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Biochemistry

Reversible import of apocytochrome *c* into mitochondria

(*Drosophila melanogaster* cytochrome *c*/high- and low-affinity uptake/protein export/mutations at heme-binding cysteines)

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ABSTRACT **^{35}S-labeled *Drosophila melanogaster* apocytochrome *c* was made by *in vitro* transcription/translation of the gene and purified to the monomeric, fully reduced form. It was found that in the presence of a wheat germ extract factor there was a high-affinity phase of the uptake into mouse liver mitochondria at 10–300 pM apocytochrome *c*, and a lower-affinity phase through 4000 pM. Without the factor, the high-affinity phase was absent. The stimulatory effect of the factor could not be elicited with various reductants, such as NADH, FMN, and ferrous protoheme IX. Conversely, when mitochondria loaded with apocytochrome *c* were resuspended in fresh medium, the protein readily reequilibrated. Successive washings depleted >95% of the associated apoprotein but removed no holoprotein. Proteases (proteinase K or trypsin) added to a suspension of mitochondria loaded with apoprotein digested an amount of apoprotein similar to that which would have been dissociated during the same time, as measured by successive washings in the absence of protease. Mitochondria loaded with apoprotein and similarly treated with protease continued exporting the apoprotein, even after the protease was inhibited and removed, suggesting that most of the apoprotein associated with the organelle was in a protease-resistant compartment. Apocytochrome *c* mutants in which serines or alanines replaced cysteines 14 and 17, which bind the prosthetic group, behaved like the cysteine-containing protein, indicating that the covalent attachment of the heme is unrelated to the translocation of the apoprotein.**

To study, in as well defined a system as possible, the mechanisms by which cytoplasmically synthesized apocytochrome *c* is taken up into mitochondria, *Drosophila melanogaster* apocytochrome *c* was biosynthetically prepared by transcription/translation of the gene *in vitro* and purified. Earlier studies have used apoprotein obtained by chemical treatment of the holoprotein (1–3) or the translation products of total cellular messenger RNA and, more recently, translation products of isolated cytochrome *c* genes, without purifying the apoprotein (3–6). An advantage of procedures yielding *in vitro* translation products is that the gene is readily available for molecular manipulation, which would be impractical if the apoprotein were to be prepared by heme removal from the holoprotein. However, unless the apoprotein is purified from the translation mixture, it is not necessarily obtained in its native monomeric, fully reduced form and it is impossible to observe any influence of the numerous materials of the cell extract used in the translation or of the numerous proteins translated when total poly(A)$^+$ RNA is employed.

The evidence that the import of apocytochrome *c* into mitochondria requires the covalent attachment of the heme prosthetic group by the cytochrome *c* heme lyase system is substantial (5–15). Thus, Nicholson *et al.* (11, 12, 14) conclude that in the absence of added reducing agents, apocytochrome *c* remains on the surface of the outer membrane. In their presence, heme is reduced, and the lyase can then add it to the peptide chain, resulting in the formation and internalization of the holoprotein. Furthermore, the mitochondria of mutants of *Neurospora crassa* (13) or of *Saccharomyces cerevisiae* (5, 15) that lack heme lyase activity do not appear to import apocytochrome *c*. Similarly, if the two cysteines that normally bind the prosthetic group (residues 14 and 17) are modified by reaction with *N*-ethylmaleimide or if one of them is replaced by tryptophan, phenylalanine, or tyrosine, the apocytochrome *c* does not penetrate the organelle (5).

With purified, biosynthetically made apocytochrome *c* and mouse liver mitochondria, the association of the protein occurred in two distinct phases. The high-affinity phase, probably representative of the physiological situation, resulted from the presence of a factor in the wheat germ extract employed for translation (WGEF). The low-affinity uptake phase was unaffected by the factor and over a wide range of apoprotein concentration did not appear to saturate. Moreover, the apoprotein associated with mitochondria was in rapid equilibrium with apoprotein free in solution. As much as 95% of the associated apoprotein could be unloaded into the medium even though most of it appeared to have been internalized by the organelle. The external medium after loading, as well as after unloading, contained apocytochrome *c* and no holocytochrome *c*. It was concluded that the uptake of apocytochrome *c* into mitochondria is unrelated to the covalent attachment of the prosthetic group; this was confirmed by showing that apocytochrome *c* in which the two cysteines serving to bind the heme were replaced by serine or alanine behaved similarly to the wild-type protein in uptake and outflow.

