First publ. in: Flavins and Flavoproteins, Proceedings of the 9th Internat. Symposium. Berlin: de Gruyter, 1987, pp. 491-500

BIOSYNTHESIS OF FLAVOPROTEINS

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Introduction

Although many investigations have been made to clarify the mechanism of reaction of flavoproteins, their biosynthesis, especially the mechanism of the incorporation of flavin into covalently bound flavoproteins, has remained to be elucidated. We intended to address this problem by using techniques such as in vitro protein synthesis and molecular cloning. Flavoproteins studied here were mitochondrial general acyl CoA dehydrogenase (GAD) and electron transfer flavoprotein (ETF), peroxisomal D-amino acid oxidase (DAO), and microsomal L-gulono-γ-lactone oxidase (GLO). Although experiments with these enzymes are still in progress in our laboratories, some useful results for a better understanding of the problem have already been obtained. This paper reports our initial findings.

Results and Discussion

Specificity of rabbit antibodies against flavoproteins

In the present study, antibodies against flavoproteins were used for detection of synthesized proteins and screening of the cDNA's. Thus, we examined the specificity of the antibodies prior to those experiments. Immunoblotting was carried out using the crude extract prepared from the homogenate, as well as mitochondrial and microsomal fractions, of kidney or liver. In the experiment using antisera against GAD, ETF, DAO, and GLO, the main band which was visualized on the blot by the peroxidase method was shown to coincide exactly with that of the respective purified flavoprotein (1-5). This indicates that the antibodies used here are specific for each flavoprotein.

In vitro synthesis of flavoproteins

For the *in vitro* synthesis of flavoproteins, total RNA's of pig kidney and liver were prepared by SDS-phenol extraction (6) and those of rat liver were prepared by guanidinium/cesium chloride extraction (7). Poly(A)$^+$RNA was separated from total RNA's by oligo-dT cellulose column chromatography.

Cell-free translation in a wheat germ extract or rabbit reticulocyte lysate system was carried out at 30°C for 60 min. Immunoprecipitation of the translational products was performed using protein A-Sepharose (8). The solution containing the immunoprecipitated products was heated at 100°C for 5 min and subjected to 10% SDS-PAGE according to the method of Laemmli (9). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R and then subjected to autoradiography or fluorography.

When translational products in the wheat germ extract system were immunoprecipitated with anti-GAD antiserum, and analyzed by SDS-PAGE/autoradiography, a main band with an approximate molecular weight of 43K was detectable, as shown in Fig. 1. This band was identified immunologically as GAD, since its appearance could be prevented by the addition of an excess

amount of the purified enzyme during immunoprecipitation. This indicates that GAD in pig kidney is synthesized as a precursor having a larger molecular weight (10).

Figure 2 shows SDS-PAGE/fluorography of GLO synthesized in the rabbit reticulocyte lysate system (11). The apparent molecular weight was estimated to be 51K, indicating that GLO was synthesized with the same molecular weight as the mature enzyme (5).

We also attempted *in vitro* synthesis of DAO and ETF using the wheat germ extract system. However, no bands corresponding to these proteins were detectable. On the other hand,

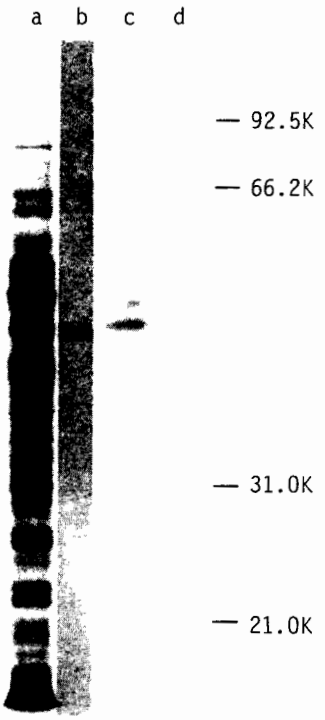

Fig. 1. *In vitro* synthesis of general acyl CoA dehydrogenase. Lane a, total translation products; b, the purified enzyme stained with Coomassie Brilliant Blue R; c, immunoprecipitates formed in the absence of the purified enzyme; d, immunoprecipitates formed in the presence of the purified enzyme.

when the rabbit reticulocyte lysate system was used for translation, a faint band with a molecular weight of 38K corresponding to DAO was detected (12). The appearance of this band was prevented by the addition of an excess amount of the purified enzyme, indicating that the synthesized protein is indeed DAO and undergoes little, if any, processing once initially synthesized. Thus, the information directing DAO to peroxisomes is likely to reside somewhere in its primary structure. Fukui et al. (13) also reported a similar result for the in vitro synthesis of pig kidney DAO.

