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Antibodies against the COOH-terminal Undecapeptide of Subunit II, but Not Those against the NH_2-terminal Decapeptide, Immunoprecipitate the Whole Human Cytochrome *c* Oxidase Complex*

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Antibodies against synthetic peptides derived from the DNA sequence of human cytochrome *c* oxidase subunit II (COII) have been tested for their capacity to immunoprecipitate the whole enzyme complex. Antibodies against the COOH-terminal undecapeptide of COII (anti-COII-C), when incubated with a Triton X-100 mitochondrial lysate from HeLa cells pulse-labeled with [^{35}S]methionine under conditions selective for mitochondrial protein synthesis and chased for 18 h in unlabeled medium, precipitated the pulse-labeled three largest subunits (mitochondrially synthesized) of cytochrome *c* oxidase in proportions close to equimolarity. Antibodies against the NH_2-terminal decapeptide of COII (anti-COII-N), although equally reactive as the anti-COII-C antibodies with the sodium dodecyl sulfate-solubilized COII, did not precipitate any of the three labeled subunits from the Triton X-100 mitochondrial lysate. In other experiments, all the 13 subunits which have been identified in the mammalian cytochrome *c* oxidase were immunoprecipitated from a Triton X-100 mitochondrial lysate of cells long-term labeled with [^{35}S]methionine by anti-COII-C antibodies, but not by anti-COII-N antibodies. By contrast, in immunoblots of total mitochondrial proteins dissociated with sodium dodecyl sulfate, the anti-COII-C antibodies reacted specifically only with COII. These results strongly suggest that, in the native cytochrome *c* oxidase complex, the epitope recognized by the anti-COII-C antibodies is in the COII subunit and that, therefore, in such complex, the COOH-terminal peptide of COII is exposed to antibodies, whereas the NH_2-terminal peptide is not accessible.

The recently introduced use of antibodies directed against synthetic peptides predicted from the DNA or protein sequence has proven to be powerful for the study of proteins encoded in known genes and for the identification of polypeptides specified by unknown reading frames (see review by Walter and Doolittle, Ref. 1). In particular, this approach has been very useful for the identification of the polypeptides encoded in the unidentified reading frames of human mitochondrial DNA (mtDNA) (2–4). A valuable extension of this approach would be the use of antipeptide antibodies for the isolation of complexes containing the specific polypeptide against which the antibodies were raised. As a preliminary to an investigation of the possible association of the products of mtDNA unidentified reading frames with one of the known or unknown complexes of the inner mitochondrial membrane, the well characterized cytochrome *c* oxidase complex was chosen as a model system for defining the appropriate conditions under which a mitochondrial complex could be immunoprecipitated in intact form by antipeptide antibodies. We report here that the cytochrome *c* oxidase complex can be immunoprecipitated from a HeLa cell Triton X-100 mitochondrial lysate by antibodies directed against the COOH-terminal undecapeptide of subunit II (COII-C), but not by antibodies directed against the NH_2-terminal decapeptide of the same subunit (COII-N).

MATERIALS AND METHODS

Preparation of Anti-peptide Antibodies—The preparation of antibodies against the COOH-terminal undecapeptide (anti-COII-C) or the NH_2-terminal decapeptide (anti-COII-N) of cytochrome *c* oxidase subunit II has been described previously (2). Each type of antiserum used derived from a single rabbit. A total γ-globulin fraction, free of antibodies against the carrier protein used in the immunizations (2), was utilized in these experiments.

In Vivo Labeling of HeLa Cell Mitochondrial Translation Products—Different labeling protocols were used. In some experiments, HeLa cells were labeled in methionine-free medium for 2 h with [^{35}S]methionine (10 μCi/ml, 1400 Ci/mmol; Amersham) in the presence of 100 μg/ml emetine, an irreversible inhibitor of cytoplasmic protein synthesis ("pulse" protocol), as previously described (2). In other experiments, the cells were labeled in the presence of 100 μg/ml cycloheximide, a reversible inhibitor of cytoplasmic protein synthesis, then centrifuged down, washed once in complete medium, resuspended in the same medium at 10^5 cells/ml, and incubated for an additional 2 h ("pulse-chase" protocol). In other experiments, HeLa cells (10^5 cells/ml) were grown in complete medium for 20–22 h in the presence of 40 μg/ml chloramphenicol, an inhibitor of mitochondrial protein synthesis, then washed in methionine-free medium, resuspended in the same medium at 2×10^6/ml, and labeled for 2 h with [^{35}S]methionine in the presence of cycloheximide, and finally chased for 2 h ("preCAP-pulse-2-h chase" protocol) or for 18 h ("preCAP-pulse-18-h chase" protocol) in complete medium. For long-term labeling, HeLa cells at 2×10^5/ml were grown for 20 h in the presence of 2×10^{-5} M methionine and 4 μCi [^{35}S]methionine/ml.

Immunoprecipitation of Cytochrome c Oxidase Complex—Isolation of the mitochondrial fraction was carried out as previously described (5). For the immunoprecipitation experiments, all operations were carried out at 4 °C. Samples of mitochondrial suspension ($1–2 \times 10^5$ cpm, 110–270 μg of protein, in the case of pulse-labeled preparations, 7×10^6 cpm, 500 μg of protein in the case of the long-term labeled preparation) in 0.01 M Tris, pH 6.7 (25 °C), 0.25 M sucrose, were made 0.5% (w/v) in Triton X-100 in a final volume of 25–80 μl (protein concentration of 3.5–6 mg/ml) and incubated for 20 min. After diluting the sample 4–10-fold with 0.005 M Tris-HCl, pH 6.7