A preliminary account of some of this work has been presented (16).

METHODS

***In Vitro* Transcription/Translation.** The *D. melanogaster* cytochrome *c* gene (DM*c*01; ref. 17) *Eco*RI–*Hin*dIII fragment was subcloned into pGEM-2 (Promega) by using these unique restriction sites (18), and the pDM*c*1.2 plasmid obtained was linearized downstream from the DM*c*01 gene with *Pst* I. Site-directed mutagenesis (19) was used to replace cysteines 14 and 17 by alanines or serines. *In vitro* transcription (Promega) employed [α-^{32}P]UTP (Amersham) and 10 μg of the template. The apocytochrome *c* was radiolabeled at its single methionine (residue 80) in the methionine-depleted wheat germ translation system [2 hr, 25°C, [^{35}S]methionine (≥40 TBq/mmol, Amersham)]. It was isolated by reversed-phase HPLC, after treatment with 5% 2-mercaptoethanol (10 min, room temperature) followed by 0.1% trifluoroacetic acid. A Waters C_{18} μBondapak column (3.9 mm × 25 cm) under a linear gradient (2%/min; 1 ml/min) from water to acetonitrile/0.1% trifluoroacetic acid was used. The outflow

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Abbreviation: WGEF, wheat germ extract factor.

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was monitored with a radioactive flow detector (Flo-one, Radiometric Instruments, Tampa, FL) or by liquid scintillation. The fully reduced monomeric apoprotein was eluted at 40% (vol/vol) acetonitrile and was concentrated not more than 2-fold by partial evaporation of the organic solvent *in vacuo* so that the pH remained acidic, minimizing oxidation to the polymer (20). Such samples could be stored at −20°C for several weeks without any change of activity with mitochondria.

Isolation of Mitochondria. Liver mitochondria were isolated from ICR mice (Harlan–Sprague–Dawley) at 4°C (21) in the presence of 1 mM phenylmethylsulfonyl fluoride with an additional centrifugation step (180,000 × *g*, 20 min, 4°C) on a discontinuous sucrose gradient [40%, 50%, 60% (wt/vol) in 10 mM Tris, pH 7.4]. The mitochondria were collected from the 40–50% interface, resuspended in 20 volumes 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), centrifuged at 7600 × *g* for 10 min, resuspended to a final concentration of 10 mg of mitochondrial protein per ml (Bio-Rad protein assay), and immediately used. Mitochondrial integrity was tested by measuring the inner-membrane succinate oxidase and cytochrome *c* oxidase activities upon addition of horse heart cytochrome *c* (12 μM and 50–100 μg of mitochondrial protein). The outer membrane is impermeable to holocytochrome *c* so that any stimulation of these sensitive activities indicates rupture of the outer membrane, allowing cytochrome *c* to permeate to the outer surface of the inner membrane (22). Oxygen uptake was determined polarographically with 10 mM succinate or with 6 mM ascorbate and 0.6 mM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (23).

Apocytochrome *c* Import. Typical import mixtures (100 μl) consisted of mitochondria (150 μg of mitochondrial protein) and various concentrations (10–4000 pM) of apocytochrome *c*. When indicated, the incubation mixture contained 2.5% (vol/vol) WGEF, obtained by centrifugation of the wheat germ lysate (314,000 × *g*, 60 min, 4°C). After 1 hr at 28°C, the mitochondria in each sample were sedimented in a microcentrifuge (5 min, 4°C), and 10-μl aliquots of the initial reaction mixture and the supernatant were taken for measurement of radioactivity by liquid scintillation. The results are presented as Scatchard graphs (24).

Dissociation of Apocytochrome *c*. Mitochondria loaded with ^{35}S-labeled apocytochrome *c* were centrifuged and resuspended in fresh mitochondrial medium at 28°C every 10 min. The apoprotein remaining associated with the organelle was determined by subtracting the amount dissociating into the medium from that initially associated. In the protease-treatment studies, the apoprotein-loaded organelles were digested with either proteinase K or trypsin (5–10% of mitochondrial protein). After 45 min on ice or at 28°C, digestion was stopped by adding a 3-fold excess of trypsin inhibitor and/or phenylmethylsulfonyl fluoride (1 mM), the mitochondria were repeatedly centrifuged and resuspended, and the associated apocytochrome *c* was determined as above.

