Biochem. J. (1983) **212**, 829–834
Printed in Great Britain

Evidence for the sequential assembly of cytochrome oxidase subunits in rat liver mitochondria

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(*Received 21 December 1982/Accepted 15 March 1983*)

The assembly of cytochrome oxidase was studied in isolated rat liver mitochondria and isolated rat hepatocytes labelled *in vitro* with L-[^{35}S]methionine. This was achieved by studying the temporal association of radioactive subunits which are immunoabsorbed with antibodies against subunits I, II and the holoenzyme. Antibodies against the holoenzyme were shown to be highly specific for subunit V. The results show that subunit I appears in the holoenzyme late in the assembly process. No radioactive subunit I is absorbed with antiserum against subunit II or the holoenzyme (subunit V) after a 30 min pulse in either isolated mitochondria or hepatocytes. However, both antisera absorb radioactive subunits I after a 150 min chase in isolated hepatocytes. This was confirmed using antibodies against subunit I, which absorbed only radioactive subunit I after a 30 min pulse but absorbed radioactive subunits I–III and VI after a 150 min chase. Thus, the late assembly of radioactive subunit I is explained by a temporal sequence in the assembly process and not by the presence of a large, non-radioactive pool of subunit I. Using the above approach and the three specific antisera, the following temporal sequence in the assembly of cytochrome oxidase was established. Subunits II and III assemble rapidly with each other or with cytoplasmically translated subunit VI. This complex of three peptides in turn assembles slowly with subunit I or with the other cytoplasmically translated subunits. The early association of subunit VI with the mitochondrially translated subunits II and III suggests a possible role of the former in integration of the holoenzyme.

The mechanisms regulating the assembly of holocytochrome oxidase are poorly understood. This is obviously a complex process involving both the translation and integration of the various subunits. Studies on lower eukaryotic cells suggest that assembly of the mitochondrially translated subunits (I–III) is dependent on certain cytoplasmically translated subunits (Poyton & Kavanagh, 1976) or haem (Saltzgaber-Müller & Schatz, 1978). In contrast, little is known about the assembly of mammalian cytochrome oxidase. In previous studies, we have suggested that assembly of rat liver cytochrome oxidase subunits follows a certain temporal sequence (Kolarov *et al.*, 1981; Wielburski *et al.*, 1982). Assembly was defined in these experiments as the time-dependent association of labelled subunits with the chemically isolated holoenzyme or with immunoprecipitates obtained with antibodies against the holoenzyme. Such experiments do not, however, yield information about assembly of subunits prior to their integration into the holoenzyme. In the present study, we have approached this problem by using monospecific antibodies directed against individual subunits of rat liver cytochrome oxidase.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and methods

Materials

Rat liver cytochrome oxidase was purified according to the method of Ades & Cascarano (1977) with modifications described previously (Wielburski *et al.*, 1982). Cytochrome oxidase subunits I, II and III were isolated electrophoretically according to the method of Mendel-Hartvig (1982). Mitochondria were isolated from rat livers as described by Johnson & Lardy (1967), and from isolated hepatocytes as described by Gellerfors & Nelson (1979). Rat hepatocytes were isolated from 180 g male Sprague–Dawley rats as described by Gellerfors & Nelson (1979). Protein A from *Staphylococcus aureus* and

protein A–Sepharose were purchased from Pharmacia, Sweden. L-[^{35}S]Methionine (sp. radioactivity >800 Ci/mmol) and Na^{125}I (sp. radioactivity 13.0 mCi/μg of I) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. The scintillation solution (Luma Gel) was from Lumac, Sweden. All other chemicals were analytical grade.

Production and characterization of antisera

Antibodies against rat liver cytochrome oxidase were produced in rabbits as described by Mendel-Hartvig & Nelson (1977). The immunization scheme used for isolated subunits I and II was as follows. Antigen (200 μg of protein) was mixed with solid urea (final concn. 6 M), diluted with an equal volume of Freund's complete adjuvant, and injected subcutaneously. A single booster injection was given after 21 days using 100–150 μg of antigen in 6 M-urea emulsified with Freund's incomplete adjuvant. Rabbits were bled after 10 days. Control serum was collected prior to the first injection.