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(25 °C), 0.002 M methionine, 0.001 M phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 1 mg/ml ovalbumin (incubation buffer, IB), methanol was added to 2% final concentration, and the mixtures were incubated for an additional 10 min. Each sample was preadsorbed with 125 μl of a 10% (w/v) suspension in IB of formaldehyde-fixed *Staphylococcus aureus*, Cowan strain I (Zysorbin, Zymed Laboratories) for 30 min and then centrifuged in an Eppendorf microfuge for 2 min. To each supernatant 250 μg of the γ-globulin fraction from the antiserum or normal serum were added, and the mixtures were incubated for 1 h. After addition of a 10-fold protein excess of a 0.5% Triton X-100 mitochondrial lysate from unlabeled cells (7–50 mg/ml) and of SDS[1] to a final concentration of 0.1%, the samples were mixed with 125 μl of *S. aureus* suspension, incubated for 30 min, and then centrifuged in the microfuge for 2 min. The pellets were washed by centrifugation one time with IB containing 0.1% SDS, one time with IB, and one time with 0.01 M Tris-HCl, pH 6.7, and were then extracted with 40 or 60 μl of 8 M urea, 4% SDS, 0.01 M Tris-HCl, pH 7.3, 0.1% mercaptoethanol for 20–60 min at 37 °C and 5–10 min at 50 °C. A portion of each extract was run on an SDS-urea-polyacrylamide gel.

Isolation of Beef Heart and Human Placenta Cytochrome c Oxidase—Reference is made to published procedures for the purification of cytochrome *c* oxidase from beef heart (6) and from human placenta (7).

Gel Electrophoresis—The immunoprecipitated samples were run on SDS-15% polyacrylamide (1:50 bisacrylamide), 8 M urea slab gels (8, 9). The gels were prepared for fluorography as described (10). In one experiment, the gel was stained with silver as described (11).

RESULTS

Conditions of Immunoprecipitation of Cytochrome c Oxidase by Anti-COII Antibodies—Like other enzyme complexes of the oxidative phosphorylation system, the cytochrome *c* oxidase complex has a chimeric structure, consisting of subunits in part synthesized in mitochondria and encoded in mtDNA, in part synthesized in the cytoplasm and encoded in the nucleus. In the mammalian enzyme, which contains 13 different polypeptides (12–14), the 3 largest subunits are of mitochondrial origin and the other 10 subunits of nuclear origin (7). Fig. 1 shows the sequence of human COII, as derived from the DNA sequence (15), and the NH_2-terminal and COOH-terminal peptides which were used for the preparation of antibodies. In previous work (2), it was shown that the anti-COII-C and anti-COII-N antibodies were equally effective in precipitating COII, when incubated with an SDS mitochondrial lysate from HeLa cells labeled for 2 h with [^{35}S]methionine in the presence of 100 μg/ml emetine. The reaction was absolutely specific, since no other mitochondrial translation

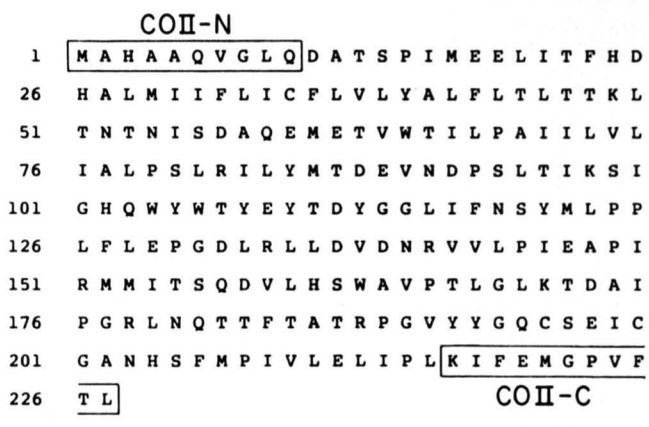

FIG. 1. **Amino acid sequence of human COII, derived from the DNA sequence (15).** The NH_2-terminal and COOH-terminal peptides which were chemically synthesized and coupled to bovine serum albumin for the preparation of antibodies are *boxed*.

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

product reacted with these antibodies, with the exception of an NH_2-terminal fragment of COII, which reacted weakly with the anti-COII-N antibodies. In the present work, immunoprecipitation experiments were carried out using a Triton X-100 mitochondrial lysate from cells labeled and chased according to different protocols and γ-globulins from the chosen peptide-specific antiserum or from normal serum. Control experiments showed that the amount of anti-COII-C or anti-COII-N antibodies employed in the present experiments was in excess over that required to precipitate the great majority of the labeled COII subunits from SDS-solubilized or Triton X-100-solubilized mitochondria.

Immunoprecipitation by Anti-COII Antibodies of Pulse-labeled Mitochondrially Synthesized Subunits of Cytochrome c Oxidase—Because of the low level of labeling attainable in the cytochrome *c* oxidase subunits after long-term labeling with [^{35}S]methionine of exponentially growing HeLa cells (due to the dilution of the label by the unlabeled methionine necessary to support cell growth), the initial experiments of immunoprecipitation of the whole complex by anti-COII-C or anti-COII-N antibodies described in this work were carried out by using material from cells labeled for 2 h with [^{35}S]methionine in the presence of an inhibitor of cytoplasmic protein synthesis, emetine, or cycloheximide, *i.e.* under conditions under which only the mitochondrially synthesized subunits would be labeled. Due to the strict dependence of the synthesis and stability of these subunits and of their integration into a complex on the synthesis and import into mitochondria of the extramitochondrially synthesized subunits (16), the cells were subjected to different treatments before and after the 2-h [^{35}S]methionine pulse, as specified below, in an effort to promote the incorporation into the complex of the subunits labeled during the pulse.

