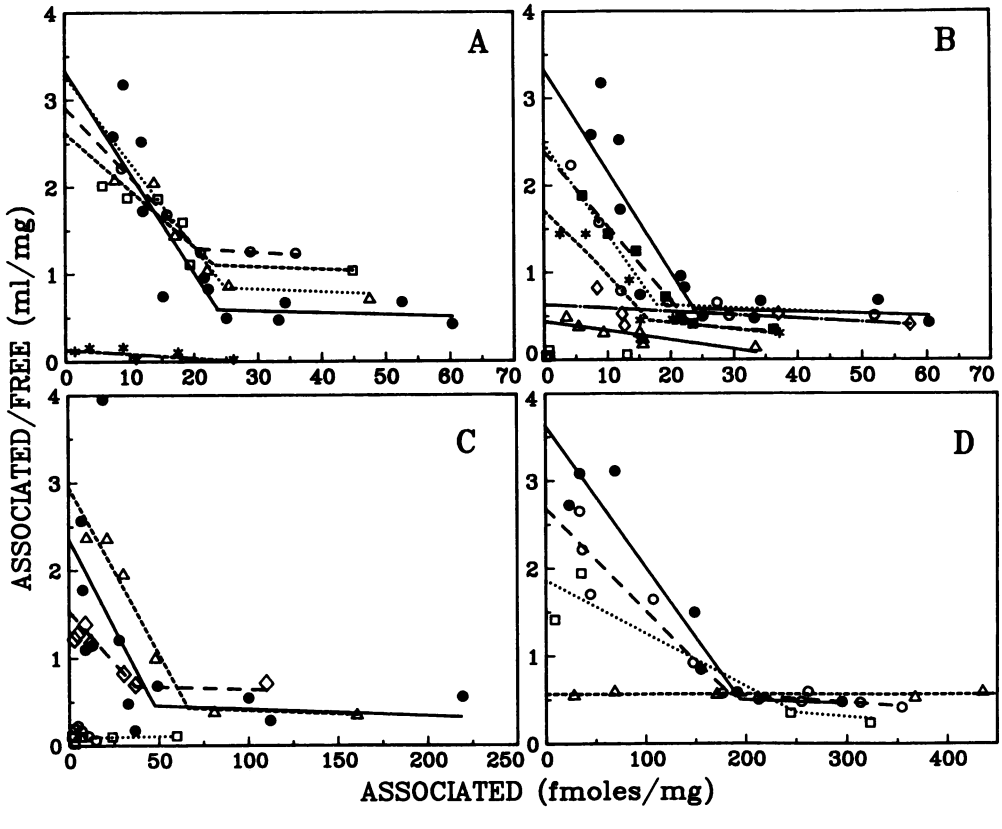

FIG. 3. Association of apocytochrome *c* constructs with mouse liver mitochondria in the presence of WGEF. The binding of the native apoprotein in the presence of WGEF is shown (●—●) and in its absence in *B* (◇–·–·–◇). The lines represent the best fit by linear regression. (*A*) [^{35}S]Cysteine-labeled apoproteins. □---□, DM*c*90; △···△, DM*c*HP3a; ○—○, DM*c*HP3s; *– – –*, *N*-ethylmaleimide-adduct of native apocytochrome *c*. (*B*) Truncated apocytochromes *c* labeled with [^{35}S]cysteine. ○···○, DM*c*77; *- - -*, DM*c*71; ■—■, DM*c*58; △—△, DM*c*50; □ □, DM*c*36. (*C*) [^{35}S]Cysteine-labeled apoproteins. △- - -△, DM*c*35Δ66; ○ ○, DM*c*36NC; □···□, DM*c*K5,7,8,QEN35Δ66; ◇—◇, rat apocytochrome *c*. (*D*) [^{35}S]Methionine-labeled apoproteins. △- - -△, DM*c*K5,7,8QEN; ○—○, DM*c*C14S; □···□, DM*c*C17S.

terminal segment, residues 90–120 (DM*c*HP3a), was also found to exhibit normal association. Thus, the C-terminal region appears to have little or no influence on this phenomenon.

