Biosynthesis of cytochrome *c* oxidase by isolated liver mitochondria of the tadpole, *Rana catesbeiana*

Naoki Amuro, Yoshitaka Goto and Ryoiti Shukuya

Department of Biochemistry, Nippon Medical School, Bunkyo-ku, Sendagi 1-1-5, Tokyo 113, Japan

Received 23 August 1982

Mitochondrial biogenesis *Protein biosynthesis* *Cytochrome c oxidase* *Liver mitochondria*
Metamorphosis (Rana catesbeiana)

1. INTRODUCTION

It is now clear that mitochondrial biogenesis is controled by the two separated and functionally distinct protein synthetic systems, mitochondrial and cytoplasmic [1–6]. While the cytoplasmic protein synthetic system contributes both soluble and membrane-bound mitochondrial proteins [4–6], the mitochondrial protein synthetic system appears to contribute the production of 10–12 polypeptides which reside in the inner mitochondrial membrane [4–6]. Some of the mitochondrial membrane-bound proteins are assembled by subunits which are collaboratively produced by the 2 separate protein synthetic systems [4–6]. Cytochrome *c* oxidase, located in the inner membrane of mitochondria, is a hetero-oligomeric protein consisting of ≥ 6 subunits [6–12]; the 3 largest subunits have been identified as mitochondrial and the others as cytoplasmic translation products [1–6]. Thus, we have examined the interaction between the 2 separate protein synthetic systems. However, the mechanism by which the 2 separated protein synthetic systems coordinate mitochondrial protein synthesis remains obscure.

We have found in the liver mitochondria of tadpoles undergoing metamorphosis a promoted mitochondrial biogenesis [13–16], an appearance of heterogeneous mitochondria in the density [15] and an accelerated turnover rate of cytochrome *c* oxidase [16]. To examine the coordination of the 2 separated protein synthetic systems, cytoplasmic and mitochondrial, the mitochondria from tadpole treated with and without cycloheximide were studied in vitro for the ability of mitochondrial protein synthesis. The results obtained are as follows:

(1) The ability of mitochondria to synthesize cytochrome *c* oxidase is higher in the tadpoles at metamorphic climax than at pre-metamorphosis;

(2) When tadpoles are pretreated with cycloheximide, the mitochondrial protein synthesis is profoundly reduced in the mitochondria of tadpoles at both stages, pre-metamorphosis and climax.

However, the relative rate of synthesis of cytochrome *c* oxidase is markedly higher in the mitochondria of tadpoles pretreated with cycloheximide than in that of normal tadpoles.

Abbreviations: EDTA, ethylendiaminetetraacetic acid; SDS, sodium dodecylsulfate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid

Enzyme: Cytochrome *c* oxidase [ferrocytochrome *c*:O_2 oxidoreductase (EC 1.9.3.1)]

2. MATERIALS AND METHODS

Tadpoles were obtained commercially from an experimental animal supplier in Tokyo. The tadpoles were classified according to the developmental stages defined in [17,18]. *Rana catesbeiana* tadpole remains in pre-metamorphic stage (before stage XI) during which the tadpole grows considerably, but with little other morphological change.

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

Then the pro-metamorphic stage (stages XI–XIX) and subsequently the metamorphic climax (stages XX–XXV) begins; the former is characterized by a rapid growth of hind limbs and the latter by a rapid transformation.

The following materials were purchased from the source indicated. Triton X-100, HEPES and cycloheximide (Wako); L-[4,5-^{3}H]leucine (spec. act. 140 Ci/mmol) (RCC Amersham); Whatman glass fiber disc GF/C (Whatman); *Staphylococcus aureus* Cowan type I was a gift from Dr Takeuchi (Department of Bacteriology, Nippon Medical School).