Fig. 2, *lane a*, shows the labeling pattern of HeLa cell

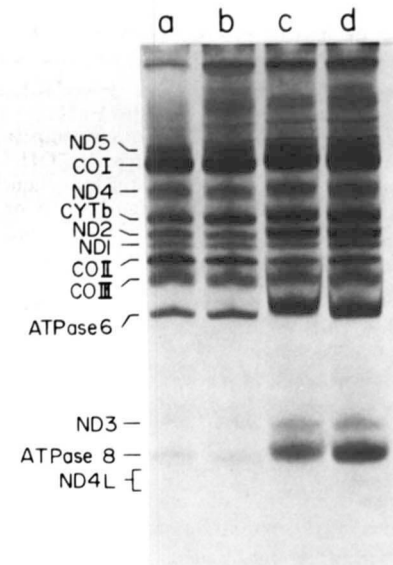

FIG. 2. **Patterns of mitochondrial translation products from HeLa cells labeled under different conditions.** Samples (~20,000 cpm) of SDS mitochondrial lysates from cells labeled according to the pulse protocol (*lane a*), or the pulse-chase protocol (*lane b*), or the preCAP-pulse-2-h chase protocol (*lane c*), or the preCAP-pulse-18-h chase protocol (*lane d*) were run in parallel on an SDS-urea-polyacrylamide gel. *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, and *ND5*, mitochondrially synthesized subunits of the respiratory chain NADH dehydrogenase (17); *COI*, *COII*, *COIII*, subunits of cytochrome *c* oxidase; *CYTb*: cytochrome b; *ATPase 6* and *ATPase 8*, subunits of [H^+] ATPase. See text for details.

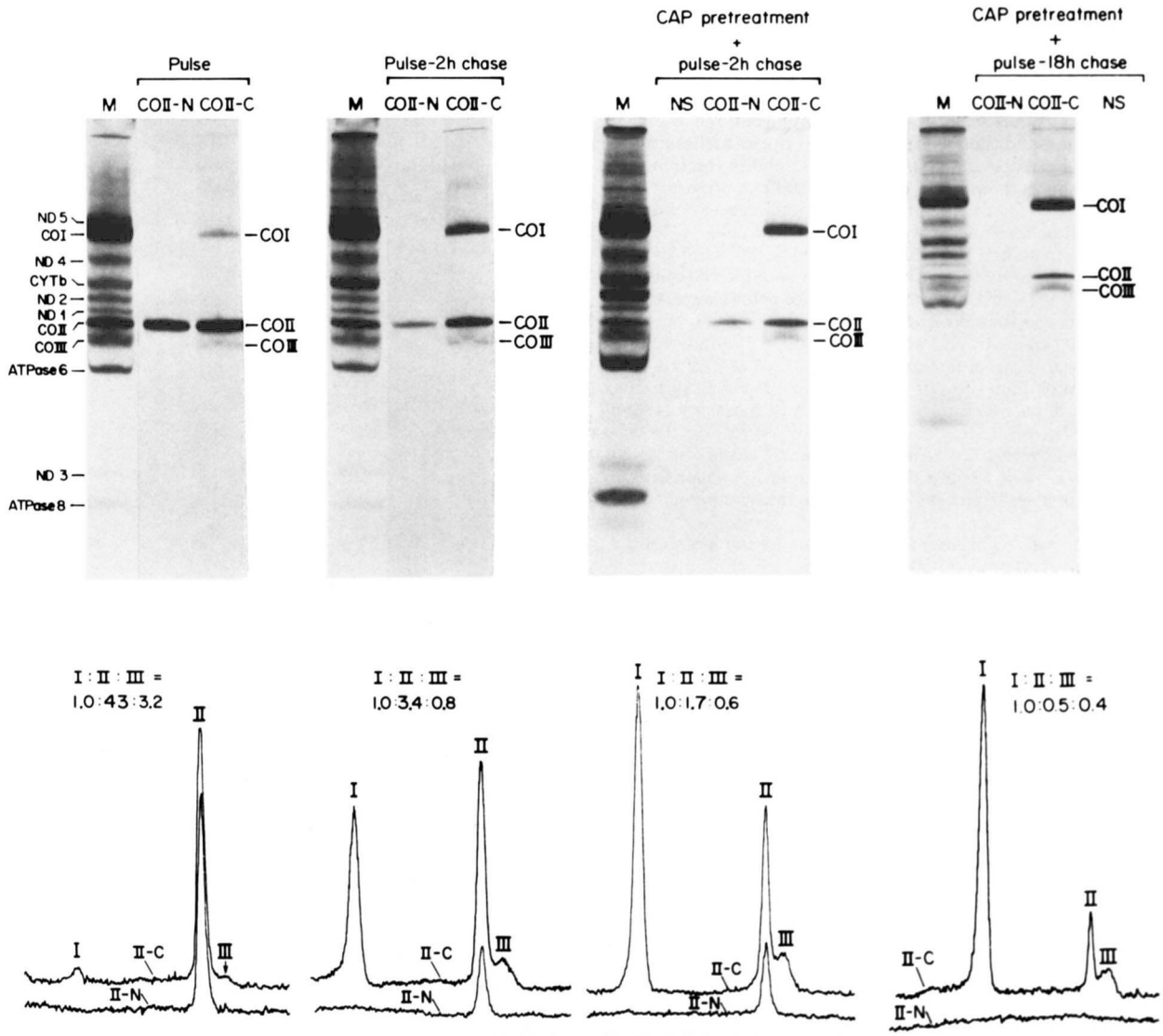

FIG. 3. **Pattern of immunoprecipitation of mitochondrially synthesized cytochrome c oxidase subunits by anti-COII-N and anti-COII-C antibodies under different labeling and chase conditions.** *Top panels,* samples of Triton X-100 mitochondrial lysates from cells labeled according to the pulse protocol (*1st panel*), or the pulse-chase protocol (*2nd panel*), or the preCAP-pulse-2-h chase protocol (*3rd panel*), or the preCAP-pulse-18-h chase protocol (*4th panel*) were immunoprecipitated with anti-COII-N or anti-COII-C antibodies, as described under "Materials and Methods" and run on SDS-urea-polyacrylamide gels. *M,* pattern of mitochondrial translation products present in the lysate used in each experiment; *NS,* normal serum γ-globulin control. *Bottom panels,* densitometric tracings of appropriate exposures of the autoradiograms. In each panel, the molar ratios of labeled COI, COII, and COIII in the complexes precipitated by anti-COII-C antibodies, calculated as explained in the text, are indicated.

