Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 235-240

CELL-FREE SYNTHESIS OF SUCCINATE DEHYDROGENASE AND MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE OF SWEET POTATO

Tsukaho Hattori, Yukimoto Iwasaki, Shigeru Sakajo
and Tadashi Asahi
Laboratory of Biochemistry, Faculty of Agriculture, Nagoya University,
Chikusa, Nagoya 464, JAPAN

Received April 18, 1983

Polyadenylated mRNA was isolated from aged slices of sweet potato root tissue and translated in a wheat germ cell-free system. The synthesis of apoprotein of the flavoprotein subunit of succinate dehydrogenase and two of the subunits of mitochondrial adenosine triphosphatase were detected by indirect immunoprecipitation. The molecular weights of the immunologically identified products were 3,000 and 8,000-9,000 daltons larger than the mature flavoprotein subunit of succinate dehydrogenase and the mature subunits of adenosine triphosphatase, respectively.

Most of the mitochondrial proteins are synthesized in cytoplasm and imported into mitochondria, although the cell organelles have their own genetic and protein-synthetic apparatus (1-3). Yeast, Neurospora and mammalian systems have been mostly employed to determine whether individual mitochondrial proteins are synthesized inside or outside mitochondria. Recently, detailed mechanisms of the import from cytosol into mitochondria of proteins have been extensively studied using yeast (4-10). Concerning higher plant mitochondria, however, little information is available as to the site of synthesis of their proteins as well as their biogenesis.

When slices of sweet potato root tissue are aged under moist conditions, the mitochondria proliferates accompanied by increases in the activities of mitochondrial enzymes (11). Succinate dehydrogenase (SDH) is one of the enzymes, of which the activity increases during aging of the tissue slices (11, 12). The

Abbreviations used: SDH, succinate dehydrogenase; SD_{fp}, flavoprotein subunit of succinate dehydrogenase; F_1ATPase, soluble form of mitochondrial adenosine triphosphatase; SDS, sodium dodecylsulfate; IgG, immunoglobulin G.

0006-291X/83 $1.50

increase can be suppressed by either cycloheximide or chloramphenicol (12). Sweet potato SDH is composed of two different subunits with molecular weights of 26,000 and 65,000: the latter is a flavoprotein (13). The amount of the flavoprotein subunit (SD_{fp}) increases with the increase in activity during aging of the tissue slices and is inhibited by either cycloheximide or chloramphenicol (12). Inhibition by chloramphenicol was unexpected because yeast SDH has been reported to be coded by nuclear DNA and synthesized on cytoplasmic ribosomes (14, 15).

We used a different approach to determine whether sweet potato SDH, particularly the SD_{fp}, is synthesized on cytoplasmic ribosomes like the yeast enzyme, in other words, whether the effect of chloramphenicol really indicates regulation of the synthesis or import into the mitochondria of the enzyme by a mitochondrially produced protein(s). This communication reports the synthesis of SD_{fp} with a wheat germ cell-free system and poly(A)$^+$RNA from aged slices of sweet potato root tissue, which indicates the synthesis of this subunit on cytoplasmic ribosomes.

All subunits of F_1ATPase have been established to be synthesized on cytoplasmic ribosomes (1), although no evidence for this has been presented for the higher plant enzyme. We found that the subunits of F_1ATPase are also produced in the cell-free system, which indicates that the cytoplasmic origin of mitochondrial proteins is actually synthesized in the cell-free system.

MATERIALS AND METHODS

Plant Material. Roots of sweet potato (Ipomoea batatas Kokei No. 14) harvested in midsummer to autumn were stored at 13-16°C until use. Slices of the parenchymatous tissue were incubated as described elsewhere (12).