The specificities of various antisera were determined by electrophoretic transfer onto nitrocellulose according to Towbin *et al.* (1979). The transfer buffer contained 0.02% SDS. Antigen–immunoglobulin G complexes were detected with ^{125}I-labelled protein A. Labelled protein A (sp. radioactivity approx. 5.6×10^8 c.p.m./mg of protein) was prepared by the Iodogen method (Fraker & Speck, 1978). Labelled protein A was diluted to 560000 c.p.m./ml in saline containing 5% (w/v) bovine serum albumin and 0.5% (w/v) NaN_3.

Protein synthesis

Labelling of isolated rat liver mitchondria and hepatocytes was performed as described by Wielburski *et al.* (1982). Solubilization of labelled mitochondria and immunoabsorption was done as described by Kolarov *et al.* (1981). Measurement of radioactivity incorporated into protein was done as described by Wielburski *et al.* (1982).

Miscellaneous methods

Electrophoresis was carried out on 12.5% or 16% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS using the buffer system of Laemmli (1970), either in the presence or absence of 3.6 M-urea and 13% (w/v) glycerol (Merle & Kadenbach, 1980). Proteins were detected by using the silver stain method described by Ansorge (1982). Gels used for fluorography were processed in 1 M-sodium salicylate (Chamberlain, 1979), and exposed for 5–10 days to X-Omat R Kodak film stored at −80°C. Protein determinations were done according to the method of Lowry *et al.* (1951).

Results

Characterization of antigens

The peptide composition of the antigen samples used for immunization are shown in Fig. 1. The samples were resolved on 16% polyacrylamide gels under conditions described by Merle & Kadenbach (1980). In our hands, holocytochrome oxidase was resolved into eight peptides. However, subunits VII and VIII (Fig. 1) probably contain unresolved peptides corresponding to subunits VIa, VIb and VIc and VIIa, VIIb and VIIc, respectively, of Merle & Kadenbach (1980). Electrophoretic patterns similar to those in Fig. 1 were observed with samples resolved in the absence of urea and glycerol. The apparent lack of resolution of bands VII and VIII (cf. Merle & Kadenbach, 1980) is not critical to the present experiment, which focuses upon the largest subunits of the enzyme. The apparent M_r values of the subunits are estimated to be: (I) 45000 ± 500, (II) 25600 ± 600, (III) 23500 ± 500, (IV) 16800 ± 800, (V) 12000 ± 600, (VI) 10500 ± 700, (VII) 7600 ± 700, (VIII) 5300 ± 400.

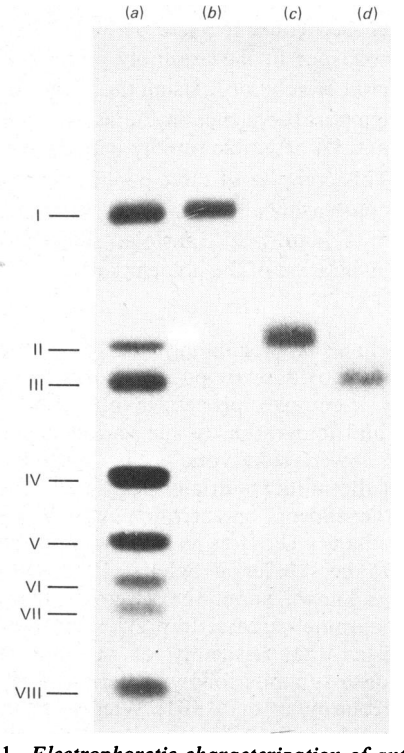

Fig. 1. *Electrophoretic characterization of antigens used in production of antisera*

(*a*) Rat liver cytochrome oxidase, (*b*) subunit I, (*c*) subunit II, and (*d*) subunit III. Electrophoresis was carried out on a 16% SDS/polyacrylamide gel containing 3.6 M-urea and 13% (w/v) glycerol. The peptides were detected by using the silver stain method.

Fig. 1 also demonstrates the purity of the isolated subunits used in the production of monospecific antisera. These were purified by electrophoretic elution from SDS/polyacrylamide gels as described in the Materials and methods section. No contaminating peptides were resolved upon re-electrophoresis of these peptides in the Laemmli buffer system or urea gels (Merle & Kadenbach, 1980), or after staining with either Coomassie Blue or silver stain.