The band corresponding to ETF was scarcely detectable.

Effect of methylmercury hydroxide on the biosynthesis of DAO and ETF

Payvar and Schimke (14) have reported that treatment of conalbumin mRNA with the nucleic acid denaturant methylmercury hydroxide prior to translation improved the translational efficiency. We, therefore, examined the effect of the reagent on the translational activity of pig kidney and liver poly(A)$^+$-RNA in order to enhance the translational yield. As shown in Fig. 3, translational activity of pig kidney poly(A)$^+$RNA was

Fig. 2. In vitro synthesis of L-gulono-γ-lactone oxidase. Lane a, immunoprecipitates formed with anti-GLO antiserum; b, immunoprecipitates formed with normal rabbit serum.

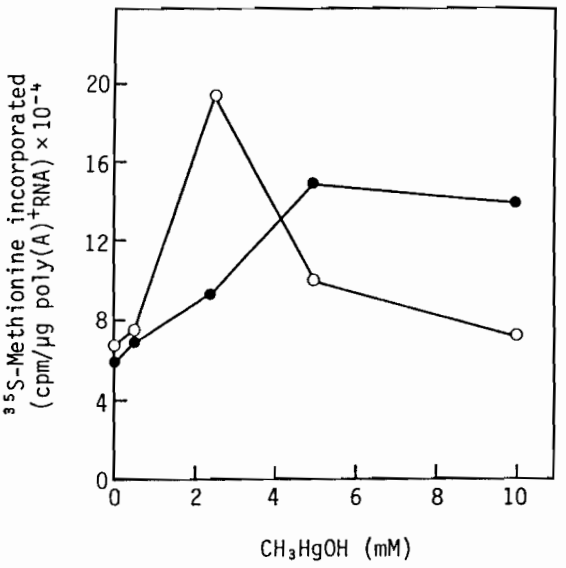

Fig. 3. Effect of methylmercury hydroxide on the translational activity of pig kidney and liver poly(A)$^+$RNA. The activity was assayed with the rabbit reticulocyte lysate. Open circles, pig kidney poly(A)$^+$RNA; closed circles, pig liver poly(A)$^+$RNA.

increased about 3-fold at 2.5 mM and the activity of pig liver poly(A)$^+$RNA was increased 2-fold at 5 mM.

After pig kidney poly(A)$^+$RNA was treated with 2.5 mM methylmercury hydroxide, it was incubated in the rabbit reticulocyte lysate system; and the translational products were immunoprecipitated with anti-DAO antiserum. The immunoprecipitates were analyzed by SDS-PAGE/fluorography. The band of DAO became much more distinct by this treatment (12).

Immunoprecipitates obtained with anti-ETF antiserum from the total translational products in the rabbit reticulocyte lysate system after the treatment of pig liver poly(A)$^+$RNA with methylmercury hydroxide were also analyzed by SDS-PAGE/fluorography. As shown in Fig. 4, lane b, three main bands were detected and their molecular weights were estimated to be 37K, 31K, and 29K, respectively. The bands became more distinct when the poly(A)$^+$RNA was denatured with higher concentrations

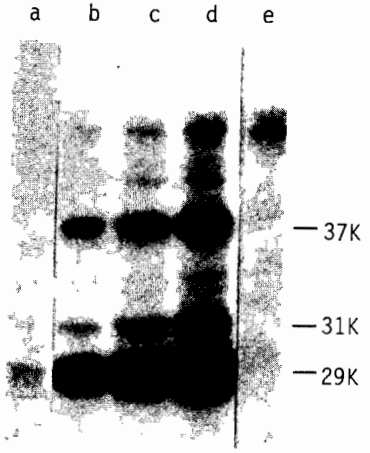

Fig. 4. Effect of methylmercury hydroxide on the production of electron transfer flavoprotein directed in rabbit reticulocyte lysate by poly(A)$^+$RNA from pig liver. Translation was performed in the presence of 0 (a), 0.5 (b), 2.5 (c), and 5 mM (d) methylmercury hydroxide. In lane e, the translation was performed in the presence of 2.5 mM methylmercury hydroxide and immunoprecipitation was carried out in the presence of the purified ETF.

of methylmercury hydroxide (lanes c and d). It was noted that the appearance of these three bands was prevented by the addition of an excess amount of the purified ETF, indicating that all these proteins synthesized were immunologically assigned to ETF. From these results we assume that the synthesized protein having a molecular weight of 37K would be a precursor of the α-subunit (mature protein: ∿34K) and that both or one of the two proteins synthesized having a molecular weight of 31K or 29K would be responsible to the β-subunit (mature protein: ∿27-31K). Ikeda et al. (15) reported on rat liver ETF that the α-subunit was synthesized as a precursor and the β-subunit as the mature protein.