Preparation of poly(A)$^+$RNA. About 100 g of the tissue slices incubated for 1 day were homogenized in a medium composed of 150 ml of 0.1 M Tris-HCl buffer (pH 9.0) containing 0.1 M NaCl, 2 mM Na_2EDTA, 2 mM $MgCl_2$, and 1% (w/v) SDS, 150 ml of a mixture of phenol/chloroform/isoamyl alcohol [25/24/1, (v/v/v)] and 30 ml of 2-mercaptoethanol. The aqueous phase was washed twice with the phenol/chloroform/isoamyl alcohol mixture, then RNA was precipitated by adding one third the volume of 1 M NaCl and 2.5-fold the volume of ethanol and then allowing the mixture to stand at -20°C overnight. The precipitate was collected by centrifugation and washed twice with 70% (v/v) ethanol. The washed precipitate was dried and dissolved in 20 ml of 10 mM Tis-HCl buffer (pH 7.5) containing 0.5 M NaCl. The solution was applied to an oligo-dT cellulose (Collaborative Research Inc.) column. After the column had been washed with the same buffer, the bound RNA was eluted with 10 mM Tris-HCl buffer (pH 7.5). The poly(A)$^+$RNA eluted from the column was precipitated with ethanol as described above, and the precipitate was dried and dissolved in a minimum volume of water.

Cell-Free Translation. Wheat germ extract was prepared by the method of Marcu and Dudock (16). The reaction mixture for the translation contained 20 mM Hepes-KOH (pH 7.5), 74 mM KCl, 1 mM $MgCl_2$, 0.6 mM spermidine, 1 mM ATP, 8 mM creatine phosphate, 5 µg/ml creatine kinase, 20 µM GTP, 1 mM dithiothreitol, 50 µM each of 19 amino acids (excluding methionine), 2.5 mCi/ml [^{35}S]methionine (1,100 Ci/mmol, New England Nuclear), and 150 µg/ml the poly(A)$^+$RNA. The translation reaction was carried out at 30°C for 1 h and stopped by adding 10% (w/v) SDS at a final concentration of 2%.

Isolation of Sweet Potato SD_{fp} and F_1ATPase and Preparation of Their Antibodies. SD_{fp} and its antibody were prepared as described before (12). The procedure for purification of F_1ATPase from sweet potato root mitochondria and preparation of its antibody will be described elsewhere (Iwasaki and Asahi, manuscript in preparation). IgG fractions were prepared from anti-SD_{fp}, anti-F_1ATPase and nonimmunized sera as described previously (17).

Indirect immunoprecipitation. Indirect immunoprecipitation of the translation products using protein A-Sepharose CL-4B (Pharmacia) was carried out according to the method of Watanabe and Price (18).

SDS-Polyacrylamide Gel Electrophoresis and Fluorography. The antigen-antibody complex bound to protein A-Sepharose beads was eluted with 30 µl of 0.125 M Tris-HCl buffer (pH 6.8) containing 4% SDS, 4% 2-mercaptoethanol and 40% glycerol. The beads were washed with 30 µl of water, which was then combined with the eluate. The combined solution underwent electrophoresis on a 10% polyacrylamide slab gel containing 0.1% SDS with the discontinuous buffer system as described by Laemmli (19). After electrophoresis, the gel was stained with Coomassie brilliant blue R, destained and then processed for fluorography using EN^{3}HANCE (New England Nuclear). The dried gel was exposed to a medical X-ray film (Fuji RX, Fuji Phto Film, Tokyo) at -80°C.

RESULTS

The activities of mitochondrial enzymes in sweet potato root tissue, including SDH, increase after a lag phase lasting for about 10 h and tend to attain maximal values after 2 days during incubation of the slices (12). Therefore, poly(A)$^+$RNA was isolated from the tissue slices incubated for 1 day, when the activities were markedly increasing.

SD_{fp} was synthesized in the cell-free system with the poly(A)$^+$RNA as mRNA (Fig. 1). Several translation products were immunoreactive to anti-SD_{fp} IgG, but most also reacted to preimmune IgG. Only one translation product was specifically reactive to anti-SD_{fp} IgG. It migrated at a slower rate in the SDS-polyacrylamide gel than the purified SD_{fp}. Its molecular weight was calculated to be approximately 3,000 daltons more than the purified SD_{fp}. To confirm that the product was really SD_{fp}, indirect immunoprecipitation with anti-SD_{fp} IgG was performed in the presence and absence of an excess of purified SD_{fp} (Fig. 2). The SD_{fp} competed immunologically with the translation product.

