FAD-dependent Regulation of Transcription, Translation, Post-translational Processing, and Post-processing Stability of Various Mitochondrial Acyl-CoA Dehydrogenases and of Electron Transfer Flavoprotein and the Site of Holoenzyme Formation*

(Received for publication, March 30, 1992)

Masayoshi Nagao and Kay Tanaka‡

From the Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

The most prominent biochemical consequence of riboflavin deficiency in rats is a drastic decrease in various acyl-CoA dehydrogenase activities, especially that of short chain and isovaleryl-CoA dehydrogenase (IVD). As a result, oxidation of fatty acids and leucine is severely inhibited. We studied the effects of FAD at various stages of acyl-CoA dehydrogenase biogenesis. Immunoblot revealed severe losses of various acyl-CoA dehydrogenases and electron transfer flavoprotein in riboflavin-deficient rat liver mitochondria. The decreases in IVD and short chain acyl-CoA dehydrogenase were particularly severe, reaching values of 17 and 34% of controls, respectively. With the exception of IVD, the rate of in vitro transcription of the respective genes and the amounts of mRNAs of these flavoproteins in tissues increased 3-8.5-fold over controls. The amount of IVD mRNA and its transcription rate remained unchanged, suggesting that IVD expression is regulated separately from other acyl-CoA dehydrogenases. When riboflavin was depleted, in vitro translation of acyl-CoA dehydrogenase and electron transfer flavoprotein α-subunit mRNAs was moderately inhibited. Translation of non-flavoproteins was also inhibited. The stability of precursor acyl-CoA dehydrogenases and their mitochondrial import/processing were unaffected. However, mature acyl-CoA dehydrogenases degraded markedly faster in deficient mitochondria than in controls. Regardless of whether precursors were translated under riboflavin-depleted or riboflavin replete conditions, mature acyl-CoA dehydrogenases survived well when imported into normal mitochondria but degraded faster when imported into deficient mitochondria. These findings indicate that FAD ligand binds to mature acyl-CoA dehydrogenase inside the mitochondria.

When rats were deprived of riboflavin, the first and most striking effect was the severe impairment of the mitochondrial oxidation of fatty acids and branched chain amino acids. This phenomenon was caused primarily by a drastic decrease in various acyl-CoA dehydrogenase activities (1-5). The loss of acyl-CoA dehydrogenase activities occurred earlier and was more severe than for any other flavin enzymes studied (1, 2, 5). Among various acyl-CoA dehydrogenases, the decreases in short chain acyl-CoA (SCAD) and isovaleryl-CoA dehydrogenase (IVD) activities were the most pronounced (2-5). FAD addition to the mitochondrial homogenates from riboflavin-deficient rat liver restored only 10-25% of the lost activity. Hence, the loss of activity was mainly due to the loss of apoenzymes (2, 3). If the apoenzymes were present, they should have been readily converted to the holoenzymes on FAD addition (6). These observations suggested either that in the absence of FAD-ligand, the synthesis of acyl-CoA dehydrogenases may be inhibited in riboflavin deficiency, or alternatively, that apoenzymes are unstable in the mitochondria.

The findings from the animal experiments are important in view of the recent clinical observations that in tissues of patients with riboflavin-responsive glutaric aciduria type II, various acyl-CoA dehydrogenase activities and their proteins were severely reduced but could be returned to normal levels by treatment with a large amount of riboflavin (7). At present, the biochemical basis of riboflavin-responsive glutaric aciduria II remains unknown. However, the similarity of the findings to those in riboflavin-deficient rats suggested that riboflavin-dependent G11 patients are riboflavin-deficient in spite of their normal food intake. Hence, a riboflavin transport defect or an enhanced loss of riboflavin has been suspected as the likely mechanism for this disease (8).

