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IN VITRO SYNTHESIS OF ADRENODOXIN AND ADRENODOXIN REDUCTASE: EXISTENCE OF A PUTATIVE LARGE PRECURSOR FORM OF ADRENODOXIN

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SUMMARY: Cytoplasmic free and bound polysomes were isolated from bovine adrenal cortex, and used to program in vitro protein synthesis in rat liver cell sap and wheat germ lysate systems. Synthesis of adrenodoxin(Ad) and adrenodoxin reductase(AdR) in the cell-free systems was determined by immunoprecipitation using monospecific antibodies, and the sizes of the in vitro products were analyzed by SDS-polyacrylamide gel electrophoresis. Ad was synthesized by both free and bound polysomes as a putative large precursor having molecular weight of approximately 20,000 daltons, which was processed to mature size Ad (MW 12,000 daltons) by in vitro incubation with adrenal cortex mitochondria. On the other hand, AdR was synthesized only by free polysomes apparently as the mature size product.

Mitochondrial matrix proteins are coded for by nuclear genes, and synthesized on cytoplasmic ribosomes (1). Therefore, they must be translocated across the outer and inner membranes of mitochondria in order to reach their ultimate destination in the organelles. It is not yet clearly understood, however, which types of cytoplasmic ribosomes, free or membrane-bound, synthesize mitochondrial matrix proteins in mammalian cells, and how newly synthesized proteins are translocated from their cytoplasmic sites of synthesis to mitochondrial matrix compartment. Recently, a post-translational mechanism involving extra-mitochondrial precursors has been suggested for the import of proteins into mitochondria (2-4). Some of the extra-mitochondrial precursors were found to be larger than the mature proteins (4-11), while such does not seem to be the case for some others (12-15).

We studied (16) the site of synthesis of adrenodoxin (Ad) and adrenodoxin reductase (AdR), two matrix proteins of adrenal cortex mitochondria

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(17-19), by examining the presence of their nascent peptides on cytoplasmic free and bound ribosomes prepared from bovine adrenal cortex, and we found that Ad nascent peptides were associated with both free and bound ribosomes, whereas AdR nascent peptides were associated only with free ribosomes. In this communication, we report *in vitro* synthesis of Ad and AdR by free and bound polysomes isolated from bovine adrenal cortex. We found that Ad was synthesized by both free and bound polysomes as a putative large precursor, whereas AdR was synthesized by free polysomes apparently as a mature size product.

MATERIALS AND METHODS:

Free and bound polysomes were prepared from bovine adrenal cortex by a procedure (20) modified from a method described by Chyn, *et al*. (21). Rat liver high speed supernatant and wheat germ lysates supernatant were prepared according to the methods of Chyn, *et al*. (21) and Roberts and Peterson (22), respectively. Cell-free protein synthesis programmed by bovine adrenal cortex polysomes was performed in 500 µl translation mixture containing; 28 mM Hepes buffer (pH 7.6), 110 mM potassium acetate, 4 mM magnesium acetate, 3.2 mM DTT, 1 mM ATP, 0.4 mM GTP, 8 mM phosphocreatine, 160 µg creatine kinase, 100 µg Aprotinin, 10 µg Pepstatin, 10 µg Leupeptin, 40 µM each of all essential amino acids except leucine, 40 µCi L-[4,5-^{3}H] leucine (136 Ci/mmole), 10 A_{260} polysomes, and 200 µl of rat liver supernatant or wheat germ lysates supernatant. Protein synthesis was carried out at 30°C for 90 min, and terminated by adding an equal volume of cold Tris-HCl buffer (pH 7.6) containing 1 % Triton X-100, 1 % sodium deoxycholate, 0.2 % SDS, 1.8 % NaCl, and 5 mM L-leucine.

Rabbit antibodies to Ad and AdR, both purified from bovine adrenal cortex, were prepared and characterized as described earlier (23). Ad and AdR peptides were isolated from *in vitro* translation products by immunoadsorption method (24) with some modifications. Processing of *in vitro* synthesized Ad precursor by adrenal cortex mitochondria in a cell-free system was carried out according to Maccecchini *et al*. (7) with some modifications.

