Heme *a* induces assembly of rat liver cytochrome *c* oxidase subunits I–III in isolated mitochondria

Antek Wielburski and B. Dean Nelson

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

Received 28 September 1984

The assembly of cytochrome *c* oxidase subunits I–III was studied in vitro in isolated rat liver mitochondria pre-labeled with [^{35}S]methionine. Individual subunits were immunoabsorbed with monospecific antibodies. Isolated heme *a* from rat liver mitochondria, when added to radiolabeled mitochondria, induced assembly of subunit I with subunits II and III. Assembly of these subunits was not observed in mitochondria incubated in the presence of heme *b*(hemin) or in the absence of heme. Quantitative analysis of immunoabsorbed, radiolabeled subunits suggests that the predominant effect of heme *a* is on the assembly of subunit I with subunit III.

Cytochrome oxidase assembly *Heme a requirement* *(Rat liver mitochondria)*

1. INTRODUCTION

Based on studies on isolated hepatocytes and isolated rat liver mitochondria [1,2], we suggested that the assembly of mammalian cytochrome *c* oxidase subunits follows a specific temporal sequence. Although the 3 mitochondrially translated subunits are synthesized simultaneously and in stoichiometric amounts [1,2], there is a lag in assembly of subunit I with subunits II and III and with the holoenzyme. The late assembly of subunit I is of interest since this subunit binds heme in the holoenzyme [3], raising the possibility that the assembly of subunit I could be dependent upon heme binding. The requirement for heme in the integration of cytochrome *c* oxidase subunits [4,5] and cytochrome *b* [5] is well documented in lower eukaryotic cells. Here we demonstrate that assembly of cytochrome *c* oxidase subunit I in mammalian mitochondria is a heme *a*-dependent process.

2. MATERIALS AND METHODS

2.1. *Materials*

Rat liver cytochrome *c* oxidase was purified as in [1]. Cytochrome *c* oxidase subunits I and II were isolated by electrophoretic elution from SDS-polyacrylamide gels [7]. Monospecific antisera against subunits I and II were produced as in [2]. Rat liver mitochondria were isolated from 180-g male Sprague–Dawley rats according to [8]. Hemin was purchased from Sigma. Protein A–Sepharose was purchased from Pharmacia, Sweden. L-[^{35}S]Methionine was purchased (spec. act. 800 mCi/ml) from The Radiochemical Centre, Amersham. All other chemicals were analytical grade.

2.2. *Protein synthesis*

Rat liver mitochondria were labeled in vitro with L-[^{35}S]methionine as in [1]. Solubilization of labeled mitochondria and immunoabsorption of radiolabeled antigens was done according to [9].

2.3. *Isolation of heme* a

Heme *a* was prepared from rat liver mitochondria by acid-acetone extraction [10]. Hemin (referred as heme *b*) and heme *a* were dissolved in 10 mM NaOH and the alkaline solutions were centrifuged for 15 min at 8000 × *g* to remove insoluble material. Aliquots of clear solutions were used for

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

determination of heme concentration by the hemochromogen method [11]. The extinction coefficients of 27.4 $mM^{-1} \cdot cm^{-1}$ at 587 nm and of 34.4 $mM^{-1} \cdot cm^{-1}$ at 556 nm served to calculate the concentrations of heme *a* and heme *b*, respectively [12].

2.4. *Treatment of labeled rat liver mitochondria with heme*

Labeled rat liver mitochondria (3 mg protein) were suspended in 1 ml of 0.2 M KPO_4 buffer, pH 7.5, and allowed to swell for 10 min at room temperature. Heme *a* or hemin (2 nmol/mg mitochondria) were added to the swollen mitochondria under vigorous shaking. Control samples were incubated under the same conditions except that 10 mM NaOH was added. After 60 min incubation at 25°C in the dark, the samples were solubilized, clarified and treated with antisera against subunits I and II of cytochrome *c* oxidase as in [9].

2.5. *Miscellaneous methods*

Electrophoresis was carried out on 12.5% (w/v) polyacrylamide gels containing 0.1% SDS (w/v) using the buffer system of [13]. Dried gels were fluorographed as in [14]. Fluorographs were scanned with a densitometric scanner and relative ratios in labeling of subunits I–III of cytochrome *c* oxidase were calculated. Protein determinations were done according to [15].