mitochondrial translation products isolated from cells labeled for 2 h with [^{35}S]methionine in the presence of 100 μg/ml emetine. The individual products have been assigned to the reading frames of human mtDNA and are designated according to their functional identification (17). This assignment was made in most cases on the basis of experiments using antibodies directed against peptides predicted from the DNA sequence (2–4, 17); COI, COII, and COIII and cytochrome *b* were identified by analysis of the site of synthesis and electrophoretic properties of the subunits of purified human cytochrome *c* oxidase complex (7) and, respectively, purified human cytochrome $b \cdot c_1$ complex.[2]

Fig. 3, *top, 1st panel,* shows the electrophoretic pattern of the immunoprecipitate obtained by incubating a 0.5% Triton X-100 mitochondrial lysate from the 2-h pulse-labeled HeLa cells with anti-COII-C or anti-COII-N γ-globulins. One can see that, while the anti-COII-N antibodies precipitated from among the pulse-labeled components only COII, the anti-COII-C antibodies precipitated all three mitochondrially synthesized subunits (COI, COII, and COIII), with a strong preponderance, however, of COII. No bands of precipitated material were visible in the control carried out with normal serum γ-globulins (not shown). A reasonable interpretation of these results is that, while the anti-COII-N antibodies precipitated from the Triton X-100-solubilized mitochondria only newly synthesized COII subunits which were not associated in complexes with labeled COI and/or COIII subunits, the anti-COII-C antibodies precipitated COII subunits assem-

[2] C. Doersen and G. Attardi, manuscript in preparation.

bled with labeled COI and/or COIII subunits, and possibly with other subunits, as well as any free COII subunits. From densitometric tracings of appropriate exposures of the autoradiogram and from the methionine content of the three subunits, it could be estimated that, in the material precipitated by anti-COII-C antibodies, the newly synthesized COI, COII, and COIII are present in proportions very different from equimolarity, with a large excess of COII and a small excess of COIII (Fig. 3, *bottom, 1st panel*).

When HeLa cells were labeled for 2 h with [^{35}S]methionine in the presence of 100 μg/ml cycloheximide, and then chased for 2 h in unlabeled medium, to allow time for integration of the newly synthesized subunits into the complex, the labeling pattern of the mitochondrial translation products was not significantly different from that obtained from nonchased cells (Fig. 2, *lane b*). However, the relative amount of labeled COII precipitated from this material by anti-COII-N antibodies was much smaller than that observed with material from nonchased cells (Fig. 3, *top, 1st panel*); furthermore, the labeled COI, COII, and COIII specifically precipitated by anti-COII-C antibodies were in proportions much closer to equimolarity (Fig. 3, *bottom, 2nd panel*).

Experiments were carried out to test whether the integration of the mitochondrially synthesized subunits into complexes could be promoted by a preincubation of the cells with chloramphenicol, an inhibitor of mitochondrial protein synthesis, so as to allow accumulation of cytoplasmic subunits in the cytosol and/or mitochondria, as has been reported to occur in *Saccharomyces cerevisiae* (18) and *Neurospora crassa* (19). For this purpose, HeLa cells were grown in the presence of 40 μg/ml chloramphenicol for 22 h before the 2-h [^{35}S] methionine pulse and a 2- or 18-h chase. The above conditions of chloramphenicol treatment have previously been shown to inhibit mitochondrial protein synthesis in HeLa cells by more than 90%, while allowing growth of the cells at a normal rate for 2 days (20). The chloramphenicol pretreatment had a marked effect on the relative labeling of the various polypeptides. Thus, the relative intensities of the bands corresponding to the ATPase 6 (3) and ATPase 8 subunits (2, 21) were much higher in the material from cells pulse-chased after growth in the presence of chloramphenicol (Fig. 2, *lanes c* and *d*) than in the material from nonchloramphenicol-pretreated cells (Fig. 2, *lanes a* and *b*). Similarly, the band corresponding to the ND2 product increased dramatically in the preCAP-pulse-chase protocols relative to the pulse and pulse-chase protocols. This marked increase in the relative labeling of the above-mentioned mitochondrial translation products may reflect an increase in their metabolic stabilities, possibly related to the greater availability of cytoplasmic polypeptides with which the mitochondrially synthesized polypeptides become associated to form various complexes.

When anti-COII-C antibodies were incubated with Triton X-100 mitochondrial lysates from chloramphenicol-pretreated cells chased for 2 h, the relative amounts of labeled COI, COII, and COIII in the precipitated complexes were found to be much closer to equimolarity (Fig. 3, *top, 3rd panel* and *bottom, 3rd panel*), as compared to the precipitates obtained with material from nonchloramphenicol-pretreated cells. Similar results were obtained with material from chloramphenicol-pretreated cells chased for 18 h (Fig. 3, *top, 4th panel* and *bottom, 4th panel*); furthermore, there was a complete disappearance of labeled COII in the immunoprecipitate obtained with anti-COII-N antibodies after the 18-h chase (Fig. 3, *top, 4th panel*).