Currently, five acyl-CoA dehydrogenases are known in mammals (6, 9-12). Three of them are involved in fatty acid oxidation, catalyzing the first reaction in each of the β-oxidation cycles. They are SCAD, medium chain, and long chain acyl-CoA dehydrogenases (6, 9, 10). Two others are IVD (9-11) and 2-methyl-branched chain acyl-CoA dehydrogenase (9, 12), which catalyze the third reaction in the leucine and valine/isoleucine pathways, respectively. Acyl-CoA dehydrogenases are localized in the mitochondrial matrix (9). They all catalyze the α,β-dehydrogenation of acyl-CoA and transfer electrons to electron transfer flavoprotein (ETF). Catalytically, acyl-CoA dehydrogenases differ from each other mainly in the chain length and configuration of their acyl-CoA substrates. Each acyl-CoA dehydrogenase is a tetramer of a 41.7-44.5 kDa subunit that contains 1 mol of non-covalently bound FAD/subunit (6, 11, 12). In addition, glutaryl-CoA dehydrogenase is similar to acyl-CoA dehydrogen-
as in many aspects. However, it differs in that its \( \alpha,\beta\)-dehydrogenation of glutaryl-CoA is coupled to the subsequent decarboxylation of the \( \gamma\)-carboxyl of the diacid semi-ester (13). ETF is a heterodimer, consisting of an \( \alpha\) (\( \alpha\)-ETF) and a \( \beta\)-subunit (\( \beta\)-ETF). The molecular size of \( \alpha\) and \( \beta\)-ETFs are 32 and 27 kDa, respectively (9, 14, 15).

With the exception of 2-methyl branched chain acyl-CoA dehydrogenase and \( \beta\)-ETF, all other acyl-CoA dehydrogenases and \( \alpha\)-ETF cDNAs from both rat and human have been cloned and sequenced (16-19). Sequence comparison of four acyl-CoA dehydrogenases demonstrated that all acyl-CoA dehydrogenases share homologous sequences, belonging to a gene family (17). Each acyl-CoA dehydrogenase is coded in a nuclear gene and is synthesized in the cytosol as a precursor (p) that contains 2.5-3.5-kDa leader peptide as an extension of the amino terminus of the mature enzyme. The acyl-CoA dehydrogenase precursor is imported into the mitochondria where it undergoes proteolytic leader peptide cleavage and tetramer formation (20). \( \alpha\)-ETF is synthesized as a 35-kDa precursor, and \( \beta\)-ETF does not change its size after mitochondrial import (21). It has been suggested that \( \beta\)-ETF binds FAD (22, 23). Currently, the stage at which FAD is incorporated into the acyl-CoA dehydrogenase and ETF molecules is unknown. Also, the effects of FAD on various stages of acyl-CoA dehydrogenase and ETF biosynthesis, including transcription, translation, and post-translational processing, have not been studied.

In this report, we studied the FAD-dependent transcriptional and post-transcriptional regulation of the various acyl-CoA dehydrogenase and ETF genes in vivo and in vitro. The stability of apo- and holoenzyme of various acyl-CoA dehydrogenases and ETF before and after mitochondrial import and the site of holoenzyme formation were also studied.

**EXPERIMENTAL PROCEDURES**

**Preparation of Riboflavin-deficient Rats, Isolation of Mitochondria, and Assay of Acyl-CoA Dehydrogenase Activities—Weaning male Wistar rats, weighing 50-60 g, were obtained from Charles River (Wilmington, MA) and were randomly divided into two groups. The deficient group was fed a riboflavin-deficient diet (ICN Pharmaceuticals, Cleveland, OH) ad libitum. The control group received the same diet, to which 22 mg of riboflavin was added per kg. Rats were housed in wire bottom cages and were maintained on their assigned diet for 6 weeks unless otherwise mentioned. At the end of 6 weeks, rats were sacrificed by a blow to the head, immediately followed by decapitation. The livers were rapidly removed, weighed, and used for further procedures. Mitochondria were isolated from rat livers by the method of Schmitt and Greenwald (24). Acyl-CoA dehydrogenase activities were measured spectrophotometrically in the presence of 0.1 mM FAD as previously described (9).

**Sources of Antibodies and cDNA and Preparation of Probes—** Purification of rat acyl-CoA dehydrogenases (SCAD, medium chain acyl-CoA dehydrogenase, long chain acyl-CoA dehydrogenase, and IVD) (6, 11) and \( \alpha\)-ETF (11), and the preparation of antibodies against these enzymes has previously been reported. Anti-orimthine transcarbamylase antibody was a gift from Dr. F. Kalousek of this department. Sources of various cDNA probes are as follows: rat SCAD (17), medium chain acyl-CoA dehydrogenase (16), long chain acyl-CoA dehydrogenase (17), IVD (17), human \( \alpha\)-subunit of ETF (\( \alpha\)-ETF) (19), and ornithine transcarbamylase (25). For use as probe, cDNAs were radiolabeled with [\( \alpha\]-32P]dCTP (3000 Ci/mmol, Amer-sham Corp.) using the random primer DNA-labeling method (26).