Ferrous protoheme IX was obtained by reduction of hemin in pyridine with mercury under argon (5 min, room temperature). Restriction enzymes and SP6 polymerase were from Promega; proteinase K, antipain, and leupeptin from Boehringer Mannheim; and other materials from Sigma.

RESULTS

Purification of Apocytochrome *c*. To measure the association of apocytochrome *c* with mitochondria and to determine the influence of added substances, it was necessary to obtain the biosynthesized protein in pure form. Fig. 1 shows the HPLC purification of the *D. melanogaster* apoprotein and

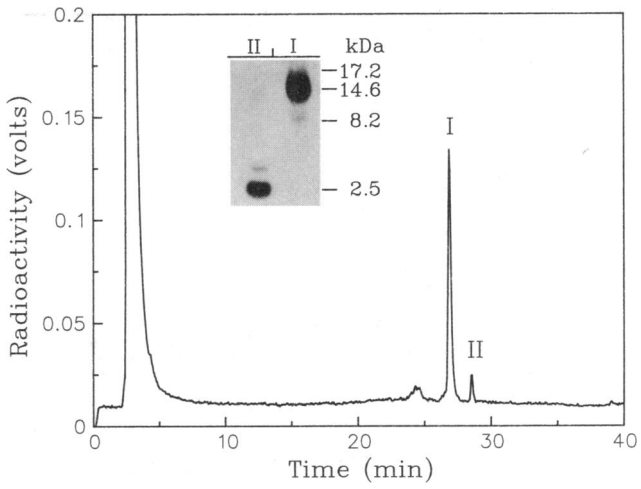

FIG. 1. Reversed-phase HPLC purification of biosynthetically made *D. melanogaster* [^{35}S]methionine-labeled apocytochrome *c*. The area under peak I represents 5% of the total radioactivity. (*Inset*) A fluorogram of the 2,5-diphenyloxazole-impregnated NaDodSO$_4$/urea/polyacrylamide gel (31) with equal amounts of radioactivity loaded for fraction I, the purified fully reduced monomeric apocytochrome *c*, and for fraction II, the out-of-frame, minor translation product that did not bind to mitochondria. Coomassie brilliant blue staining gave identical results.

emphasizes the small proportion of the total radioactivity incorporated into the monomeric, fully reduced apoprotein. In the absence of unlabeled methionine, the radioactivity of the apoprotein was a direct measure of its quantity. The apoprotein had the correct molecular weight and was judged to be pure by PAGE.

It appeared that the translation mixture contained a considerable proportion of disulfide-polymerized apocytochrome *c*, since the yield of monomeric protein was always larger when a reducing agent was added prior to HPLC. When most of the acetonitrile and trifluoroacetic acid were removed and the monomeric apoprotein fraction was concentrated to <75% of its original elution volume, the apoprotein polymerized and the polymeric material was eluted from the C_{18} reversed-phase column at the same position (5 min) as unincorporated label in the translation mixture (Fig. 1). 2-Mercaptoethanol reduced the polymeric material to the monomer (25).

Association of Apocytochrome *c* with Mitochondria. The *D. melanogaster* apoprotein associated with freshly prepared mouse liver mitochondria in amounts directly proportional to its initial concentration, from 10 pM up to 4000 pM. Treating this association as an equilibrium binding gave an apparent K_d of $\approx 10^{-8}$ M (Fig. 2). However, in the presence of WGEF, dramatically higher associations were observed at low concentrations of apocytochrome *c*. This high-affinity phase occurred between 10 pM and its saturation at 200–500 pM, with an apparent K_d of $\approx 10^{-10}$ M. The optimal proportion of wheat germ extract yielding the typical biphasic binding curve was 2–3% of the total volume. At concentrations of apoprotein beyond those saturating the high-affinity association, adding more WGEF had no effect. In its absence no high-affinity phase was detected, and the binding was indistinguishable from that at low affinity in its presence. Disulfide-polymerized apocytochrome *c* showed only low-affinity association with the mitochondria, even in the presence of WGEF. In the usual incubations, only monomeric apocytochrome *c*, little or no polymerized apocytochrome *c*, no holocytochrome *c*, and no cytochrome *c* fragments occurred in the supernatant following centrifugation of the mitochondria, as determined by HPLC (25).