Characterization of antisera

Antisera specificity was characterized by 'Western blotting' (Towbin *et al.*, 1979) of electrophoretically resolved mitochondria or purified cytochrome oxidase. Fig. 2 shows a nitrocellulose sheet onto which purified cytochrome oxidase had been transferred and which had been treated with antisera against (*a*) holocytochrome oxidase, (*b*) isolated subunit I and (*c*) isolated subunit II. To date no antibodies have been detected against subunit III. Antisera raised against subunits I and II react specifically with their respective antigens. Similar results were obtained with mitochondria.

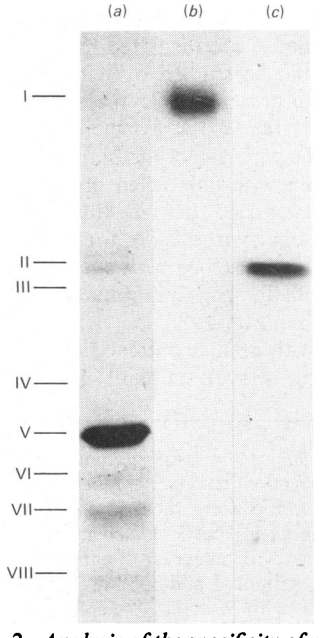

Fig. 2. *Analysis of the specificity of various antisera*
Isolated rat liver cytochrome oxidase was resolved by electrophoresis on 12.5% SDS/polyacrylamide gel, and then transferred onto nitrocellulose sheets. The nitrocellulose was incubated with antisera against: (*a*) holocytochrome oxidase, (*b*) subunit I, and (*c*) subunit II. Antigen–immunoglobulin G complex was detected autoradiographically after binding ^{125}I-labelled protein A. The locations of the subunits of cytochrome oxidase are indicated by Roman numerals.

Antisera against holocytochrome oxidase contained antibodies against most of the subunits, but primarily against subunit V (Fig. 2). This was assessed by cutting the radioactive bands from the nitrocellulose strips and counting for radioactivity. Approx. 65% of total radioactivity is located in subunit V (Table 1). The remaining subunits each contained between 4 and 10% of the total recoverable radioactivity. Since this experiment was done under conditions in which all antibodies were in excess, we can conclude that the antibody titre to subunit V is 7–20 times higher than that of the other antibodies. Antiserum against holocytochrome oxidase can, thus, be used as relatively specific antiserum against subunit V.

Assembly of cytochrome oxidase in isolated mitochondria

Assembly of cytochrome oxidase was studied in isolated rat liver mitochondria pulsed with L-[^{35}S]-methionine for 20min and chased with unlabelled methionine for 10min in order to clear the ribosomes of nascent polypeptide chains (Kużela *et al.*, 1981). Mitochondria were immunoabsorbed with antisera against holocytochrome oxidase (subunit V) and against subunits I and II. Fig. 3 shows the fluorographs of samples separated by SDS/polyacrylamide-gel electrophoresis. Newly labelled subunits II and III are immunoabsorbed with antisera against holocytochrome oxidase (Fig. 3*b*).

Table 1. *Quantitative analysis of the different antibodies produced against holocytochrome oxidase*
Isolated rat liver cytochrome oxidase was resolved by electrophoresis on SDS/polyacrylamide gels and transferred onto nitrocellulose sheet as in Fig. 2. The nitrocellulose was treated with antiserum against holocytochrome oxidase. Conditions were chosen in which doubling the antigen concentration doubled the amount of the immunoglobulin G bound. Thus, antibody to each subunit is in excess. Specific antigen–immunoglobulin G complexes were located autoradiographically after binding ^{125}I-labelled protein A. The bands were then cut from the nitrocellulose strip and counted. Background corrections were made by counting sections of the nitrocellulose strip which contained no antigen–immunoglobulin G complex. Abbreviation: n.d., not detected.