2.1. *Preparation of anti-cytochrome* c *oxidase IgG*

The IgG against cytochrome *c* oxidase purified from the frog liver was prepared as in [16]. The IgG thus obtained reacted with the whole molecule of cytochrome *c* oxidase to form a single precipitin line on an agarose gel plate. Control IgG fraction was prepared from the rabbit serum which was collected prior to the immunization.

2.2. *Pretreatment with cycloheximide*

Tadpoles were injected with cycloheximide (50 μg/g body wt) into the peritoneal cavities; control tadpoles with a 0.75% saline solution. After 2 h, the tadpoles were killed.

2.3. *Preparation of mitochondria*

Tadpole livers (35–45 g) were homogenized in 3 vol. buffer mixture (2 mM HEPES, pH 7.4, 220 mM mannitol, 70 mM sucrose, 2 mM EDTA and 10 mM mercaptoethanol). The mitochondria were isolated according to [19] which was better than the other method for obtaining intact mitochondria in high yield.

2.4. *Mitochondrial protein synthesis*

The mitochondria (60 mg protein) were incubated with 1 mCi of L-[4,5-^{3}H]leucine (spec. act. 140 Ci/mmol) at 37°C in 5 ml medium which contained 50 mM HEPES, 5 mM KH_2PO_4, 1 mM EDTA, 70 mM sucrose, 220 mM mannitol, 1 mM NaCl, 10 mM $MgCl_2$, 10 mM α-ketoglutarate (pH 7.4), 20 000 units of penicillin G and 0.72 mM cycloheximide. The incubation was carried out in a 250 ml flask for 40 min under shaking at 120 cycles/min.

An aliquot (50 μl) of the incubation mixture was transferred into a centrifugal tube containing 1 μmol leucine and was mixed with 0.5 ml ethanol and 3 ml 10% (w/v) trichloroacetic acid, successively. The sample was heated in a boiling water bath and was centrifuged to collect the precipitate formed after chilling in an ice bath for 30 min. The precipitate was dissolved in 0.5 ml 1 N NaOH and again precipitated with 10% (w/v) trichloroacetic acid after neutralization with 0.5 ml 1 N HCl. The precipitate was trapped and washed on a Whatman glass fiber disc GF/C with 50 ml 5% trichloroacetic acid. After drying the radioactivity trapped on the glass fiber disc was counted.

The residual incubation mixture was mixed with 100 μmol leucine and chilled in an ice bath. The mitochondria were lysed by an addition of 1% Triton X-100 and centrifuged at 5000 × *g* for 30 min. The resultant supernatant was incubated with 1.5-fold excess of anti-cytochrome *c* oxidase-IgG at 4°C for 10 h. The immunoprecipitate formed was quantitatively collected by using formaldehyde-inactivated staphylococcal cells as in [20]. The cytochrome *c* oxidase was dissociated from the precipitated complex and denatured in 200 μl 60 mM Tris–HCl (pH 8.8) containing 2 mM EDTA, 5 mM dithiothreitol and 6% SDS. The solubilized enzyme was alkylated with 10 mM iodoacetamide prior to the electrophoresis. Control was 0.1 mg purified cytochrome *c* oxidase which was denatured and alkylated. Electrophoresis of the cytochrome *c* oxidase on a SDS–polyacrylamide gel and determination of the radioactivity incorporated into the mitochondrial subunits were as in [14–16].

2.5. *Protein determination*

Protein was determined as in [21] using bovine serum albumin as a standard. When detergent was used to solubilize mitochondria, the method in [22] was used.