**RNA Preparation and Isolation of Poly(A)* RNA—** Total RNA was prepared from rat liver by the guanidine-isothiocyanate method (27). Poly(A)* RNA was isolated using the PolyATtract mRNA isolation system (Promega Biotec).

**Preparation of FAD-depleted Reticulocyte Lysate Using Apogluco- lase Oxidase (GOD)—** Holo-GOD from Aspergillus niger was purchased from Boehringer Mannheim. Apogluco was prepared from holo-GOD according to the method of Swohoda (28). When stored at 0°C, the apogluco was stable for at least several weeks, and it could be almost fully reactivated by the addition of FAD. For depleting FAD, rabbit reticulocyte lysate (35 µl) was incubated with 5 µl of apogluco (80 µM) at 30°C for 1 h.

**In Vitro Transcription/Translation of Individual Proteins and Total Protein Synthesis—** cDNA inserts, each containing the entire coding region for acyl-CoA dehydrogenases and ETF, were translated in vitro using rabbit reticulocyte lysate translation kit (Promega Biotec). The reaction mixture, containing [\( \alpha\]-methionine labeled pSCAD mRNA, and precursors orimthine transcarbamylase, were sub- cloned into pGEM blue or pBluescript transcription vector. In vivo transcription of cDNAs was carried out using T7 or SP6 RNA polymerase (Promega Biotec). The synthesized mRNA was translated in vitro using the rabbit reticulocyte lysate translation kit (Promega Biotec). The reaction mixture, containing [\( \beta\]-methionine labeled pSCAD mRNA, and appropriately prepared reticulocyte lysate, was incubated for 1 h at 30°C. The translated polypeptides were analyzed, either directly or after immunoprecipitation, by SDS-PAGE on 8% gels to the method previously described (30). The stability of the mature enzymes, the reaction mixture was centrifuged for 5 min at 8000 x g. The isolated mitochondrial pellet was resuspended in 10 µl of HBS buffer and further incubated at 37°C for varying periods of time, and then the samples were treated with 5 µg of trypsin/mg protein for 10 min at 4°C. After trypsin treatment, soybean trypsin inhibitor (8 mg/ml) was added, and the mitochondrial extracts were analyzed with SDS-PAGE and fluorography.

**Dot-blot Hybridization—** Poly(A)* RNA was denatured with 1 ml glyoxal in 10 mM sodium phosphate buffer, pH 6.8, at 50°C for 1 h. The RNAs were prepared by applying the glyoxalated poly(A)* RNAs to a nylon membrane using a microsample filtration manifold (Schleicher & Schuell). The nylon membrane was first baked at 80°C and prehybridized with sonicated/denatured salmon sperm DNA (250 µg/ml) in prehybridization buffer consisting of 50% formamide, 5 X SSC, 50 mM sodium phosphate buffer, pH 6.5, and 1 X Denhardt's solution for 24 h at 45°C. The prepared nylon membrane was then hybridized with an appropriate radiolabeled cDNA. After hybridization, the membrane was washed with four changes of 2 X SSC, 0.1% SDS for 15 min each at room temperature and then with two changes of 0.2 X SSC, 0.1% SDS for 30 min each at 64°C, and was exposed to an x-ray film at -70°C.

**In Vitro Transcription and Use of Nuclei from Rat Liver for Transcription and Analysis of Specific RNA Transcripts—** Nuclei were isolated from rat liver according to the method of Marzluff and Huang (32). The prepared nuclei were suspended in a storage buffer at a concentration of 1-2 x 10⁶ nuclei/ml of buffer (50 mM Tris-HCl, pH 8.0, containing 25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 5.0 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) and were stored at -70°C. The yield of this procedure was approximately 5 x 10⁶ nuclei/g of liver.