In vitro-synthesized Ad and AdR isolated by immunoadsorption were analyzed by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (25). 15 % and 10 % separation gels were used in the analysis of Ad and AdR, respectively. After the electrophoresis, the gel was cut into 2 mm slices, and the slices were dissolved in NCS solubilizer, and the radioactivity was counted in toluene-Triton X-100-PPO scintillant.

RESULTS:

The *in vitro* synthesis of Ad and AdR was detected by immunoprecipitation from total translation products programmed by free and bound polysomes. Ad antibodies immunoprecipitated about 0.3 % each of total translation products programmed by free and bound polysomes in rat liver cell sap system.

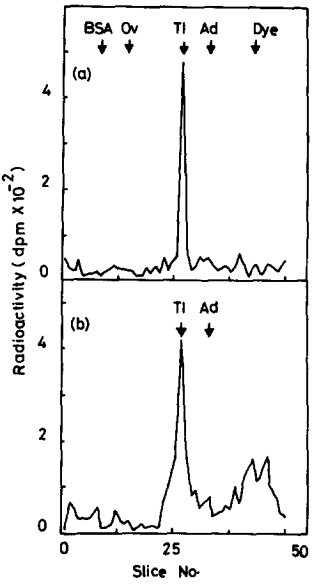

Fig. 1, SDS-Polyacrylamide Gel Electrophoretic Analysis of Ad Peptides Synthesized by Free (a) and Bound (b) Polysomes in Wheat Germ Lysate System.
Ad peptides were isolated from in vitro translation products by immunoadsorption using Protein A-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis as described in MATERIALS AND METHODS. Molecular weight markers were ; bovine serum albumin (BSA, 67,000), ovalbumin (Ov, 45,000), trypsin inhibitor (TI, 20,000), and adrenodoxin (Ad, 12,000).

On the other hand, AdR antibodies immunoprecipitated about 0.2 % of total translation products programmed by free polysomes, whereas negligible radioactivity was immunoprecipitated from those of bound polysomes.

The sizes of in vitro synthesized Ad and AdR peptides, isolated by immunoadsorption using Protein A-Sepharose, were examined by SDS-polyacrylamide gel electrophoresis. The peptides immunoadsorbed to Ad antibodies from total translation products programmed by either free and bound polysomes in rat liver cell sap system showed two radioactivity peaks, one corresponding to a molecular weight of about 20,000 daltons and another to that of mature Ad (12,000 daltons). When free and bound polysomes were read out in wheat germ lysate system, however, the products immunoadsorbed to Ad antibodies showed only a single component of polypeptide having a molecular weight of 20,000 daltons (Fig. 1). When cold Ad was added to the translation products before addition of Ad antibodies, the radioactivity

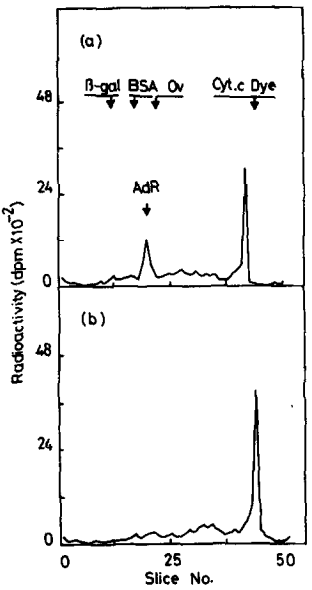

Fig. 2, SDS-Polyacrylamide Gel Electrophoretic Analysis of AdR Peptides Synthesized by Free (a) and Bound (b) Polysomes in Rat Liver Cell Sap System. AdR peptides were isolated by immunoadsorption using Protein A-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis as described in MATERIALS AND METHODS. "AdR" in the figure denotes the position of authentic AdR as determined by staining the gel for protein. Molecular weight markers were the same as in the legend to Fig. 1 except β-galactosidase (β-gal, 130,000) was also used.

peak at 20,000 dalton molecular weight diminished (data not shown), indicating that the large in vitro product competed with Ad for immunoreaction with the antibodies.