The presently observed enhancement of the synthesis of ETF by treatment of poly(A)$^+$RNA with methylmercury hydroxide could be ascribed to the structure of the mRNA in its isolated form or to its aggregation during preparation, as in the case of

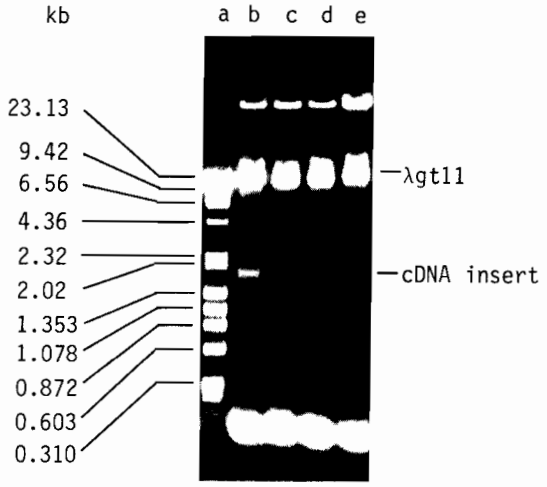

Fig. 5. Agarose gel electrophoresis of λ-GAD 1, 2, and 3 after digestion with EcoRI. Lane a, marker DNA; b, λ-GAD 1; c, λ-GAD 2; d, λ-GAD 3; e, λgt11 alone.

DAO (12,16). This seems not to be the case for GAD, since the synthesis of GAD was not enhanced by the treatment with this reagent at 2.5 mM.

Isolation of the cDNA clones for GAD and DAO

The cDNA clones for GAD and DAO were isolated by immunoscreening a rat liver cDNA library in λgt11, in which antisera against the respective flavoproteins were used according to the method of Young and Davis (17). Immunopositive clones were visualized by the horseradish peroxidase method using goat anti-rabbit IgG-horseradish peroxidase conjugates.

Three positive clones for GAD were obtained after the third immunoscreening. In order to determine the insert sizes of the cDNA clones, the phage DNA's were digested with EcoRI and subjected to 1% agarose gel electrophoresis. From this, the sizes of the inserts were estimated to be 1.5-1.6 kb (Fig. 5).

For the analysis of the fusion protein, E. coil Y1090 was infected with plaque-purified immunopositive phage and induced with 10 mM isopropylthiogalactoside for fusion protein produc-

tion. Proteins extracted from bacteria were subjected to SDS-PAGE. After electrophoresis, the proteins on the gel was transferred to a nitrocellulose membrane and the blot was subjected to immunostaining. Fusion proteins of approximately 155-160K produced in the bacteria were detected on the immunoblots. As the fusion protein consists of GAD and β-galactosidase (114K), the net molecular weight of GAD was estimated to be 40-50K. This indicates that the cDNA clones have presumably a full length of the GAD structure gene, because the molecular weight of the precursor was shown to be approximately 43K (see Fig. 1).

By the same immunoscreening procedure, we isolated two positive clones for DAO. Immunoblotting analysis showed that the cDNA's having 1.65-1.7 kb have only a partial structure gene for DAO followed by a relatively long-tailed 3' non-coding region of 1.3-1.4 kb.

In Table 1, the results of the present study are summarized.

Table 1. In vitro synthesis of flavoproteins and molecular cloning of their cDNA's

	Molecular weight		Translation		Enhancement with methyl-mercury hydroxide	Size of cDNA (kb)
	Mature	Synthesized	Wheat germ	Rabbit reticulocyte		
GAD	42K	43K	+	+	No	1.6
ETF	34K	37K	-	+	Yes	ND
	27-31K	27,31K	-	+	Yes	
DAO	38K	38K	-	+	Yes	1.65-1.7 (partial) 3.0-3.5 (putatively native form)
GLO	51K	51K	ND	+	ND	ND

ND, not determined.

GAD was synthesized as a precursor protein having a slightly larger molecular weight than that of the mature form and the size of the cDNA was 1.6 kb. ETF was shown to be quite different in that its α-subunit was synthesized as a protein having a larger molecular weight, whereas its β-subunit was synthesized as protein(s) having the same molecular weight as the mature protein. Furthermore, the biosynthetic efficiency was affected markedly by denaturation of the mRNA. DAO was synthesized as a protein having the same molecular weight as that of the mature protein and its biosynthesis was also enhanced by denaturation of the mRNA, which was found to have a long-tailed 3' non-coding region. GLO was synthesized as a protein having the same molecular weight as that of the mature enzyme.

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