In Table I, the molar proportions of labeled COI, COII, and COIII in the complexes specifically precipitated by anti-COII-C antibodies from cells labeled and chased according to different protocols are compared to the molar ratios of the same polypeptides among the mitochondrial translation products present in the SDS mitochondrial lysate. It is clear that, in all cases, except in the case of cells pretreated with CAP and chased for 18 h after the pulse, the three labeled subunits are present in different proportions in the complexes specifically precipitated by anti-COII-C antibodies and in the SDS mitochondrial lysate. Thus, the relative intensities of the COI, COII, and COIII bands in the pattern of 2-h-pulse-labeled mitochondrial translation products correspond to approximately equimolar amounts of newly synthesized COI and COII and a somewhat lower molar amount of newly synthesized COIII, probably reflecting the relative rates of synthesis of the three subunits. By contrast, in the pattern of the immunoprecipitated complexes, there is a large excess of labeled COII and a much smaller excess of labeled COIII, indicating a delay in the incorporation of the newly synthesized COI and, to a lower degree, of COIII into the complexes.

TABLE I

Relative molar amounts of pulse-labeled COI, COII, and COIII in the SDS mitochondrial lysate and in the complexes immunoprecipitated by anti-COII-C antibodies

	Conditions of labeling			
	Pulse I:II:III[a]	Pulse-chase I:II:III	preCAP-pulse 2-h chase I:II:III	preCAP-pulse 18-h chase I:II:III
SDS mitochondrial lysate	1:1:0.4	1:0.6:0.4	1:0.4:0.5	1:0.5:0.4
Immunoprecipitated complexes	1:43:3.2	1:3.4:0.8	1:1.7:0.6	1:0.5:0.4

[a] The molar proportions of COI, COII, and COIII were determined from densitometric measurements made on appropriate exposure of the autoradiograms, after correction for the different methionine content of the three subunits.

The decrease in the relative labeling of COII in the pattern of mitochondrial translation products from cells chased for 2 h after a 2-h pulse, relative to the pattern from the 2-h-pulse-labeled nonchased cells, suggests a higher rate of decay of the labeled COII relative to the other two subunits during the 2-h chase. The discrepancy between the relative molar amounts of newly synthesized subunits in the immunoprecipitates and in SDS mitochondrial lysates from 2-h-chased cells presumably reflects the fact that different proportions of the labeled COI, COII, and COIII subunits are incorporated into complexes specifically precipitated by anti-COII-C antibodies. By contrast, the identity of the molar proportions of labeled subunits in the precipitated complex and in the SDS mitochondrial lysate from 18-h-chased cells (Table I), combined with the observation of the absence of labeled COII precipitable by anti-COII-N antibodies in the Triton X-100 mitochondrial lysate, suggests that all three labeled subunits are completely incorporated into complexes after that length of chase. The relatively modest deviations from equimolarity of the labeled subunits in both the whole SDS lysate and the immunoprecipitated complex may reflect small differences in the rate of synthesis and/or stability of the three subunits.

Fig. 4 shows that 5 μg of the NH_2-terminal decapeptide or, respectively, the COOH-terminal undecapeptide of COII competed completely with this subunit, free or incorporated into complexes, in the precipitation by the anti-COII-N or, respectively, anti-COII-C antibodies.

Immunoprecipitation by Anti-COII Antibodies of Long-term-labeled Subunits of Cytochrome c Oxidase—The results reported in the previous section indicated that anti-COII-C antibodies were able to precipitate all three mitochondrially

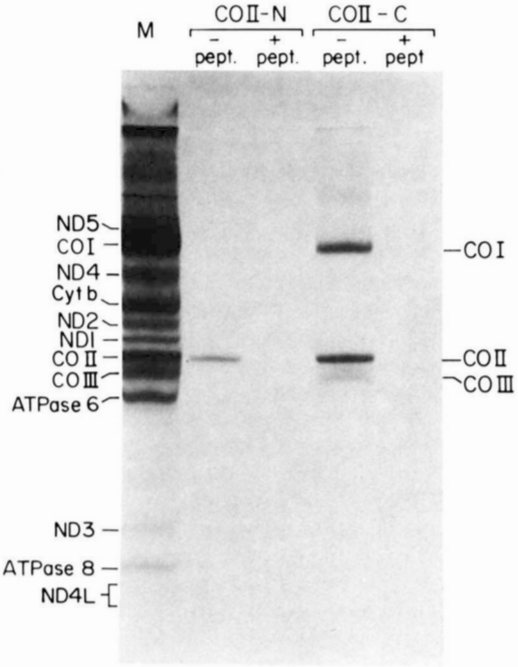

FIG. 4. **Specific peptide inhibition of immunoprecipitation of COI, COII, and COIII by anti-COII-N and anti-COII-C antibodies.** Samples of a Triton X-100 mitochondrial lysate (1×10^5 cpm, 125 μg) from cells labeled under the pulse-2-h chase protocol were immunoprecipitated by anti-COII-N or anti-COII-C γ-globulins (250 μg) in the absence or presence of 5 μg of COII-N or, respectively, COII-C peptide. *M*, pattern of mitochondrial translation products.

synthesized subunits of cytochrome *c* oxidase from a Triton X-100 mitochondrial lysate. After an 18-h chase, the three subunits labeled during the 2-h [^{35}S]methionine pulse appeared to be incorporated in proportions close to equimolarity into complexes precipitable by anti-COII-C antibodies. This suggested that the whole intact cytochrome *c* oxidase complex was precipitated by anti-COII-C antibodies. In order to confirm this interpretation, the same antibodies were tested on a Triton X-100 mitochondrial lysate from exponentially growing cells labeled for 22 h with [^{35}S]methionine in the absence of inhibitors.