In the case of AdR, a radioactivity peak corresponding to the mature size of the enzyme appeared when the products immunoadsorbed to AdR antibodies from the translation mixture programmed by free polysomes in rat liver cell sap system was analyzed, whereas no radioactivity peak at the corresponding position appeared in the case of bound polysomes (Fig. 2). The radioactivity peak seems to represent AdR peptides, since it was diminished when competing cold AdR was added to the translation products before addition of AdR antibodies (data not shown).

These results demonstrate that both free and bound polysomes synthesize Ad as a large precursor form (pre-adrenodoxin: pAd), whereas AdR is synthesized as the mature size product by free polysomes in cell-free read out

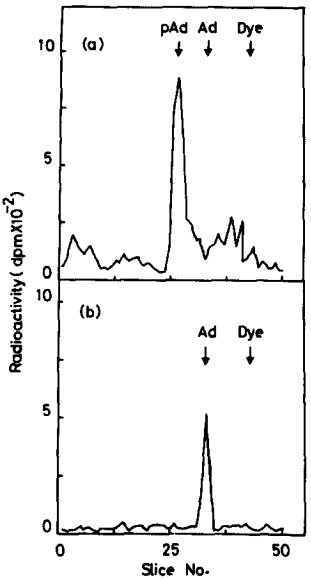

Fig. 3, Processing of pre-Adrenodoxin (pAd) by Bovine Adrenal Cortex Mitochondria in a Cell Free System.

(a) Ad peptides synthesized in vitro by adrenal cortex total polysomes in wheat germ lysate system were isolated by immunoadsorption using Protein A-Sepharose, and analyzed by SDS-polyacrylamide gel electrophoresis. (b) After in vitro translation, polysomes were removed by centrifugation, and the supernatant was incubated with freshly prepared bovine adrenal cortex mitochondria at 28°C for 30 min. Mitochondria were then recovered by centrifugation, and solubilized by 1 % Triton X-100. Ad peptides were isolated from the solubilized mitochondria using Protein A-Sepharose, and analyzed by SDS-polyacrylamide gel electrophoresis.

systems. Since Aprotinin, Leupeptin, and Pepstatin were used during the translation and immunoprecipitation, some proteolytic activities not inhibited by these protease inhibitors were possibly present in rat liver cell sap. which converted pAd to the mature form of Ad.

To confirm the precursor nature of the 20,000 dalton product immunoadsorbed to Ad antibodies, the cell-free translation products were incubated in vitro with bovine adrenal cortex mitochondria. As shown in Fig. 3, the incubation of the translation products with the mitochondria resulted in a complete processing of pAd into the mature form of Ad. The processing of pAd was accompanied by its import into the inside of mitochondria, since the processed product was associated with mitochondria and resistant to the digestion by externally added proteases (data not shown).

DISCUSSION:

Present study confirms the synthesis of Ad as a large precursor form by both free and bound polysomes of bovine adrenal cortex in cell-free systems. On the other hand, AdR was synthesized apparently as the mature size form only by free polysomes. However, we can not rule out the possibility of the synthesis of AdR as a large precursor form since a small difference in the molecular size between the in vitro product and mature AdR may not be detectable by the technique used.

Since pAd competed with authentic Ad for immunoreaction with Ad antibodies, and was processed in vitro to the mature size of Ad by the incubation with adrenal cortex mitochondria, we conclude that Ad is synthesized as a precursor which is about 8,000 daltons larger than the mature form. The existence of the large precursor form of Ad was also confirmed by RNA-dependent protein synthesis in a wheat germ lysate system using bovine adrenal cortex RNA (data not shown). The synthesis of several mitochondrial proteins as large precursors have recently been reported (4-11). Large precursor forms of ferredoxin (26) and the small subunit of ribulose biphosphate carboxylase (27-30), two chloroplast stroma proteins, have also been reported to be the primary translation products.

Since our free and bound polysome preparations were not grossly cross-contaminated (16), the synthesis of Ad by both free and bound polysomes can not be attributed to the contamination of bound polysome fraction by free ribosomes. We have evidence (16) suggesting that the synthesis of Ad peptides starts on free ribosomes, and the ribosomes attach to the membrane of endoplasmic reticulum before the completion of Ad peptides. Our present observations suggest that all mitochondrial matrix proteins are not exclusively synthesized by free ribosomes.