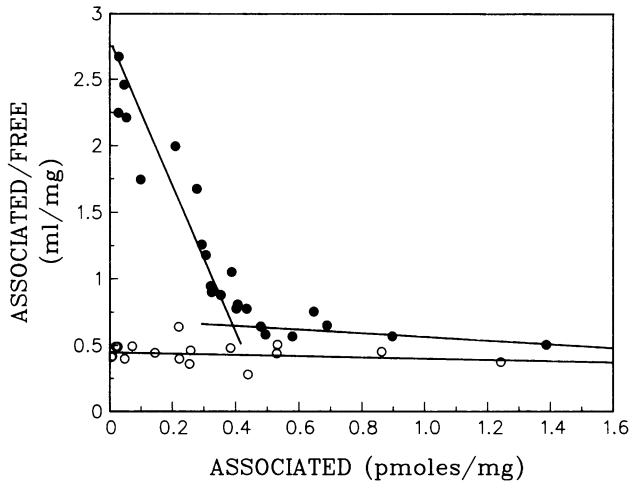

FIG. 2. High- and low-affinity association of [^{35}S]methionine-labeled apocytochrome *c* with mouse liver mitochondria. Purified fully reduced monomeric [^{35}S]methionine-containing apocytochrome *c* was incubated with mitochondria (10–4000 pM apoprotein per 150 μg of mitochondrial protein) in the presence (●) or absence (○) of WGEF.

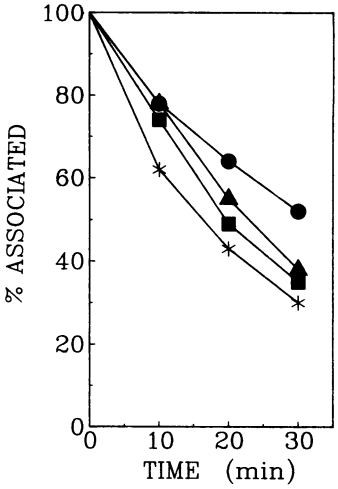

FIG. 3. Dissociation of apocytochrome *c* from mitochondria. Multiple aliquots of mitochondria (950 μg of protein) loaded at 80 pM apoprotein, in the presence or absence of WGEF, were resuspended repetitively every 10 min in fresh medium (1000 μl) in the presence or absence of WGEF. The amount of radioactivity remaining associated with the organelle was determined as given under *Methods*. The curves represent experiments in which WGEF was present during loading and unloading (●), loading only (▲), unloading only (■), or neither (*).

The association reaction was rapid and reached equilibrium in <5 min, as binding curves measured after a 5-min incubation were the same as after an incubation of 60 min. After such prolonged incubations, the outer mitochondrial membrane was intact as judged by the lack of effect of added holocytochrome *c* on the activities of succinate oxidase and cytochrome *c* oxidase. No proteolytic effects were observed in the incubation of the mouse liver mitochondria with apocytochrome *c* and wheat germ extract, since addition of phenylmethylsulfonyl fluoride (1 mM), leupeptin (1 μg/ml), and antipain (1 μg/ml) to the incubation medium had no influence on the association curves. Similarly, the small amount (<2%) of acetonitrile added with the apoprotein had no effect on the associations.

To determine whether the active substances in the wheat germ extract were among the materials reported to affect the mitochondrial formation of holoprotein (11, 12, 14, 26), association curves were obtained, with and without WGEF, in the presence of millimolar concentrations of NADH, NADPH, FMN, NADH and FMN, ferrous heme, deuterohemin, dithionite, or mercaptoethanol. Also tested were glutathione, dithiothreitol, ATP, ADP and phosphate, GTP, oxaloacetate, citrate, spermidine, and azolectin (ref. 25 and data not shown). None could replace the extract, and many of the reducing agents inhibited binding in the presence of WGEF. Deuterohemin inhibited association at concentrations 20-fold higher than those reported to prevent the formation of holocytochrome *c* (9).