For transcription, a suspension of 1 x 10⁶ nuclei in 10 µl was incubated in 100 µl of reaction mixture containing 100 µl of [\( \alpha\]-P]UTP (800 Ci/mmol), 1 µg each of ATP, CTP, and GTP, 240 mM KCl, and 5 mM magnesium acetate at 25°C for 30 min. The reaction was stopped by adding 2 ml of a solution of 1% SDS, 10 mM EDTA, pH 7.0 (32). Radiolabeled RNA was then purified by using a Quick-Spin-Column (Seephiadex G-50, Boehringer). Each species of the prepared RNA was analyzed by hybridization with the cDNA probe specific for each RNA species or with a pool of all cDNA probes (5 µg) that had been bound to nylon membrane (32). Before RNA hybridization, prehybridization was performed at
Riboflavin-dependent Expression of Acyl-CoA Dehydrogenases

42 °C for 12 h in 10 mM Tris-HCl, pH 7.5, containing 50% deionized formamide, 5 × SSC, 0.1% SDS, 1 mM EDTA, 5 ng of poly(A), 5 × Denhardt's solution, and 250 μg/ml of salmon sperm DNA. After prehybridization, [32P]RNA was added in the same buffer (total volume 0.5 ml), and hybridization was carried out at 42 °C for 36 h. The membrane was then washed at 64 °C four times with gentle shaking in 2 × SSC, 0.1% SDS for 15 min and then twice with 0.1 × SSC, 0.1% SDS for 30 min. The amount of hybridized RNA was determined by autoradiography and by punching out the spots and counting them in a liquid scintillation counter.

**Immunoblot Analysis—** Mitochondria were solubilized by boiling for 3 min with a 10-fold volume of buffer containing 4% SDS, 125 mM Tris-HCl, pH 6.8, 20% glycerol, 0.01% bromophenol blue, and 10% 2-mercaptoethanol. Samples were electrophoresed in a 12.5% SDS-polyacrylamide gel with a 4% stacking gel. After electrophoresis, the gel was electroblotted onto an Immobilon-P membrane (Millipore, Bedford, MA), according to the method of Towbin et al. (34). Immunoreactive protein was detected using the Protoblot Western Blot AP system (Promega Biotec). The relative amount of protein bands in the blot was determined densitometrically using a Bioimage system (Millipore). For the determination of each protein in riboflavin-deficient mitochondria, the densities of bands in each of five lanes for different dilutions were compared to the corresponding band from control mitochondria and were expressed in percentage of the respective control. The values of the five bands were then averaged to produce the final data.

**RESULTS**

**Immunoblot Analysis of Various Acyl-CoA Dehydrogenases, ETF, and Ornithine Transcarbamylase in Normal and Riboflavin-deficient Rat Liver Mitochondria—** After being fed a riboflavin-deficient diet for 6 weeks, deficient rats weighed 100–140 g whereas controls weighed 280–340 g. Mitochondria from five normal and five deficient rats were separately pooled and used for enzyme assay and immunoblot analysis. Enzyme assays revealed that various acyl-CoA dehydrogenase activities, particularly those of IVD and SCAD, in the riboflavin-deficient liver mitochondria were severely reduced. The levels of activities/milligram of protein in the deficient rat mitochondria, as expressed in percent of control, were: SCAD (62%), medium chain acyl-CoA dehydrogenase (21.4%), long chain acyl-CoA dehydrogenase (30.0%), and IVD (0.7%). In contrast, the ornithine transcarbamylase activity was essentially the same as that of control.

In order to elucidate whether the decreased acyl-CoA dehydrogenase activities were caused by deficient conversion of apoprotein to holo-protein, or by a decreased amount of apoproteins, we estimated the amount of enzyme proteins in the mitochondria using immunoblot analysis. The amounts of acyl-CoA dehydrogenases and ETF in riboflavin-deficient rat liver mitochondria were all decreased to varying degrees as compared to controls (Table I). The decreases in IVD and SCAD proteins were particularly severe, reaching to 17 and 34% of controls, respectively. The amount of medium chain acyl-CoA dehydrogenase was 58% of control. The decrease of long chain acyl-CoA dehydrogenase and of both subunits of ETF was much less than that of SCAD and IVD. The amount of ornithine transcarbamylase in deficient mitochondria was essentially the same as control.