As shown in Fig. 5*a*, the precipitate obtained with anti-COII-C antibodies revealed 12 bands, which corresponded well in mobility to the 13 polypeptides which have been identified in mammalian cytochrome *c* oxidase (14). (The VIIa and VIIb subunits could be resolved in some of the electrophoretic runs.) In fact, these bands aligned well with the subunits detected by silver staining in purified beef heart cytochrome *c* oxidase (Fig. 5*b*). (Similar results were obtained with the human placenta cytochrome *c* oxidase, although components VIa, VIb, and VIc and VIIa, VIIb, and VIIc were not well resolved in this sample (not shown).) The somewhat slower migration of human COI and the faster migration of human COII relative to the homologous bovine subunits have been previously documented (7). The 13 polypeptides precipitated by anti-COII-C antibodies were absent in the precipitate obtained in the presence of the specific peptide. Again, as observed in the experiments with pulse-labeled material, no cytochrome *c* oxidase-specific polypeptides were precipitated by anti-COII-N antibodies. In the experiment shown in Fig. 5, a few bands which are present in the precipitate obtained with anti-COII-C or anti-COII-N antibodies or with normal serum γ-globulins, in most cases with the same intensity, represent minor contaminants precipitated or adsorbed on the *S. aureus* immunoadsorbent nonspecifically.

Assay of Blots of Total Mitochondrial Proteins with Anti-COII-C Antibodies—The experiments described above clearly showed that the anti-COII-C antibodies precipitated specifically the whole cytochrome *c* oxidase complex from a Triton X-100 mitochondrial lysate. No other mitochondrial protein was precipitated by these antibodies, indicating that the epitope that the latter recognized pertained to one of the subunits of the cytochrome *c* oxidase complex. The previously reported evidence on the specificity of the anti-COII-C antibodies (2) and the absence of any significant homology of the COII-C peptide with COI and COIII (2) argued against the presence in the latter subunits of an epitope cross-reacting with the anti-COII-C antibodies in the intact complex. To exclude the presence of such a cross-reacting epitope in any of the cytoplasmically synthesized subunits of the complex, total mitochondrial proteins, denatured with SDS, separated on an SDS-urea-polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane, were tested with anti-COII-C antibodies, using peroxidase-conjugated goat anti-rabbit IgG for the detection of the reacting proteins (22). As shown in Fig. 5*c*, the anti-COII-C antibodies reacted very strongly and specifically with COII. No such a reaction was observed in parallel lanes assayed with anti-URF3-C (3) or anti-URF4L-C antibodies (4) (not shown). The very faint bands migrating faster or slower than COII result from nonspecific reaction, since they were also present in the control lanes. These results, therefore, strongly suggest that the epitope recognized by anti-COII-C antibodies in the intact cytochrome *c* oxidase complex is in the COII subunit itself.

The monospecificity of the anti-COII-C antibodies was also observed in immunoprecipitation experiments utilizing an SDS mitochondrial lysate from cells long-term-labeled with [^{35}S]methionine in the absence of inhibitors (not shown).

DISCUSSION

The results reported above indicate that antibodies directed against the COOH-terminal undecapeptide of COII can precipitate from a Triton X-100 mitochondrial lysate the entire cytochrome *c* oxidase complex in a substantially pure form. The finding that the three mitochondrially synthesized subunits labeled during a 2-h pulse in the presence of cycloheximide and then chased for 18 h in the absence of inhibitors were precipitated in close to equimolar proportion by anti-COII-C antibodies, in the absence of any precipitation of COII by anti-COII-N antibodies, strongly suggests that the complex released by Triton X-100 lysis of the organelles and immunoprecipitated was not appreciably disrupted. The presence of the 10 cytoplasmically synthesized subunits, besides the mitochondrially synthesized ones, in the complex immunoprecipitated from long-term-labeled cells is consistent with the above conclusion.

The main observation reported in this paper is that antibodies directed against the COOH-terminal undecapeptide of COII react with this epitope when the subunit is integrated in the cytochrome *c* oxidase complex, whereas antibodies directed against the NH_2-terminal decapeptide of COII, when tested on the intact complex, do not recognize this epitope. This observation may have interesting implications for the molecular structure and subunit organization of this complex. Since the two above-mentioned synthetic peptides were equally immunogenic in rabbits (2), and since each of the two types of antibodies was capable of recognizing the corresponding peptide of COII in an SDS mitochondrial lysate (2), it must be concluded that, in the complex immunoprecipitated from a Triton X-100 lysate, the NH_2-terminal peptide of COII