Dissociation of Apocytochrome *c* from Mitochondria. The binding of apocytochrome *c* to mitochondria is an equilibrium reaction, since apoprotein taken up is rapidly released into medium lacking apocytochrome *c*. Repeated centrifugation of the mitochondria and resuspension into fresh medium removed up to 95% of the associated apoprotein (see Figs. 3 and 5). This equilibration appeared to be as rapid as the association, since 10- and 60-min dissociations were equally effective. The influence of WGEF on dissociation was much smaller than its large stimulatory effect on association. The decrease in apoprotein dissociation was largest when the extract was present during both association and dissociation, but smaller effects were observed when it was present during either one (Fig. 3). In all cases, the material dissociating was shown by HPLC to consist entirely of monomeric apocytochrome *c*. There was no holocytochrome *c* and no protein fragments.

It should be noted that the concentration of wheat germ extract employed (2.5% vol/vol) represents a 300-fold dilution of the original cellular material (Promega). Thus, it is possible that at normal cellular concentrations, the inhibitory effect on apoprotein dissociation is of physiological significance.

Associated Apocytochrome *c* Is Internalized. The accepted approach for determining that a protein precursor is internalized is to demonstrate that added proteases fail to digest it. This type of experiment cannot be employed directly in the present case, since apocytochrome *c* dissociates from mitochondria as readily as it associates, and it was shown that the amount of apoprotein removed by treatment with proteinase K or trypsin was only somewhat larger than dissociated into free solution under the same conditions (Fig. 4). This demonstrates that proteinase K-digested apocytochrome *c* is no longer in the mitochondrial association–dissociation equilibrium, and the presence of an externally added protease merely pulls that equilibrium in the direction of dissociation. The 10-min equilibration periods were slightly less effective than the protease, presumably because equilibration occurred in even shorter times. This conclusion is supported by the observation that agarose-immobilized proteinase K, which would hydrolyze organelle-bound apoprotein inefficiently, nevertheless digested as much apoprotein as the free enzyme (Fig. 4 *Inset*).

To circumvent this problem, mitochondria loaded with apocytochrome *c* were treated with proteinase K, at concentrations that digested maximal amounts of apoprotein (Fig. 4 *Inset*). After removal of the protease, the remaining apoprotein continued to dissociate to the same extent as from mitochondria that had not been treated with protease (Fig. 4). The same results were obtained by digestion at 4°C and 28°C, as well as with trypsin. This indicates that the apoprotein is flowing out from a protease-resistant compartment in the organelle, presumably through a pore, since permeation through the membrane would be blocked at the lower temperature (27). In these experiments, the outer mitochondrial membrane was not disrupted as indicated by the lack of

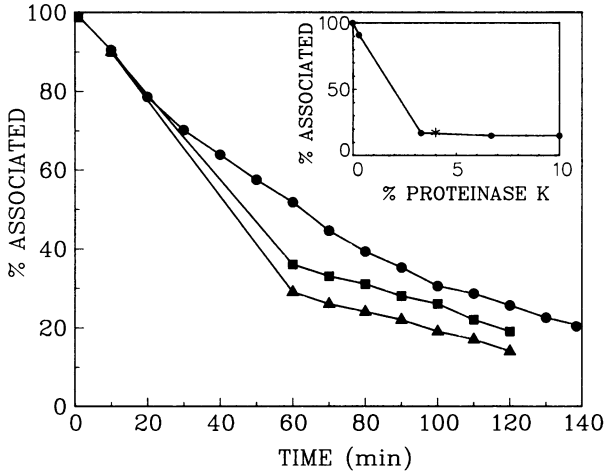

FIG. 4. Dissociation of apocytochrome *c* following protease treatment of mitochondria. Mitochondria (1200 μg of protein), loaded at 40 pM apocytochrome *c* and in the presence of WGEF, were resuspended in 1500 μl of fresh mitochondrial medium for 10 min at 28°C. Thereafter equal aliquots (400 μg of protein) of the organelles were resuspended every 10 min at 28°C in fresh medium (●) or in medium containing proteinase K (8% of mitochondrial protein) at 28°C (▲) or at 4°C (■). Proteolysis was stopped after 50 min with 1 mM phenylmethylsulfonyl fluoride and the organelles were subsequently resuspended every 10 min in mitochondrial medium at 28°C. (*Inset*) Influence of proteinase K concentration on removal of apoprotein from mitochondria loaded at 30 pM apocytochrome c, resulting from a 50-min incubation. Also shown is the result obtained with the indicated amount of immobilized proteinase K (*). This amount was calculated from the proteolytic activity (Sigma).