Sweet potato F_1ATPase is composed of six different subunits (α, β, γ, δ, δ′ and ε) (Iwasaki and Asahi, unpublished data). Two translation products

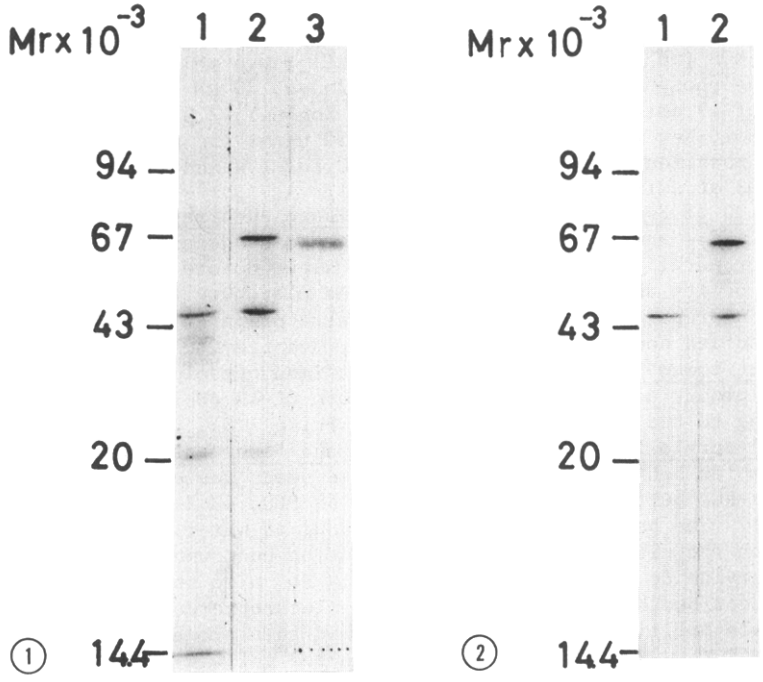

Fig. 1. Synthesis of the apoprotein of SD_{fp} in a cell-free protein-synthesizing system from wheat germ with poly(A)+RNA from sweet potato root tissue as mRNA. Lanes 1 and 2, fluorograms of the immunoprecipitates from 150 µl of the reaction mixture with control and anti-SD_{fp} IgG, respectively; lane 3, electrophorogram of purified SD_{fp} (stained for protein with Coomassie brilliant blue R). Electrophoresis and fluorography were done as described in MATERIALS AND METHODS. The gel was calibrated for molecular weights (Mr) with ^{14}C-labelled marker proteins (Amersham): phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), trypsin inhibitor (20,000) and lactoalbumin (14,400).

Fig. 2. Competition of translation products with the purified SD_{fp} for antibody to SD_{fp}. Twenty micrograms of anti-SD_{fp} IgG was incubated with (lane 1) and without (lane 2) 20 µg of the purified SD_{fp} before being added to the reaction mixture (75 µl). Conditions of electrophoresis and fluorography are given in Fig. 1.

reacted specifically to the antibody to the purified F_1ATPase (Fig. 3). These products had apparent molecular weights of about 63,000 and 61,000 which were larger than those of the α and β subunits (55,000 and 52,000, respectively). We suppose that the translation products were precursors of the α and β subunits, although sufficient evidence is lacking. Our proposition is based on the fact the α and β subunits of yeast F_1ATPase are synthesized as precursors of sizes larger by 6,000 and 2,000 daltons, respectively.

DISCUSSION

The present work shows that SD_{fp} was synthesized in a cell-free system from wheat germ with poly(A)$^+$RNA from sweet potato root tissue as mRNA.

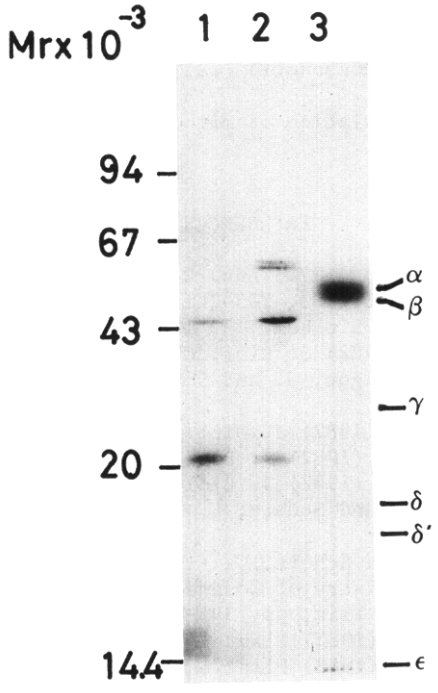

Fig. 3. Synthesis of F_1ATPase subunits in a cell-free protein-synthesizing system programmed with poly(A)$^+$RNA from sweet potato root tissue. Lanes 1 and 2, fluorograms of the immunoprecipitates from 75 µl of the reaction mixture with control and anti-F_1ATPase IgG, respectively; lane 3, electrophorogram of the purified F_1ATPase from sweet potato, stained for protein. α, β, γ, δ, δ' and ε denote the subunits of F_1ATPase. Electrophoresis and fluorography were carried out as described in Fig. 1.