3. RESULTS AND DISCUSSION

Liver mitochondria from normal and cycloheximide-pretreated tadpoles were incubated with L-[4,5-^{3}H]leucine in the presence of α-ketoglutarate. The radioactivity incorporated into the total mitochondrial protein was plotted as a function of the incubation time. The incorporating activity was markedly reduced in the mitochondria from the

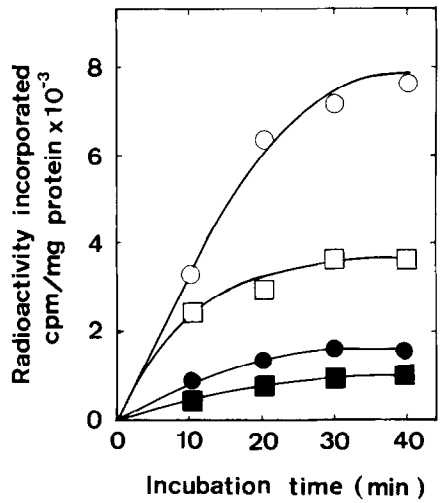

Fig.1. Incorporation of L-[4,5-^{3}H]leucine in vitro into total mitochondrial protein by isolated mitochondria. Mitochondria were isolated from the liver of tadpoles with or without cycloheximide-pretreatment and incubated with L-[4,5-^{3}H]leucine. Radioactivity incorporated into the total mitochondrial protein was determined at the indicated time. Details are described in section 2: (•, ○) mitochondria from the tadpoles at premetamorphic stage with and without cycloheximide-pretreatment, respectively; (▪, □), mitochondria from the tadpoles at metamorphic climax with and without cycloheximide-pretreatment, respectively.

tadpoles treated with cycloheximide at both stages, premetamorphic stage and metamorphic climax (fig.1). The decrease in the activity of mitochondrial protein synthesis indicates that cycloheximide given to tadpoles depletes proteins which are made in the cytoplasm and needed for the activity of mitochondrial protein synthesis. The mitochondria isolated from tadpoles at metamorphic climax when the mitochondrial biogenesis is markedly promoted, have shown unexpectedly lower incorporating activity than those at pre-metamorphosis. This low incorporating activity may be resulted from that the tadpole at metamorphic climax has a large pool of leucine as noted in [23].

We determined the radioactivity incorporated into cytochrome *c* oxidase to examine whether cycloheximide given to tadpole is repressive for the synthesis in vitro of all the mitochondrial proteins. The effect of the leucine pool size on the incorporating activity into cytochrome *c* oxidase was eliminated by taking the relative rate of synthesis; the ratio (*B*/*A*) of the radioactivity incorporated into cytochrome *c* oxidase (*B*) to the radioactivity incorporated into the total mitochondrial protein (*A*). The relative rate of cytochrome *c* oxidase synthesis is found to be ~6-times higher in the tadpoles at metamorphic climax than at pre-metamorphic stage (table 1), inconsistent with the finding showing that the synthesis of cytochrome *c* oxidase is specifically elevated in the liver of tadpole undergoing metamorphosis [16]. However, it is

Table 1

Relative rate of synthesis of cytochrome *c* oxidase in the isolated mitochondria from tadpoles pretreated with cycloheximide

	cpm incorporated/mg of: mitochondrial protein (*A*)		cytochrome *c* oxidase (*B*)		Relative rate of synthesis of cytochrome *c* oxidase ($B/A \times 10^2$)	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
Premetamorphic stage	7770	1530	13	305	0.17	19.9
Metamorphic climax	5380	1040	63	486	1.17	46.7

Tadpoles at both pre-metamorphic stage and metamorphic climax were injected with cycloheximide. Control tadpoles at each stage with 0.75% saline solution. The mitochondria were incubated with L-[4,5-^{3}H]leucine in the presence of α-ketoglutarate and cycloheximide. Radioactivities incorporated into the cytochrome *c* oxidase and the total mitochondrial protein were determined as in section 2

characteristic that the mitochondria isolated from tadpoles pre-treated with cycloheximide have an extremely high incorporating activity into cytochrome *c* oxidase, whereas they have a lowered incorporating activity into the total mitochondrial protein. The relative rate of synthesis of cytochrome *c* oxidase in the mitochondria from tadpoles pretreated with cycloheximide is 117-fold of that from untreated tadpole at pre-metamorphic stage and 40-fold at metamorphic climax.