To explore this possibility further, truncated apoproteins of various lengths were prepared. In the presence of WGEF, apoproteins nominally truncated at residues 90, 77, 71, or 58 exhibited binding affinities indistinguishable from those of native apocytochrome *c* (Fig. 3*B*). However, the apoprotein truncated at residue 50 only showed low-affinity association, whereas that truncated at residue 36 displayed neither high- nor low-affinity association. These results suggested that either (*i*) the region between residues 50 and 58 is crucial for the association reaction and constitutes a so-called targeting sequence or (*ii*) a minimum length of polypeptide is required.

To determine if a defined targeting sequence is present, a deletion mutant was prepared in which the region between residues 35 and 66 was removed. This mutant, DM*c*35Δ66, is 76 residues long and consists of the native C-terminal sequence from residue 66 to residue 103 joined to the native N-terminal sequence at residue 35. This deletion mutant was found to exhibit normal high- and low-affinity association with mitochondria (Fig. 3*C*). However, when a noncytochrome segment of 51 residues was joined to the same N-terminal polypeptide at residue 36, no association could be observed.

By contrast, the N-terminal region of the apoprotein and the area of the two heme binding cysteines appear to have a larger influence on the association phenomenon. A mutant (DM*c*K5,7,8QEN) in which lysine-5, -7, and -8 are changed to glutamine, glutamic acid, and asparagine fails to exhibit the high-affinity association (Fig. 3*D*), while the *N*-ethylmaleimide adduct of cysteine-14 and -17 shows no high-affinity and decreased low-affinity association (Fig. 3*A*). However, changing one or the other cysteine to serine does not affect the association in the presence or absence of WGEF and, as was shown (8), changing both cysteines to either serines or alanines had no effect. Furthermore, the influence of the loss of the three N-terminal segment lysines could also be observed with the apoprotein lacking the segment of residues 36–65 (DM*c*K5,7,8QEN35Δ66) (Fig. 3*C*).

To examine the species specificity of the association, rat apocytochrome *c* was prepared. This protein has the same amino acid sequence as the mouse protein (32). It was found to bind to mouse mitochondria like the *Drosophila* apoprotein (Fig. 3*C*), which differs from it by 20 residues, indicating evolutionary conservation of the relevant properties of apocytochrome *c*.

DISCUSSION

The present studies on the uptake of apocytochrome *c* by mitochondria were undertaken to develop a system in which it would be possible to examine the details of the relations between the structure of the protein and its ability to penetrate into the organelle to where the heme lyase could turn it into mature cytochrome *c*. The understanding of these structure–function relations was considered essential to the interpretation of tests of function of mutant holocytochromes *c*, particularly in view of the relatively common occurrence of mutations at evolutionarily invariant sites that exhibit little or no obvious functional or structural disturbances (33–36). After the purification of biosynthesized *D. melanogaster* apocytochrome *c*, it became possible to examine directly its uptake by mitochondria *in vitro*. This overcame the complications arising from the presence of the complex tissue extract used for translation of mRNA and the difficulty inherent to *in vivo* biosynthesis experiments of distinguishing between translocation of the apoprotein into the organelle, reaction of the heme lyase, and functional insufficiency of the holoprotein (37). The *in vitro* system developed has been described (8). It led to the discovery of a factor in the wheat germ extract that increases substantially the uptake of apoprotein by mitochondria and made it possible to show that the apoprotein is in equilibrium with the mitochondria, even though it is internalized.

The present experiments were designed to examine how much of the apocytochrome *c* is needed to allow import into mitochondria. The following results were obtained. (*i*) The C-terminal region from about residue 58 has little or no influence on the association with mitochondria when the native N-terminal half of the apoprotein is present. (*ii*) The N-terminal segment to residue 36 by itself does not sustain the association phenomenon. This is not due to a requirement for size, since an additional noncytochrome *c* sequence is ineffective; however, the association can be restored by an additional sequence from the apoprotein, provided either by the adjacent region, up to residue 58, or by the segment beyond residue 66. (*iii*) The N-terminal sequence is much more critical to the association, since changing lysine-5, -7, and -8 to acidic or neutral residues had a large effect. (*iv*) Replacing one or the other of the heme binding cysteines by serine or both by serines or alanines has no influence on the association, whereas the *N*-ethylmaleimide adduct of the cysteines exhibits little association. (*v*) The system operates well with components having a large degree of species heterogeneity.