Mitochondrial mRNAs cannot be translated in such a wheat germ system (21, 22). Therefore, we inferred that sweet potato SD_{fp} is synthesized on cytoplasmic ribosomes as reported with yeast SDH (14, 15). Thus the fact that the increase in the amount of SD_{fp} during incubation of slices of sweet potato root tissue is inhibitied by either cycloheximide or chloramphenicol (12) suggests that the synthesis or import into the mitochondria of SD_{fp} is controlled by a mitochondrially produced protein(s).

This study is the first to show that the apoprotein of SD_{fp} is synthesized in a larger precursor like most mitochondrial proteins. Also, in sweet potato, like in yeast, the subunits (probably α and β subunits) of F_1ATPase are synthesized in larger precursors on the cytoplasmic ribosomes. The precursors are 8,000-9,000 daltons larger than the mature polypeptides in the case of sweet potato, although they are 2,000-6,000 daltons larger in yeast. There has been

only one report for watermelon malate dehydrogenase on the *in vitro* synthesis of proteins in higher plant mitochondria (23). Thus, this communication is the second to report *in vitro* translation of mitochondrial proteins using higher plant cells.

REFERENCES

1. Schatz, G. and Mason, T. L. (1974) Ann. Rev. Biochem. 43, 51-87.
2. Schatz, G. (1979) FEBS Lett. 103, 203-211.
3. Ades, Z. (1982) Mol. Cell. Biochem. 43, 113-127.
4. Daum, G. and Schatz, G. (1982) J. Biol. Chem. 257, 13034-13041.
5. Ohashi, A., Gibson, J., Gregor, J. and Schatz, G. (1982) J. Biol. Chem. 257, 13042-13047.
6. Suissa, M. and Schatz, G. (1982) J. Biol. Chem. 257, 13048-13055.
7. Reid, G. A. and Schatz, G. (1982) J. Biol. Chem. 257, 13056-13061.
8. Reid, G. A. and Schatz, G. (1982) J. Biol. Chem. 257, 13062-13067.
9. Reid, G. A., Yonetani, T. and Schatz, G. (1982) J. Biol. Chem. 257, 13068-13074.
10. Daum, G., Gasser, S. M. and Schatz, G. (1982) J. Biol. Chem. 257, 13075-13080.
11. T. Asahi (1978) in Biochemistry of Wounded Plant Tissues, ed. G. Kahl, Walter de Gruyter & Co., Berlin, pp. 391-419.
12. Hattori, T. and Asahi, T. (1982) Plant & Cell Physiol. 23, 515-523.
13. Hattori, T. and Asahi, T. (1982) Plant & Cell Physiol. 23, 525-532.
14. Kim, I. C. and Beattie, D. S. (1973) Eur. J. Biochem. 36, 509-518.
15. Lin, L. F., Kim, I. C. and Beattie, D. S. (1974) Arch. Biochem. Biophys. 160, 458-464.
16. Marcu, K. and Dudock, B. (1974) Nucl. Acids Res. 1, 1385-1397.
17. Maeshima, M. and Asahi T. (1981) J. Biochem. 90, 391-397.
18. Watanabe, A. and Price, C. A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6304-6308.
19. Laemmli, U. K. (1970) Nature 227, 680-685.
20. Maccecchini, M. L., Rudin, Y., Blobel, G. and Schatz, G. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 343-347.
21. Leaver, C. J. and Gray, M. W. (1982) Ann. Rev. Plant Physiol. 33, 373-402.
22. Fox, T. D. and Leaver, C. J. (1982) Cell, 26, 315-323.
23. Gietl, C. and Hock, B. (1982) Plant Physiol. 70, 483-487.