Since the antibody used in this study was prepared against the holocytochrome *c* oxidase purified from the frog liver, it is considered (as in [24–26]), that the precipitated cytochrome *c* oxidase is a holoenzyme assembled with de novo-synthesized mitochondrial subunits and cytoplasmically made subunits. Consequently, the high radioactivity found in the immunochemically precipitated cytochrome *c* oxidase implies that the mitochondria from cycloheximide-pretreated tadpole have a sufficient amount of cytoplasmically made subunits with which de novo-synthesized mitochondrial subunits associate. In other words, this means that the synthesis of cytochrome *c* oxidase is specifically regulated by the cytoplasmically made subunits in a different way from that of the other mitochondrial proteins. An elevated amount of cytochrome *c* oxidase was reported in *Neurospora carassa* after treatment with cycloheximide, being ascribed to decrease of mitochondrial protease [27].

REFERENCES

[1] Galper, J.B. and Darnell, J.E. (1971) J. Mol. Biol. 57, 363–367.
[2] Coote, J.L. and Work, T.S. (1971) Eur. J. Biochem. 23, 564–574.
[3] Tzagoloff, A., Rubin, M.S. and Sierra, M.F. (1973) Biochim. Biophys. Acta 301, 71–104.
[4] Schatz, G. and Mason, T.L. (1974) Annu. Rev. Biochem. 43, 51–87.
[5] Tzagoloff, A. and Macino, G. (1979) Annu. Rev. Biochem. 48, 419–441.
[6] Poyton, R.O. (1980) Curr. Top. Cell. Reg. 17, 231–295.
[7] Weisse, H., Sebald, W. and Bücher, Th. (1971) Eur. J. Biochem. 22, 19–26.
[8] Mason, T.L., Poyton, R.O., Wharton, D.C. and Schatz, G. (1973) J. Biol. Chem. 248, 1346–1354.
[9] Rubin, M.S. and Tzagoloff, A. (1973) J. Biol. Chem. 248, 4269–4274.
[10] Werner, S. (1977) Eur. J. Biochem. 79, 103–110.
[11] Malmström, B.G. (1979) Biochim. Biophys. Acta 541, 281–301.
[12] Merle, P. and Kadenbach, B. (1980) Eur. J. Biochem. 105, 499–507.
[13] Goto, Y., Ohki, Y., Shimizu, J. and Shukuya, R. (1981) Biochim. Biophys. Acta 657, 383–389.
[14] Ohki, Y., Goto, Y. and Shukuya, R. (1981) Biochim. Biophys. Acta 661, 230–234.
[15] Goto, Y., Amuro, N., Ohki, Y. and Shukuya, R. (1982) Comp. Biochem. Physiol. 73B, 637–640.
[16] Goto, Y., Amuro, N. and Shukuya, R. (1982) Biochim. Biophys. Acta, in press.
[17] Taylor, A.C. and Kollros, J.J. (1946) Anat. Rec. 94, 7–23.
[18] White, B.A. and Nicoll, C.S. (1981) in: Metamorphosis (Gilbert, L.I. and Frieden, E. eds) pp. 363–366, Plenum, London, New York.
[19] Bustamante, E., Soper, J.W. and Pedersen, P.L. (1977) Anal. Biochem. 80, 401–408.
[20] Kessler, S.W. (1975) J. Immunol. 115, 1617–1624.
[21] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[22] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[23] Smith-Gill, S.L. and Carver, V. (1981) in: Metamorphosis (Gilbert, L.I. and Frieden, E. eds) Plenum, London, New York.
[24] Bertrand, H. and Werner, S. (1977) Eur. J. Biochem. 79, 599–606.
[25] Bertrand, H. and Werner, S. (1979) Eur. J. Biochem. 98, 9–18.
[26] Brambl, R. (1980) J. Biol. Chem. 255, 7673–7680.
[27] Kumar, C.C. and Padmanabam, G. (1981) Biochem. Biophys. Res. Commun. 100, 576–583.