In vitro synthesis of pig kidney general acyl CoA dehydrogenase

ARTICLE *in* BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS · JULY 1986

Impact Factor: 2.3 · DOI: 10.1016/0006-291X(86)90331-1 · Source: PubMed

7 AUTHORS, INCLUDING:

Sandro Ghisla
Universität Konstanz
384 PUBLICATIONS **6,581** CITATIONS

IN VITRO SYNTHESIS OF PIG KIDNEY GENERAL ACYL COA DEHYDROGENASE

Taisuke Inagaki[1,2], Norihiro Tsukagoshi[1], Chikako Ichihara[2], Nobuko Ohishi[2], Shigezo Udaka[1], Sandro Ghisla[3], and Kunio Yagi[2,*]

[1]Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Nagoya 464, Japan

[2]Institute of Applied Biochemistry, Mitake, Gifu 505-01, Japan

[3]Fakultät für Biologie, Universität Konstanz, Konstanz, FRG

In vitro synthesis of general acyl CoA dehydrogenase [EC 1.3.99.3], one of the mitochondrial flavoenzymes, was carried out to elucidate its biosynthetic mechanism. Poly(A)$^+$ RNA isolated from pig kidney was translated *in vitro* using wheat germ lysate system and the synthesized enzyme was immunoprecipitated by the antibody against purified pig kidney general acyl CoA dehydrogenase. The apparent molecular weight of the synthesized protein was estimated to be approximately 1,000 daltons larger than that of the mature enzyme, indicating that general acyl CoA dehydrogenase in pig kidney is synthesized as a precursor with a larger molecular weight.

General acyl CoA dehydrogenase [EC 1.3.99.3] is a mitochondrial flavoenzyme catalyzing the first step of β-oxidation of fatty acids. Many investigations have been made to clarify the mechanism of the enzymatic action. Recently, the enzyme was completely purified from various sources such as pig kidney (1), bovine liver (2), and rat liver (3). Preliminary X-ray data of the crystalline pig liver enzyme were also reported (4). On the other hand, the mechanism of biosynthesis of the enzyme is still equivocal. We therefore attempted *in vitro* synthesis of pig kidney general acyl CoA dehydrogenase. In this communication, we describe that the enzyme in pig kidney is synthesized as a precursor having a larger molecular weight than that of the mature enzyme.

EXPERIMENTAL PROCEDURES

Preparation of rabbit anti-general acyl CoA dehydrogenase antibody. Pig kidney general acyl CoA dehydrogenase was purified by the procedure of Thorpe *et al.* (1). Antibodies were raised against the purified enzyme in a rabbit. The antigen (100 μg), emulsified with an equal volume of complete Freund's adjuvant, was injected subcutaneously into the back of a rabbit at weekly inter-

* To whom correspondence should be addressed.

Abbreviation: SDS, sodium dodecylsulfate.

vals. The antiserum obtained after the fourth booster was used for the following experiments.

Preparation of poly(A)$^+$ RNA. Total RNA's were prepared from freshly excised pig kidney by SDS-phenol extraction (5); this extract was precipitated with 2 M lithium chloride twice and then extracted with chloroform/phenol (1:1, v/v) three times. Poly(A)$^+$ RNA was separated from total RNA's by oligo-dT cellulose (Pharmacia, Uppsala) column chromatography. The poly(A)$^+$ RNA eluted from the column was precipitated by the addition of 0.1-volume of 3 M Na-acetate (pH 5.2) and 2.5-volume of ethanol, and stood at -20°C overnight. The precipitate was dried, dissolved in 50 µl of water, and kept at -80°C until used.

Cell-free translation. Wheat germ lysate was a generous gift from Dr. A. Watanabe (Research Institute of Biochemical Regulation, Nagoya University). The reaction mixture contained 20 mM Hepes-KOH (pH 7.5), a mixture of amino acids (each 50 µM), 1 mM dithiothreitol, 1 mM ATP, 20 µM GTP, 8 mM creatine phosphate, 10 µg/ml of creatine kinase, 90 mM KCl, 2 mM $MgCl_2$, 0.6 mM spermidine, poly(A)$^+$ RNA (200 µg/ml), [^{35}S]methionine (about 200 µCi/ml, Radiochemical Centre, Amersham), and wheat germ lysate in a total volume of 20 µl. The translation was carried out at 30°C for 60 min.

Immunoprecipitation and immunoblotting. Immunoprecipitation of the translation products was performed using protein A-Sepharose (Pharmacia) (6). The translation products were incubated with 1 µl of the antiserum at 37°C for 60 min and further incubated with protein A-Sepharose (50 µl suspension) at 37°C for 60 min. The antigen-antibody complex bound to protein A-Sepharose beads in a mini-column was eluted with 40 µl of 0.125 M Tris-HCl (pH 6.8) containing 4% SDS, 4% 2-mercaptoethanol, and 50% glycerol. The solution containing the immunoprecipitated products was heated at 100°C for 5 min and subjected to 10% SDS-polyacrylamide slab gel electrophoresis according to the method of Laemmli (7). After electrophoresis, the dried gel was stained with Coomassie Brilliant Blue R. For autoradiography, the dried gel was exposed to an X-ray film (Fuji RX, Fuji Photo Film, Tokyo) at -80°C for 7 days.