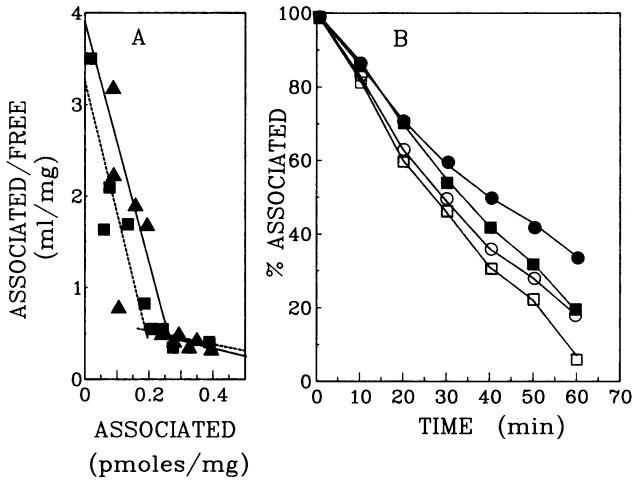

FIG. 5. High-affinity association of mutant apocytochromes *c*, in which both cysteine residues were replaced with serine (DM*c*$C_{14,17}$S) (▲—▲) or alanine (DM*c*$C_{14,17}$A) (■---■), with mouse liver mitochondria in the presence of WGEF. (*B*) Dissociation of mutant apocytochrome *c* from mitochondria. Mitochondria (3 mg of protein), incubated at 75 pM DM*c*$C_{14,17}$A (■, □) or native apoprotein (●, ○) in the presence (filled symbols) or absence (open symbols) of WGEF, were resuspended repeatedly in fresh medium every 10 min without apocytochrome *c* or WGEF, as given for Fig. 3. The amount of apoprotein remaining associated with the mitochondria was determined as given under *Methods*.

response of succinate oxidase and cytochrome oxidase activities to added holocytochrome *c*. Furthermore, the material dissociating after protease treatment consisted solely of full-length apoprotein without any proteolytic fragments, as judged by HPLC.

Association and Dissociation of Cysteine-Substituted Apocytochromes *c*. Since nearly all the apocytochrome *c* taken up by mitochondria can dissociate into free solution, it appears that the covalent attachment of the heme is not needed for import. To check this conclusion, apocytochromes *c* were prepared in which the two heme-binding cysteines were replaced by serines or by alanines. These mutant apoproteins were found to associate with (Fig. 5*A*) and dissociate from (Fig. 5*B*) the mitochondria, like native apocytochrome *c*, including the large stimulation of uptake by WGEF. Here again, the presence of WGEF diminished slightly the amount dissociated, and the native apocytochrome *c* dissociated somewhat less than the mutant apoproteins. All these differences were not larger than 10–15%.

DISCUSSION

The major differences between the conclusions with regard to the uptake of apocytochrome *c* by mitochondria reached from the present experiments and those accepted by previous workers (28, 29) are that (*i*) a factor(s) in the wheat germ extract results in a high-affinity phase of the association of apoprotein and organelle; (*ii*) this association is at least 95% readily reversible, even though the present evidence indicates that the apoprotein is mostly internalized in a protease-resistant compartment; (*iii*) a variety of substances, all of them reducing agents, previously shown to be required for the formation of the holoprotein (11, 12, 14, 26) are of no effect on the uptake of the monomeric, fully reduced apoprotein; and (*iv*) this uptake occurs as well with or without the action of the heme lyase.

The high-affinity association in the presence of the tissue factor(s) has been missed because earlier experiments employed radiolabeled complete translation mixtures, all of which contain considerable amounts of the factor(s). These factors have been detected in both the wheat germ lysates and reticulocyte lysates ordinarily employed for *in vitro* translation (E.M. and D. Miller, unpublished data).