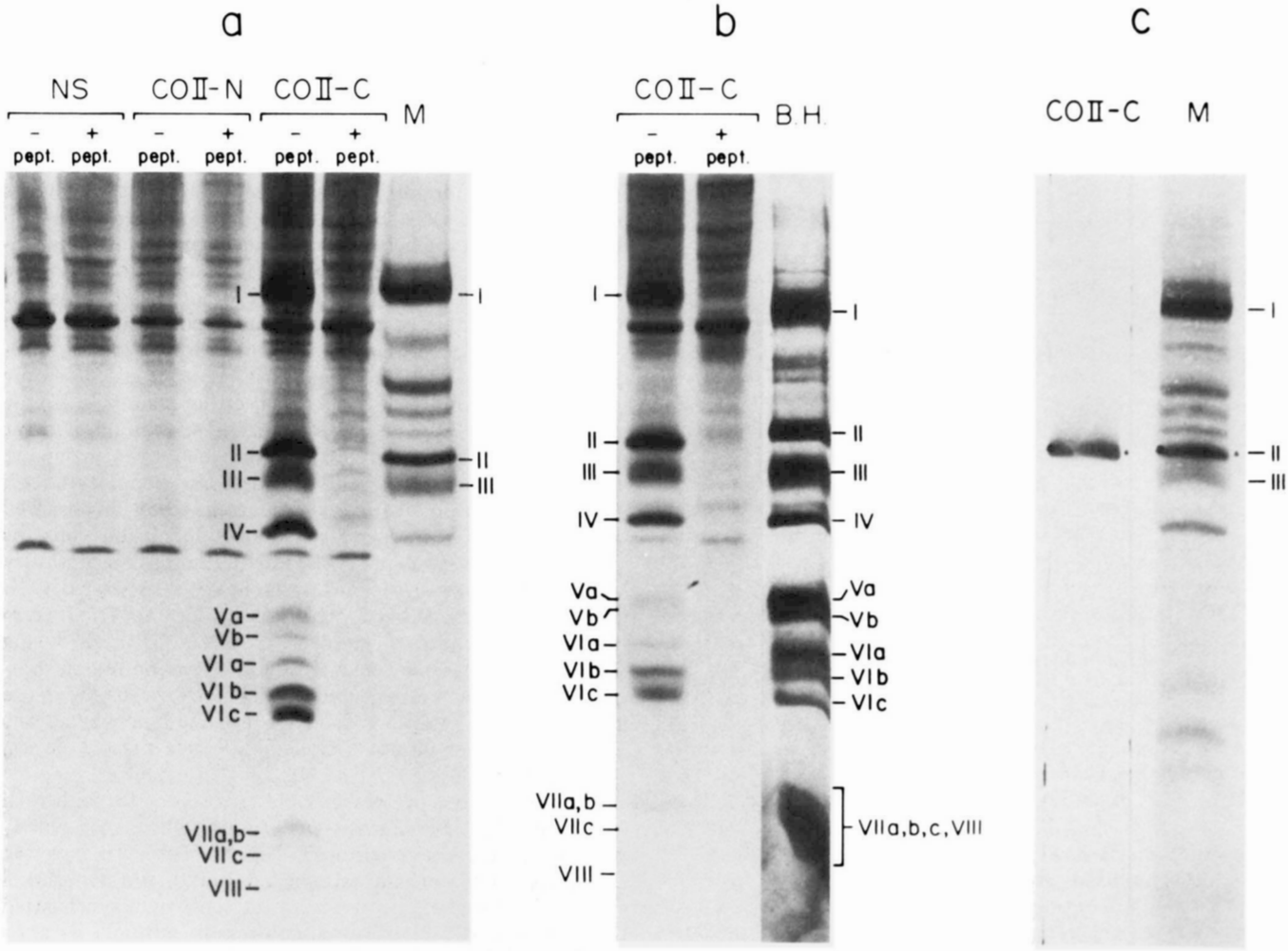

FIG. 5. **Immunoprecipitation of the cytochrome *c* oxidase complex by anti-COII-C antibodies.** *a*, samples of a Triton X-100 mitochondrial lysate from cells long-term-labeled with [^{35}S]methionine (500 μg of protein) were immunoprecipitated by 250 μg of anti-COII-C or anti-COII-N γ-globulins (in the absence or presence of 20 μg of the corresponding peptide), or by 250 μg of normal serum (NS) γ-globulins (in the absence or presence of 20 μg each of COII-C and COII-N), and run on an SDS-urea-polyacrylamide gel. *M*, pattern of mitochondrial translation products. *b*, a sample of purified cytochrome *c* oxidase from beef heart (*BH*) was run on an SDS-urea-polyacrylamide gel in parallel with samples of a Triton X-100 mitochondrial lysate from long-term-labeled cells immunoprecipitated by anti-COII-C antibodies in the absence or presence of the corresponding peptide and its subunit pattern revealed by silver staining. *c*, a sample of an SDS mitochondrial lysate (150 μg protein) from HeLa cells labeled with [^{35}S]methionine as in the experiment of Fig. 2, *lane a*, was run on an SDS-urea-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schuell, BA85) with a Bio-Rad apparatus. The blot was sequentially incubated with anti-COII-C γ-globulins and with peroxidase-conjugated goat anti-rabbit-IgG and then processed for the peroxidase reaction according to a published protocol (22) (*COII-C*). After drying, the COII band was marked with radioactive ink, and the blot was exposed for autoradiography (*M*).

is masked by one or more of the other subunits of the complex, or by Triton X-100 or phospholipids, or that the conformation of COII is changed in such a way that its NH_2-terminal peptide is no longer exposed or, in any case, recognizable by the antibodies. As the absence of reactivity of anti-COII-N antibodies with the complex was observed in experiments in which the complex was presumably precipitated intact, it seems likely that the exposure of the COOH-terminal peptide but not of the NH_2-terminal peptide of COII reflects the organization of the native enzyme.

The present observations should be considered in the context of what is known about the arrangement of the cytochrome *c* oxidase subunits within the complex in the inner mitochondrial membrane. Electron microscopic and image reconstruction studies have led to a model of the cytochrome *c* oxidase complex as a Y-shaped structure made of three domains: two of these (M1 and M2, corresponding to the arms of the Y) span the lipid bilayer, while the third one (C domain) extends outside the bilayer (23, 24). Cytochrome *c* (25–28) and antibody binding experiments (29) clearly indicate that the C domain is located on the cytoplasmic side of the inner mitochondrial membrane. As to the topology of COII within the complex, antibody binding (29–30) and iodination experiments (31) and labeling studies with hydrophylic protein-modifying reagents (31–34) have shown that this subunit is a part of the C domain and therefore projects into the intermembrane space; on the other hand, results obtained by the use of hydrophobic probes (34–36) have indicated that COII is in part embedded in the bilayer. These reactivities of COII can be correlated with the known amino acid sequence of this subunit (15), which is largely conserved in different mammalian species (37–39). As shown in Fig. 6 for the human