For immunoblotting, an aliquot of pig kidney extract was subjected to SDS-polyacrylamide slab gel electrophoresis followed by electroblotting onto a nitrocellulose membrane (Bio-Rad Laboratories, Richmond) (8). The blot was incubated with a solution containing the anti-general acyl CoA dehydrogenase antiserum (1:200) at room temperature for 2 hours and immunostained by the avidin/biotin method (9).

Enzyme assay. General acyl CoA dehydrogenase activity was determined by the phenazine methosulfate/dichlorophenolindophenol method using n-octanoyl CoA as substrate (1). The effect of the antibody on the enzymatic activity was determined after the mixture of the enzyme (2 µg) and diluted antiserum solution (diluted with 0.1 M Na-phosphate buffer, pH 7.4, containing 0.15 M NaCl) was incubated at 4°C for 24 hours.

RESULTS AND DISCUSSION

Specificity of the rabbit antibody against the purified general acyl CoA dehydrogenase. Prior to carrying out the *in vitro* biosynthesis experiment, we examined the specificity of the anti-general acyl CoA dehydrogenase antiserum. As shown in Fig. 1A, the activity of the enzyme (2 µg as protein) was completely inhibited by 1 µl of the antiserum, while normal rabbit serum did not affect the activity at the concentrations examined. The specificity of the antiserum was, furthermore, confirmed by immunoblotting. Fig. 1B shows the results of immunoblotting of the crude extract prepared from pig kidney homo-

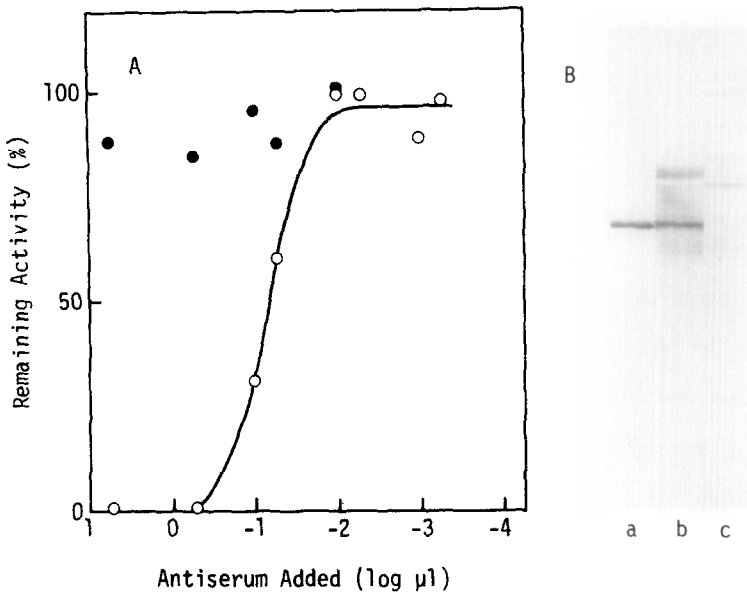

Fig. 1. Inhibition of pig kidney general acyl CoA dehydrogenase by anti-general acyl CoA dehydrogenase antiserum (A) and immunoblot of the crude extract from pig kidney (B). In A, 2 µg of the purified enzyme was incubated with the rabbit antiserum or normal serum as described in the text, and the activity was determined using n-octanoyl CoA as substrate. Open circles, with antiserum; solid circles, with normal serum. In B, pig kidney was homogenized in 5-volume of 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1% SDS using a Polytron at a maximum speed for 30 sec in an ice bath. The homogenate was centrifuged at 20,000 × g and 4°C for 30 min. The supernatant was used as crude extract. An aliquot of the crude extract was subjected to immunoblotting as described in the text. Lane a, purified enzyme (2 µg); lane b, 20 µl of crude extract; lane c, 20 µl of crude extract. In a and b, the blot was incubated with antiserum, and in c, with normal serum.

genate. When the blot was incubated with the antibody followed by immunostaining, two distinct bands were detected (lane b). The molecular weight of the lower band coincided exactly with that of the purified enzyme (42,000) in lane a, while the upper had an approximate molecular weight of 65,000. These bands were, however, not detectable when incubated with normal rabbit serum (lane c). A question arose as to whether the rabbit antibody may cross-react with other acyl CoA dehydrogenases, i.e., short and long-chain acyl CoA dehydrogenases, since those enzymes are thought to closely resemble each other. However, this possibility may be excluded by recent studies; those dehydrogenases were reported to be immunologically different from general acyl CoA dehydrogenase (3), and pig kidney is known to be a rich source of general acyl CoA dehydrogenase (1). The protein having the larger molecular weight (Fig.

1B) was not characterized, but it should be neither short nor long-chain acyl CoA dehydrogenase since the molecular weights of those dehydrogenases have been reported to be in the range from 40,000 to 45,000 (2,3). These results revealed that the antiserum used here is specific to general acyl CoA dehydrogenase but not to other acyl CoA dehydrogenases.