3. RESULTS

Rat liver mitochondria were pulsed with L-[^{35}S]methionine for 20 min and chased with unlabeled methionine (10 mM) for 10 min to clear the ribosomes of nascent polypeptide chains [16]. No assembly of radiolabeled subunit I takes place under these conditions [1,2]. Labeled mitochondria were then incubated for 60 mn in the presence or absence of heme and were immonoabsorbed with antisera against subunits I and II. Fig.1 shows the fluorographs of samples separated by SDS-polyacrylamide gel electrophoresis. As reported in [1,2], antisera against subunit I immunoabsorbed only radiolabeled subunit I from untreated mitochondria (fig.1A, lane 3). However, in mitochondria pretreated with heme *a*, antisera against subunit I also co-absorbed radiolabeled

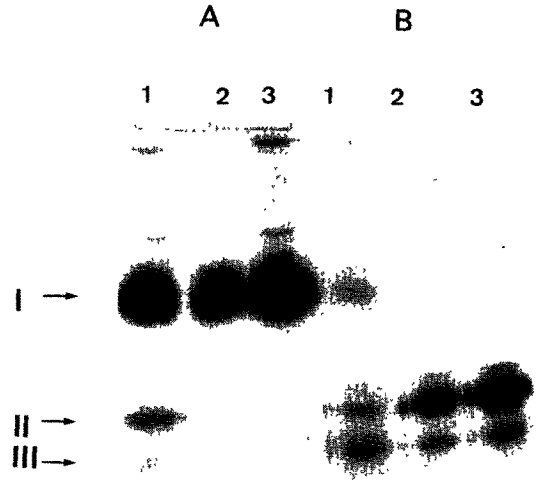

Fig.1. Heme *a* induces the assembly of rat liver cytochrome *c* oxidase subunits I–III. Radiolabeled mitochondria were incubated in the presence or absence of heme as described in section 2, and were immunoabsorbed with antibodies against: (A) subunit I, or (B) subunit II. Mitochondria were incubated with heme *a* (lane 1), heme *b* (lane 2) or without heme (lane 3). Cytochrome *c* oxidase subunits I–III are indicated with Roman numerals.

subunits II and III (fig.1A, lane 1). Subunits II and III were not co-absorbed from mitochondria pretreated with heme *b* (fig.1A, lane 2). Furthermore, heme *a* in which the pyridine-NaOH spectra were altered, presumably due to changes around the iron, was ineffective in promoting assembly of subunit I with subunits II and III (not shown). These data imply that assembly of subunit I with subunits II and III of rat liver cytochrome *c* oxidase is dependent upon heme *a* accessibility.

Analogous results were obtained using monospecific antibodies against subunit II. Here, radiolabeled subunit I was co-absorbed only from heme *a* pretreated mitochondria (fig.1B, lane 1), whereas only subunits II and III were immunoabsorbed from untreated mitochondria or mitochondria pretreated with heme *b* (fig.1B, lanes 2 and 3). Together, these data are in agreement with our previous finding that subunits II and III rapidly combine with each other [2]. Furthermore, the data are consistent with the idea that heme *a* is re-

Table 1

Quantitative analysis of the heme-induced assembly of rat liver cytochrome *c* oxidase subunits

Preparations subjected to electrophoresis	Incubation conditions	Percent of total radioactivity in		
		Subunit I	Subunit II	Subunit III
Immunoabsorbed with anti-subunit I	no heme	100	0	0
	heme *b*	100	0	0
	heme *a*	75.7 ± 1.5	10.4 ± 1.2	13.9 ± 0.8
Immunoabsorbed with anti-subunit II	no heme	0	62.5 ± 1.1	37.6 ± 1.1
	heme *b*	0	63.2 ± 1.2	36.8 ± 0.8
	heme *a*	18.9 ± 0.6	37.1 ± 1.1	44.0 ± 0.9
Rat liver mitochondria	–	61.6 ± 1.2	22.4 ± 1.4	16.0 ± 0.9

Fluorographs were scanned densimetrically and the areas under the curves were calculated for subunits I, II and III. The sum of the areas under all three peaks was taken as 100%. The values are the means ± SD of 4 different experiments

quired for the assembly of rat liver cytochrome *c* oxidase.

Table 1 shows the distribution of radioactivity among the mitochondrially translated subunits of cytochrome *c* oxidase immunoabsorbed with antisera against subunits I and II. For comparison, the distribution of radioactivity of newly synthesized subunits I–III in rat liver mitochondria was also calculated. The ratios of radioactivity in subunit I *vs* II is between 1.4 and 1.7 in mitochondria and in immunoabsorbed samples after incubation of mitochondria in the absence of heme *a*. This ratio decreases to about 0.8 in immunoabsorbed samples from heme *a*-treated mitochondria due to a relative increase in co-immunoabsorbed subunit III (table 1). These data suggest that both subunits II and III combine with subunit I in the presence of heme *a*, and that the heme-induced interaction favours an association of subunit I with subunit III.