	Radioactivity	
Subunit	(c.p.m.)	(% of total)
I	358	4.5
II	570	7.2
III	304	3.8
IV	n.d.	—
V	5191	65.2
VI	415	5.2
VII	759	9.5
VIII	345	4.6

In view of the specificity of the antisera (Table 1), it is likely that subunits II and III are associated with subunit V. This is supported by the fact that subunit I is not absorbed with anti-holoenzyme serum, even though the titre of antibodies against subunit I is as high as those against subunits II and III (Table 1). Together, these data strongly suggest that subunits II and III are rapidly assembled with subunit V or with a protein complex in common with subunit V. This is in agreement with our previous findings on chemically isolated cytochrome oxidase (Wielburski *et al.*, 1982).

The absence of assembly of subunit I with subunit II and III after a 30min incubation is further shown by the fact that antiserum against subunit I does not remove subunits II and III (Fig. 3*c*). Furthermore, antiserum against subunit II does not remove subunit I (Fig. 3*d*). Quantitative immunoabsorption shows, however, that large amounts of labelled subunit I are present (Fig. 3*c*, Table 2).

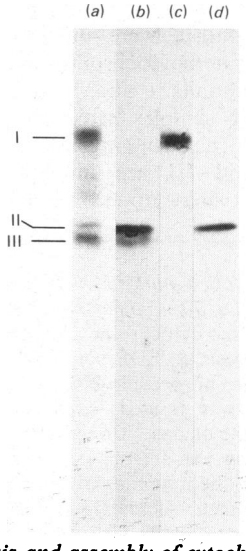

Fig. 3. *Synthesis and assembly of cytochrome oxidase in isolated liver mitochondria*

Isolated rat liver mitochondria were labelled for 20min with L-[^{35}S]methionine (30μCi/ml) and then chased for 10min with 5mM-unlabelled methionine. Incubation was terminated by addition of a solution containing 0.25M-sucrose, 10mM Tris/HCl, pH7.5, and 10mM-unlabelled methionine. Mitochondria were removed by centrifugation, resuspended in lysing buffer and treated with various antisera. The antigen–immunoglobulin G complex was immunoabsorbed with protein A–Sepharose and the absorbed samples were subjected to electrophoresis. Fluorographs are shown for: (*a*) total mitochondrial translation products, and for samples immunoabsorbed with antisera against (*b*) the holoenzyme, (*c*) subunit I and (*d*) subunit II. Cytochrome oxidase subunits I–III are indicated with Roman numerals.

Subunits II and III also appear to be bound in the same complex since the latter is absorbed by antibodies specific to subunit II (Fig. 3*d*). However, only about 30% of the newly synthesized subunit II is assembled with the holoenzyme after a 30min incubation. This is shown by the finding that antiserum against holocytochrome oxidase absorbed only one-third of the total radioactivity in subunit II (Table 2).

Table 2 also shows that subunits I and II are translated stoichiometrically. The ratio of L-[^{35}S]-methionine in immunoabsorbed subunits I and II is 2.3. This is close to the molar ratio (2.5) of methionine which was determined in these two peptides from the nucleotide sequences of the rat liver genes (Grosskopf & Feldman, 1981). The slightly lower ratio obtained with L-[^{35}S]-methionine measurements (Table 2) is probably due to the small amount of subunit III absorbed together with subunit II (Fig. 3). These findings indicate that no preferential translation of the two mRNA species occurs.

Assembly of cytochrome oxidase in isolated rat hepatocytes

Further studies on the assembly of cytochrome oxidase were carried out on isolated hepatocytes so that assembly could be studied over longer periods of time and in the presence of cytoplasmic protein synthesis. In the standard experiment, cells were labelled for 30min with L-[^{35}S]methionine and then chased for 150min with unlabelled methionine. All incubations were carried out in the absence of inhibitors of protein synthesis. Mitochondria were isolated and immunoabsorbed with specific antisera. Electrophoretic and fluorographic analysis of these samples are shown in Fig. 4.

In agreement with results obtained with isolated mitochondria (see above), subunit I of isolated

Table 2. *Distribution of the total radioactivity in cytochrome oxidase from mitochondria labelled in vitro*

Isolated mitochondria were labelled *in vitro* with L-[^{35}S]methionine as in Fig. 3. Lysed mitochondria (1mg) were treated with an excess of antisera against the holoenzyme, subunit I and subunit II, and then immunoabsorbed with protein A–Sepharose. The immunoabsorbed samples were counted directly. Specificity of the antibody was determined as in Fig. 2 by the nitrocellulose-transfer technique. Labelled subunits absorbed by a specific antibody were analysed as in Fig. 3.