Isolation of poly(A)$^+$ RNA from pig kidney

In order to obtain mRNA having a high translational activity, we attempted three different methods to prepare total RNA's from pig kidney: the phenol/SDS (5), guanidinium/cesium chloride (10), and guanidinium/lithium chloride (11) method. Poly(A)$^+$ RNA was isolated from total RNA's by oligo-dT cellulose column chromatography and translated in a wheat germ lysate cell-free system in the presence of [^{35}S]methionine. As a result, poly(A)$^+$ RNA from RNA's which had been prepared by the phenol/SDS method, followed by chloroform/phenol extraction, was found to have the highest translational activity, which was about five times higher than those of poly(A)$^+$ RNA isolated from the other total RNA's preparations.

In vitro synthesis of general acyl CoA dehydrogenase

The translation products were analyzed as described in Experimental Procedures. As shown in Fig. 2, a main band with an approximate molecular weight of 43,000 was detected by SDS-polyacrylamide gel electrophoresis/autoradiography when the products were immunoprecipitated with the specific antibody. The band was identified as a precursor of general acyl CoA dehydrogenase, because its appearance could be prevented by the addition of an excess amount of the purified enzyme during immunoprecipitation. The apparent molecular weight of the precursor was approximately 1,000 daltons larger than that of the mature enzyme (42,000 daltons). A weak band located above the precursor was shown to be a non-specific one, since it did not display competition with the purified enzyme.

In the immunoprecipitation procedure, about 0.5% of the total labeled methionine incorporated into the peptide products was recovered in the immunoprecipitates. As the autoradiograph showed that the precipitates also con-

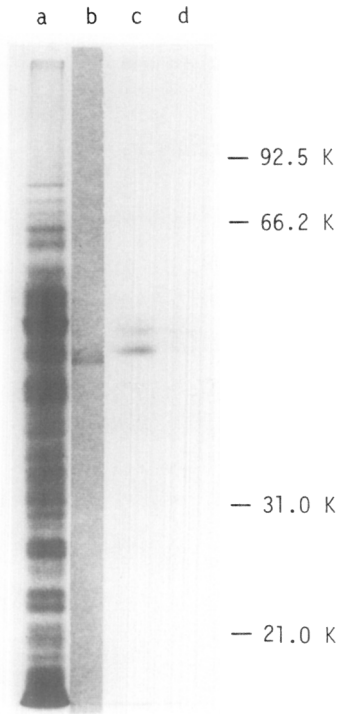

Fig. 2. In vitro synthesis of general acyl CoA dehydrogenase. Lane a, total translation products; lane b, the purified enzyme stained with Coomassie Brilliant Blue R; lane c, immunoprecipitates formed in the absence of the purified enzyme; lane d, immunoprecipitates formed in the presence of the purified enzyme (20 μg).

tained other minor products as well as free labeled methionine (Fig. 2, lane c), the amount of the precursor synthesized under the conditions used here was approximately 0.1-0.2% of the total products. This would suggest that the amount of mRNA coding for general acyl CoA dehydrogenase should be 1/500 to 1/1000 of the total mRNA in pig kidney.

Recent studies have shown that mitochondrial proteins are coded on nuclear genes, translated on cytoplasmic polysomes, and imported post-translationally into mitochondria by an energy-dependent process (12). Furthermore, most of the mitochondrial proteins were shown to be synthesized as a larger-size precursor protein except for proteins located in the outer mitochondrial membrane (12). Mori et al. (13) have demonstrated that medium-chain acyl CoA dehydrogenase in rat liver is synthesized as a larger-size protein. In the present experiments, we have also clearly demonstrated using a specific antibody to

general acyl CoA dehydrogenase that this enzyme in pig kidney is synthesized as a precursor having a larger molecular weight.

REFERENCES

1. Thorpe, C., Matthews, R. G., and Williams, C. H. Jr. (1979) Biochemistry 18, 331-337.
2. Dommes, V., and Kunau, W.-H. (1984) J. Biol. Chem. 259, 1789-1797.
3. Ikeda, Y., Ikeda, K. O., and Tanaka, K. (1985) J. Biol. Chem. 260, 1311-1325.
4. Kim, J.-J. P., Vollmer, S. H., and Frerman, F. E. (1984) J. Biol. Chem. 259, 3318-3319.
5. Palmiter, R. D. (1974) Biochemistry 17, 3606-3615.
6. Watanabe, A., and Price, C. A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6304-6308.
7. Laemmli, O. K. (1970) Nature 277, 680-685.
8. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354.
9. Hsu, S. M., Raine, L., and Fanger, H. (1981) Am. J. Clin. Pathol. 75, 347-348.
10. Glišin, V., Crkvenjakov, R., and Byus, C. (1974) Biochemistry 13, 2633-2637.
11. Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M., and Darnell, J. E. Jr. (1981) Cell 23, 731-739.
12. Hay, R., Böhni, P., and Gasser, S. (1984) Biochim. Biophys. Acta 779, 65-87.
13. Mori, M., Matsue, H., Miura, S., Tatibana, M., and Hashimoto, T. (1985) Eur. J. Biochem. 149, 181-186.