REFERENCES:

1. Schatz,G. and Mason,T.L. (1974) Ann. Rev. Biochem., 43, 51-87.
2. Hallermayer,G., Zimmerman,R. and Neupert,W. (1977) Eur. J. Biochem., 81, 523-532.

3. Harmey,M.A., Hallermayer,G., Korb,H. and Neupert,W. (1977) Eur. J. Biochem., 81, 533-544.
4. Schatz,G. (1979) FEBS Lett., 103, 203-211.
5. Maccecchini,M.L., Rudin,Y., Blobel,G. and Schatz,G. (1979) Proc. Natl. Acad. Sci. U.S.A., 76, 343-347.
6. Cote,C., Solioz,M. and Schatz,G. (1979) J. Biol. Chem., 254, 1437-1439.
7. Maccecchini,M.L., Rudin,Y. and Schatz,G. (1979) J. Biol. Chem., 254, 7468-7471.
8. Shore,G.C., Carignan,P. and Raymond,Y. (1979) J. Biol. Chem., 254, 3141-3144.
9. Harmey,M.A. and Neupert,W. (1979) FEBS Lett., 108, 385-389.
10. Mori,M., Miura,S., Tatibana,M. and Cohen,P.P. (1979) Proc. Natl. Acad. Sci. U.S.A., 76, 5071-5075.
11. Sonderegger,R., Jaussi,R. and Christen,P. (1980) Biochem. Biophys. Res. Commun., 94, 1256-1260.
12. Korb,H. and Neupert,W. (1978) Eur. J. Biochem., 91, 609-620.
13. Zimmermann,R., Paluch,U., Sprinzl,M. and Neupert,W. (1979) Eur. J. Biochem., 99, 247-252.
14. Zimmermann,R., Paluch,U. and Neupert,W. (1979) FEBS Lett., 108, 141-146.
15. Mori,M., Morris,Jr.,S.M. and Cohen,P.P. (1979) Proc. Natl. Acad. Sci. U.S.A., 76, 3179-3183.
16. Nabi,N., Ohashi,M., Ishikawa,T. and Omura,T., in preparation.
17. Omura,T., Sanders,E., Estabrook,R.W., Cooper,D.Y. and Rosenthal,O. (1966) Arch. Biochem. Biophys., 117, 660-673.
18. Nakamura,Y., Otsuka,H. and Tamaoki,B. (1966) Biochim. Biophys. Acta, 122, 34-42.
19. Kimura,T. and Suzuki,K. (1967) J. Biol. Chem., 242, 485-491.
20. Nabi,N. and Omura,T., in preparation.
21. Chyn,T.L., Martonosi,A.N., Morimoto,T. and Sabatini,D.D. (1979) Proc. Natl. Acad. Sci. U.S.A., 76, 1241-1245.
22. Roberts,B.E. and Peterson,B.M. (1973) Proc. Natl. Acad. Sci. U.S.A., 70, 2330-2334.
23. Ohashi,M. and Omura,T. (1978) J. Biochem., 83, 249-260.
24. Erickson,A.H. and Blobel,G. (1979) J. Biol. Chem., 254, 11771-11774.
25. Laemmli,U.K. (1970) Nature, 227, 680-685.
26. Huisman,J.G., Moorman,A.F.M. and Verkley,F.N. (1978) Biochem. Biophys. Res. Commun., 82, 1121-1131.
27. Dobberstein,B., Blobel,G. and Chua,N.H. (1977) Proc. Natl. Acad. Sci. U.S.A., 74, 1082-1085.
28. Cashmore,A.R., Broadhurst,M.K. and Gray,R.E. (1978) Proc. Natl. Acad. Sci. U.S.A., 75, 655-659.
29. Chua,N.H. and Schmidt,G.W. (1978) Proc. Natl. Acad. Sci. U.S.A., 75, 6110-6114.
30. Highfield,P.E. and Ellis,R.J. (1978) Nature, 271, 420-424.