The most remarkable of these results is when the apoprotein is truncated from the C-terminal end to the point that it can no longer associate with mitochondria, that function is not returned by a noncytochrome *c* sequence but can be restored not only by the adjacent segment, which had been removed, but also by segments further toward the C terminus. This is in agreement with an earlier result that indicated that the fragment of residues 81–104 of mouse cytochrome *c* inhibited the uptake of the apocytochrome into isolated mitochondria (31). Whether the repetitive characteristics of the sequence segments needed for uptake represent some signal or whether the amino acid sequence of cytochrome *c* has unknown properties essential for other possible aspects of mitochondrial uptake remains to be determined.

With the rest of the sequence present, the C-terminal segment appears to be of no consequence for the association with mitochondria. This was shown with mutants having truncated C-terminal segments, with a construct in which residues 90 and 91 had been changed and three prolines had been introduced into the region corresponding to the C-terminal α-helix of the holoprotein, and with a mutant in which the C-terminal segment from residue 90 was replaced by an extended noncytochrome *c* sequence to residue 120. However, if the association phenomenon studied here is correlated to the formation of the holoprotein, as previously argued (8) and discussed below, the current results would appear to contradict a report that an aberrant apocytochrome *c*, produced by a processing mutant of *N. crassa*, which had the 8 C-terminal residues replaced by a 27-residue noncytochrome *c* sequence, was unable either to bind to mitochondria or to form holocytochrome *c* (12). The origin of the apparent discrepancy is not known. Possibly, the cysteine at residue 106 in the mutant makes more stable disulfide polymers than do the cysteines that bind the heme in the holoprotein.

By contrast, the N-terminal segment of the apoprotein behaves rather like a prepeptide, though it remains in full in the holoprotein. Thus, replacing the three positively charged lysines in the N-terminal sequence of the protein with neutral or positively charged amino acids appears to influence dramatically the uptake into mitochondria, eliminating totally the high-affinity phase of the association. This phase occurs only in the presence of WGEF and probably represents the

physiological phenomenon. On this basis, it is tempting to consider that for most mitochondrial proteins it is only a covalent change that renders their uptake irreversible, such as the addition of the heme to apocytochrome *c* or the proteolytic cleavage of the prepeptide in many other proteins (10, 11). Before this happens, the precursor protein may remain in equilibrium with the external medium.

The observed importance of the N-terminal region of apocytochrome *c* with respect to its association with mitochondria, as compared to the relative insignificance of the C-terminal region, correlates well with the observations of De Kruijff and collaborators (16–18) on the insertion of apocytochrome *c* into artificial lipid vesicles, even though the preliminary evidence (8) seems to indicate that for the system employed here the portal for transmembrane passage of the apocytochrome *c* is likely to be a pore.

The region of the two cysteines, residues 14 and 17, which bind the heme in holocytochrome *c*, also influences the uptake of the apoprotein. As earlier shown (8) and also demonstrated above, changing the cysteines to small residues, such as serines or alanines, seems to have no influence on association, whereas, as found by Dumont *et al.* (24), changing them to a bulky amino acid such as tyrosines, phenylalanines, or tryptophans, appears to interfere with uptake. The *N*-ethylmaleimide adduct also shows very little association. Until some of the molecular detail of the mechanism of apocytochrome *c* uptake is worked out, it is unlikely that these observations can be explained.

Finally, the most striking correlations the present results exhibit are with those obtained by S. Nye and R. Scarpulla (personal communication), using a variety of truncated constructs of apocytochrome *c* fused at their C terminus to other proteins, including some whose conformations could be manipulated to allow or not allow passage through a membrane. These authors showed that constructs, which the above *in vitro* experiments show are or are not taken up, behaved in a precisely similar manner *in vivo* in yeast cells as measured by uptake into mitochondria and the formation of the holoprotein. These correlations make it likely that the mitochondrial association phenomenon studied here is indeed in the physiological pathway for the formation of holocytochrome *c* (8).

We thank Dr. R. Scarpulla (Northwestern University) for kindly providing the RC9 gene; Dr. K.-L. Ngai and the Northwestern Biotechnology Center for preparing the oligonucleotides; K. Hospodar, D. Gombos, and M. Tan for their expert assistance; and C. Orendain for typing the manuscript. This work was supported by National Institutes of Health Grant GM19121. J.R.S. was supported by Individual National Research Service Award 32 GM10755.

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