Antisera raised against:	Specificity of the antibody	Radioactive subunits absorbed	Total radioactivity absorbed (c.p.m.)
Subunit I	I	I	14850
Subunit II	II	II+III	6450
Holoenzyme	V	II+III	2140

hepatocytes is not assembled with the holoenzyme (lane 1*a*) or with subunits II or III (lanes 2*a* and 3*a*) after 30min of labelling. Subunit I assembly with subunits II, III and VI is observed, however, after a 150min chase (lanes 2*b*, 3*b*). At this time, small amounts of subunit I are also assembled with the holoenzyme (subunit V) as well as with subunit II (lane 1*b*). These findings suggest that assembly of subunit I is relatively slow and that it appears first with the other mitochondrially translated subunits II and III of cytochrome oxidase. Subunit VI is the most abundant cytoplasmically translated subunit which is absorbed with antibodies against subunits I and II. These data support an assembly sequence in which mitochondrially translated peptides first assemble with each other and with subunit VI, followed by assembly with subunit V and other cytoplasmically translated subunits. Quantitative immunoabsorption with antisera against the different subunits (Fig. 4, Table 2) indicates that only a small part of the total label in subunits I and II is associated with the holoenzyme fraction.

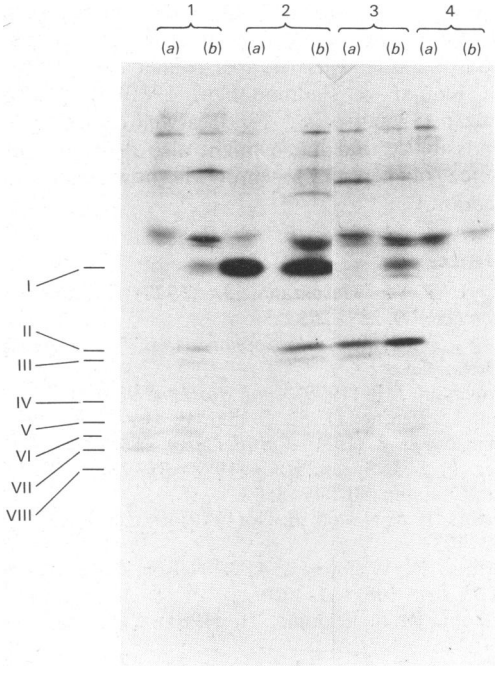

Fig. 4. *Synthesis and assembly of cytochrome oxidase in isolated rat hepatocytes*

Isolated rat hepatocytes were labelled for 30min with L-[^{35}S]methionine (40μCi/ml) and then chased with 5mM-unlabelled methionine for 150min. Samples were removed after 30 and 180min and mitochondria prepared as described (Gellerfors & Nelson, 1979). The labelled mitochondria (4mg of protein) were immunoabsorbed as in Fig. 3. Fluorographs are shown for samples immunoabsorbed with antisera against: (1) holocytochrome oxidase, (2) subunit I, (3) subunit II, and (4) pre-immune serum. For each sample, lane (*a*) represents samples labelled for 30min and lane (*b*) samples labelled for 30min and chased for 150min. The locations of the cytochrome oxidase subunits are indicated.

Discussion

A partial understanding of the sequences in the assembly of rat liver cytochrome oxidase is starting to emerge. In agreement with our earlier findings (Kolarov *et al.*, 1981; Wielburski *et al.*, 1982), subunit I assembles with the other subunits only after a lag period of 1.5–2h. It is, however, rapidly labelled. A lag in the appearance of subunit I in the holoenzyme could result from dilution of the newly synthesized peptide in a pre-existing pool of unlabelled subunits (Schwab *et al.*, 1972). This possibility is eliminated, however, by experiments utilizing an excess of anti-(subunit I) serum, which does not co-absorb labelled subunits II and III after a 30min pulse with L-[^{35}S]methionine. Under these conditions, all subunit I, including the unlabelled subunits, should be removed and its assembly with other labelled subunits should be detected.