4. DISCUSSION

Studies on lower eukaryotic cells demonstrated a role for heme [4], oxygen [17] and nuclear genes [18] in the assembly of mitochondrially and cytoplasmically translated subunits of cytochrome *c* oxidase. The task of investigating the process of assembly in mammalian cells is made difficult by the lack of mutants. However, studies on the assembly of rat liver cytochrome *c* oxidase in isolated hepatocytes and isolated rat liver mitochondria [1,2] have provided an insight into temporal events in this process. This report demonstrates that heme *a* has at least one major effect on the biogenesis of cytochrome *c* oxidase in isolated rat liver mitochondria – it promotes assembly of the mitochondrially translated subunits of the enzyme. Although it is not clear how heme *a* exerts this effect, the simplest explanation would be that it must attach to subunit I before assembly of the holoenzyme can proceed to completion. These results are in line with multiple effects of heme which have been observed on assembly of yeast mitochondria. Requirements for heme in the assembly [4] and synthesis [4,10] of cytochrome *c* oxidase subunits have been reported. Furthermore, heme is required for integration of cytochrome *b* into the cytochrome b–c_1 complex [19], and for the processing of the cytochrome c_1 [20] in lower eukaryotic cells. Although a role for heme in the synthesis of mammalian cytochrome *c* oxidase has been suggested [21,22], this study provides the first proof for direct involvement of heme *a* on the assembly of the mammalian enzyme.

ACKNOWLEDGEMENT

This work was supported by the Swedish Natural Science Research Council.

REFERENCES

[1] Wielburski, A., Kuzela, S. and Nelson, B.D. (1982) Biochem. J. 204, 239–245.

[2] Wielburski, A. and Nelson, B.D. (1983) Biochem. J. 212, 829–834.

[3] Winter, D.B., Bruyninckx, W.J., Foulke, F.G., Grinich, N.P. and Mason, S.H. (1980) J. Biol. Chem. 255, 11408–11414.

[4] Saltzgaber-Müller, J. and Schatz, G. (1978) J. Biol. Chem. 253, 305–310.

[5] Kumar, C.C. and Padmanaban, G. (1978) J. Biol. Chem. 255, 11130–11134.

[6] Clejan, L., Beattie, D.S., Gollub, E.G., Liu, K.P. and Sprinson, D.B. (1980) J. Biol. Chem. 255, 1312–1316.

[7] Mendel-Hartvig, I. (1982) Anal. Biochem. 121, 215–217.

[8] Johnson, D. and Lardy, H. (1967) Methods Enzymol. 10, 94–96.

[9] Kolarov, J., Wielburski, A., Mendel-Hartvig, I. and Nelson, B.D. (1981) Biochim. Biophys. Acta 652, 334–346.

[10] Tuppy, H. and Birkmayer, G.D. (1969) Eur. J. Biochem. 8, 237–243.

[11] Morrison, M. and Harie, S. (1965) Anal. Biochem. 12, 77–82.

[12] Falk, J.E. (1964) in: Porphyrins and Metalloporphyrins, p.240, Elsevier, Amsterdam, New York.

[13] Laemmli, U. (1970) Nature 277, 680–685.

[14] Chamberlain, J.P. (1979) Anal. Biochem. 98, 132–135.

[15] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.

[16] Kuzela, S., Wielburski, A. and Nelson, B.D. (1981) FEBS Lett. 135, 89–92.

[17] Woodrow, G. and Schatz, G. (1979) J. Biol. Chem. 254, 6088–6093.

[18] Cabral, F. and Schatz, G. (1978) J. Biol. Chem. 253, 4396–4401.

[19] Gasser, S.M., Daum, G. and Schatz, G. (1982) J. Biol. Chem. 257, 13034–13041.

[20] Ohashi, A., Gibson, J., Gregor, I. and Schatz, G. (1982) J. Biol. Chem. 257, 13042–13047.

[21] Wood, J.S. (1977) Mol. Pharmacol. 13, 50–59.

[22] Beattie, D.S. and Stuchell, R.N. (1970) Arch. Biochem. Biophys. 139, 291–297.