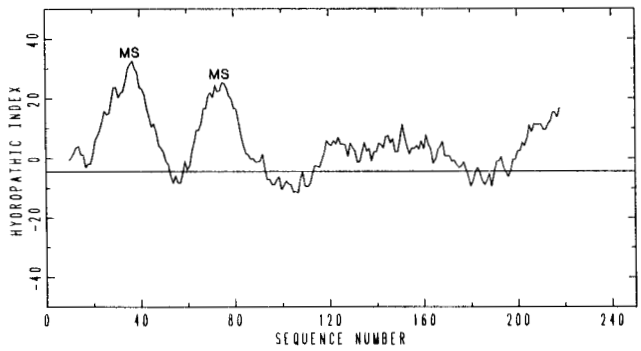

FIG. 6. **Hydropathy plot (40) of human cytochrome *c* oxidase subunit II.** A span of 19 residues was used. Note the two putative membrane-spanning segments in the NH_2-terminal third of the sequence.

COII, the hydropathy plot (40) of this polypeptide reveals two prominent peaks that are characteristic of membrane spanning segments in the NH_2 terminus proximal third of the molecule. Therefore, it is reasonable to think that these two segments cross the inner mitochondrial membrane, whereas the COOH terminus proximal portion of the subunit molecule projects out of the membrane. In agreement with this interpretation is the observation that the majority of the conserved acidic residues of COII, which have been implicated in cytochrome *c* binding, are in the COOH-terminus proximal half of the molecule (41). It has been shown that, in Triton X-100, the isolated cytochrome *c* oxidase exists in dimeric form as a detergent-phospholipid-protein complex, in which a portion of the original phospholipids have been displaced by the detergent (42). It is a reasonable assumption that, in the detergent-solubilized cytochrome *c* oxidase complex, the NH_2 terminus proximal portion of COII is masked by the detergent displacing the phospholipids, or by the residual tightly bound phospholipids, or by the interaction of this portion of COII with other subunits in the same or in the opposing monomer; by contrast, the COOH terminus of COII would be freely accessible to antibodies. The alternative possibility that the NH_2 terminus proximal peptide of COII is still exposed in the Triton X-100-solubilized complex but has become completely unreactive due to a conformational change cannot be rigorously excluded, but seems unlikely: in fact, such an exposed terminal sequence would be expected to be mobile and therefore likely to move through a range of conformations (43).

Although the definitive interpretation of the significance of the differential reactivity of anti-COII-C and anti-COII-N antibodies with the intact complex, in terms of the detailed interaction of the subunits and of the formation of functional sites, must await further information, it is clear that an extension of this approach to other segments of COII and of the other subunits of the cytochrome *c* oxidase complex can produce a detailed molecular map of the surface of this complex. Furthermore, the use of fragments of the complex obtained by cross-linking methods or by controlled disruption can make "internal" epitopes accessible to antipeptide antibodies, thus allowing a molecular mapping of the interior of the complex. In a more general context, the use of specifically targeted antibodies should prove invaluable for the analysis of the organization of multimeric complexes. Antipeptide antibodies have recently been used to map the phosphotransferase activity in the transforming protein $pp60^{src}$ and to locate the sites of this protein involved in its interaction with two cellular proteins, pp90 and pp50 (44).

A delay was observed in the appearance of newly synthesized COI and, to a lower degree, COIII, relative to COII, in the complexes immunoprecipitated from the Triton X-100-solubilized mitochondria. Previously, a different kinetics of appearance of the various pulse-labeled subunits into the cytochrome *c* oxidase complex, with COI being particularly delayed relative to COII and COIII, had been reported for *N. crassa* (45), *S. cerevisiae* (46), and rat hepatocytes (47, 48). In *N. crassa*, the different time courses of labeling of the subunits in the isolated cytochrome *c* oxidase could be correlated with different sizes of the pools of the various "precursor" polypeptides (*i.e.* the polypeptides not yet incorporated into the holoenzyme), with indications that the pool of COI precursor polypeptides consisted of two or more pools connected in series (45). Evidence that the delayed incorporation of pulse-labeled subunit I into the complex is due to a late assembly in a sequential and ordered process, rather than to a simple effect of dilution of the newly synthesized subunits by a larger precursor pool, has been recently presented for the enzyme in isolated rat liver mitochondria or isolated rat hepatocytes (47, 48). It seems reasonable to assume that the situation observed in isolated rat hepatocytes also holds for HeLa cells, as described in the present work. Furthermore, it seems very likely that the kinetic behavior of newly synthesized COI, COII, and COIII observed in HeLa cells does not result from the use of inhibitors of cytoplasmic protein synthesis during the labeling, but reflects the situation occurring in exponentially growing cells. In isolated rat hepatocytes, the lag in the incorporation of pulse-labeled COI into the holoenzyme was observed whether or not cycloheximide was present during the labeling (47).

Our finding that pretreatment of HeLa cells for 22 h with a concentration of CAP sufficient to inhibit almost completely mitochondrial protein synthesis, but still permitting growth of HeLa cells at a normal rate for 2 days (20), did not allow a substantially higher incorporation of the newly synthesized COI, COII, and COIII into complexes precipitable by anti-COII-C antibodies strongly suggests that the availability of the cytoplasmic subunits was not rate-limiting in the assembly of newly synthesized COII with COI and/or COIII; alternatively, it is conceivable that no accumulation of cytoplasmic subunits occurred during the CAP pretreatment.

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Note added in proof—Since this paper was submitted for publication, our attention has been called to a paper by Millet *et al.* (Millet, F., deJong, C., Paulson, L., and Capaldi, R. A. (1983) *Biochemistry* **22,** 546–552), in which the authors propose a folding pattern for bovine COII similar to that proposed here, with two hydrophobic stretches in the NH_2-terminal third of the molecule traversing the inner mitochondrial membrane.

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