The integration of subunit I starts with its association to subunits II, III and VI. These labelled peptides are the first to be absorbed with monospecific antisera against subunit I (Fig. 4) after a 1.5h chase. Some newly labelled subunit I is also associated with the holoenzyme after a 1.5h chase, but this is quantitatively small compared with that which remains unassembled or that which is assembled with subunits II, III and VI (Fig. 4). Thus,

Table 3. *Distribution of the total radioactivity in cytochrome oxidase from hepatocytes labelled in vitro*

Isolated rat hepatocytes were labelled with L-[^{35}S]methionine as in Fig. 4. Mitochondria were isolated after 30min of labelling and after a 150min chase with unlabelled methionine. The samples were lysed and treated with an excess of antisera and absorbed with protein A–Sepharose. The absorbed samples were counted directly. Analyses of the antibody specificity and the subunits absorbed are taken from Figs. 2 and 4, respectively.

		Radioactive subunits absorbed		Total radioactivity absorbed (c.p.m.)	
Antiserum raised against:	Specificity of antibody	30min	180min	30min	180min
Subunit I	I	I	I, II, III, VI	13600	11000
Subunit II	II	II, III, VI	I, II, III, VI	11200	7600
Holocytochrome oxidase	V	II–VIII	I–VIII	9000	7400
Pre-immune serum	–	—	—	1000	750

mitochondrially translated peptides can assemble with each other to some extent prior to assembly into the holoenzyme. Similar conclusions can be drawn from experiments using antisera against subunits II and V (holoenzyme), which show that more subunit II is associated with subunit III than with the holoenzyme after a 30 min pulse.

The early association of subunit VI with subunits II and III suggests a possible role for the former peptide in the integration of mitochondrially translated subunits into the holoenzyme. In *Neurospora*, the integration of subunits I–III into holocytochrome oxidase is dependent upon one, or perhaps two, cytoplasmically translated subunits (Michel & Neupert, 1973). Furthermore, immunoabsorption of yeast post-microsomal supernatant with monospecific antisera against subunits IV and VI prevents the cytosolic-dependent integration of subunits I–III into an immunoprecipitable form (Poyton & Kavanagh, 1976). Thus, in the organisms tested, the assembly of subunits I–III into the holoenzyme requires only one or two cytoplasmically translated subunits. This peptide corresponds to subunit VI in rat liver, and its assembly with subunits II and III appears to represent an early event in the assembly of the holoenzyme.

The above results suggest the following events in the assembly of rat liver cytochrome oxidase. Subunits I–III are synthesized rapidly, and, judging from our data on subunits I and II, in stoichiometric amounts. Subunits II and III assemble rapidly with each other and/or with subunit VI. This complex in turn assembles with subunit V and then slowly with subunit I or with other cytoplasmically translated subunits. A similar result was reported for the assembly of *Neurospora* cytochrome oxidase (Schwab *et al.*, 1972), in which subunits II, III and an 8000 kDa peptide appeared together in the chemically isolated enzyme in the early phase of labelling and on the same time scale. This was followed by integration of subunit I and then the remaining cytoplasmically made subunits. The latter experiments were interpreted, however, in terms of different pool sizes for the various subunit precursors. Our data obtained with antiserum against subunit I (see above) indicate that the delayed assembly of subunit I is not due to an enlarged pool of this subunit, but rather to its late assembly in a sequential assembly process. A sequential assembly process can, of course, in theory lead to different pool sizes of the unassembled subunits. However, assuming assembly to be a process involving a 1:1 stoichiometric association of the different subunits, only insignificant differences in the pool sizes should be expected. Additional mechanisms for generating different sized precursor pools, such as differential synthesis or breakdown of the translation products, do not appear to operate in isolated hepatocytes under conditions in which the present experiments were carried out (Gellerfors *et al.*, 1979).

The reasons for the late assembly of subunit I are not known. It is tempting to speculate, however, that it might be related to the binding of haem. It is now generally accepted that subunit I binds haem (Winter *et al.*, 1980). Furthermore, the requirement for haem in the integration of yeast cytochrome oxidase subunits (Saltzgaber-Müller & Schatz, 1978; Kumar & Padmanaban, 1980) and cytochrome *b* (Clejan *et al.*, 1980) is well documented. The possibility that haem might also determine the sequence of peptide assembly is now under investigation.

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