The reversibility of association was unexpected. In those cases in which prepeptides serve to guide protein precursors into mitochondria (28, 29), their proteolytic removal may be responsible for making the uptake irreversible. In the case of cytochrome *c*, such irreversibility may occur only when the heme is covalently attached. The apoprotein itself, at least in the amounts provided *in vitro*, which may be much larger than the amounts the heme lyase can deal with expeditiously, is free to equilibrate in and out. The apparent K_d for the high-affinity binding, 10^{-10} M, is several orders of magnitude smaller than those previously reported (3, 6) and may indicate that WGEF is somehow involved in regulating the intramitochondrial binding of the apoprotein.

The lack of requirement for heme binding in the uptake reaction was further substantiated by showing that apoproteins in which the cysteines were replaced by either serines or alanines were taken up and released just like the wild type. This conclusion is contrary to those reached by many studies, both *in vivo* and *in vitro*, in which heme lyase-deficient cells not only made no holocytochrome *c*, as expected, but also did not accumulate any apocytochrome *c* in their mitochondria (5, 13). However, this is likely to result from the reversibility of apoprotein uptake, since any apoprotein escaping into the cytoplasm would probably be rapidly degraded.

Nevertheless, the question remains whether the present system represents the physiologically effective pathway, leading to holocytochrome *c*. The indications that this system is physiologically significant are as follows. (*i*) It would be remarkable if a process of apocytochrome *c* uptake that is dramatically influenced by a tissue factor, and that operates best at very low concentrations of apoprotein likely to be in the physiological range, were of no bearing to the normal pathway. (*ii*) A small amount of radiolabeled holoprotein can

be recovered from our *in vitro* system (ref. 26 and unpublished data), representing <5% of the total apoprotein supplied. This degree of conversion is similar to those which can be calculated from the results of other experiments (11, 12). Whether this yield can be improved, or whether adult mouse liver mitochondria are unable to make a large amount of cytochrome *c* beyond the normal level, remains to be examined. (*iii*) Finally, the pattern of apocytochrome *c* uptake *in vitro*, following a variety of truncations, deletions, and site-directed mutations, reported elsewhere (30), is the same as that found by S. Nye and R. Scarpulla (personal communication), who employed various fusion protein–apocytochrome *c* constructs *in vivo* in yeast cells. The similarities are striking and show that the way these changes affect the uptake of the modified apocytochromes *c in vitro* parallels the production of holocytochrome *c in vivo*.

In our experiments, the holoprotein must be contained in the ≈5% or less of the total label that does not readily reequilibrate with the external medium. That proportion is somewhat larger in the presence of the wheat germ extract, and it is possible that a physiological role of such a factor is to keep the apoprotein inside the organelle long enough to allow the heme lyase to operate.

A remarkable feature of the present system is its heterogeneity. It consists of mouse liver mitochondria, *D. melanogaster* apocytochrome *c*, and WGEF. To examine whether the most effective apocytochrome *c* would be that made by the species providing the mitochondria, we have prepared rat apocytochrome *c* by transcription/translation *in vitro* (30). The rat and mouse cytochromes *c* have identical amino acid sequences, while that of the fruit fly differs by 20 residues. The behavior of the purified rat apocytochrome *c* with mouse liver mitochondria with respect to association could not be distinguished from that of the *Drosophila* apoprotein (30). Such wide taxonomic crossreactivities are indicative of evolutionary conservation of a system that is probably very old, since cytochrome *c* itself must have appeared at an early stage of eukaryotic development.

If the parallelism of apocytochrome *c* uptake and holoprotein formation is general, as noted above, it is not obvious why the various substances shown by earlier authors (11, 12, 14, 26) to be required for the formation of holocytochrome *c in vitro*, and which are all reducing agents, such as NADH, $FADH_2$, dithionite, and ferrous heme, have no influence on the association phenomena studied here. We employed fully reduced monomeric apocytochrome *c*. It may be significant that, in our hands, *in vitro* translation systems readily yield disulfide polymers of apocytochrome *c*. Such polymers do not undergo high-affinity uptake, even in the presence of WGEF. The reducing agents could result in an increase of the proportion of monomeric reduced apocytochrome *c* and cause the apoprotein to participate in the high-affinity pathway. If that pathway were the one to lead to the formation of holoprotein, the discrepancy would be resolved.

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