**Dot-blot Hybridization Analysis of mRNAs for Various Acyl-CoA Dehydrogenases, ETF, and Precursor Ornithine Transcarbamylase in Normal and Riboflavin-deficient Rat Liver Mitochondria—** Because mRNA preparations from six control and nine deficient rats were separately combined. Four serial diluted amounts (1.0, 0.5, 0.25, and 0.125 μg) of each poly(A)+ RNA preparation were applied to nylon membranes using a microsample filtration manifold and were hybridized with a specific radiolabeled cDNA probe (autoradiograph data not shown). Quantitation of each dot by liquid scintillation counting showed that the amounts of pSCAD, precursor medium chain acyl-CoA dehydrogenase, precursor long chain acyl-CoA dehydrogenase, and pα-ETF mRNAs increased 3–8.5-fold over control (Fig. 1). In striking contrast, the amounts of the IVD mRNA in riboflavin-deficient rat liver was essentially the same as control. This was also true of ornithine transcarbamylase mRNA.

In view of the previous paper, reporting significant decrease in acyl-CoA dehydrogenase activities in the early stage of deficiency (35), we examined the early effects of riboflavin deficiency on various mRNAs. We determined the amounts of mRNAs in rat livers during the first 7-day period after riboflavin deprivation. In order to elucidate whether the decreased acyl-CoA dehydrogenase activities were caused by deficient conversion of apoprotein to holo-protein, or by a decreased amount of apoproteins, we estimated the amount of enzyme proteins in the mitochondria using immunoblot analysis. The amounts of acyl-CoA dehydrogenases and ETF in riboflavin-deficient rat liver mitochondria were all decreased to varying degrees as compared to controls (Table I). The decreases in IVD and SCAD proteins were particularly severe, reaching to 17 and 34% of controls, respectively. The amount of medium chain acyl-CoA dehydrogenase was 58% of control. The decrease of long chain acyl-CoA dehydrogenase and of both subunits of ETF was much less than that of SCAD and IVD. The amount of ornithine transcarbamylase in deficient mitochondria was essentially the same as control.

**Dot-blot Hybridization Analysis of mRNAs for Various Acyl-CoA Dehydrogenases, ETF, and Precursor Ornithine Transcarbamylase in Normal and Riboflavin-deficient Rat Liver Mitochondria—** Because mRNA preparations from six control and nine deficient rats were separately combined. Four serial diluted amounts (1.0, 0.5, 0.25, and 0.125 μg) of each poly(A)+ RNA preparation were applied to nylon membranes using a microsample filtration manifold and were hybridized with a specific radiolabeled cDNA probe (autoradiograph data not shown). Quantitation of each dot by liquid scintillation counting showed that the amounts of pSCAD, precursor medium chain acyl-CoA dehydrogenase, precursor long chain acyl-CoA dehydrogenase, and pα-ETF mRNAs increased 3–8.5-fold over control (Fig. 1). In striking contrast, the amounts of the IVD mRNA in riboflavin-deficient rat liver was essentially the same as control. This was also true of ornithine transcarbamylase mRNA.

In view of the previous paper, reporting significant decrease in acyl-CoA dehydrogenase activities in the early stage of deficiency (35), we examined the early effects of riboflavin deficiency on various mRNAs. We determined the amounts of mRNAs in rat livers during the first 7-day period after riboflavin deprivation.
feeding of the riboflavin-deficient diet or of pair-feeding of the supplemented diet was commenced (Fig. 2). Surprisingly, the mRNAs for all four acyl-CoA dehydrogenases, including IVD, increased 2-3-fold over the control as early as the end of the second day on the deficient diet. The values on the fourth and seventh days were somewhat variable. The amount of medium chain acyl-CoA dehydrogenase mRNA further increased to a level of four times greater than that of controls on day 7. The amount of SCAD mRNA remained two to three times greater than that of the controls, whereas long chain acyl-CoA dehydrogenase and IVD mRNAs decreased to 1.4-1.6 times the control value. In contrast, the amount of ornithine transcarbamylase mRNA in riboflavin-deficient rat livers and SCAD mRNA in pair-fed control rat livers remained unchanged during the 7 days.

Effects of FAD on in Vitro Transcription of the Genes for Various Acyl-CoA Dehydrogenases and α-ETF—Nuclei, isolated from three normal and three riboflavin-deficient rat livers, were separately combined, and used for the determination of the relative rate of transcription of various acyl-CoA dehydrogenase and α-ETF genes. The transcription rates of the SCAD, medium chain acyl-CoA dehydrogenase, long chain acyl-CoA dehydrogenase, and α-ETF genes were markedly increased in degrees that well correlated to the changes in the amount of the respective mRNA species in vivo (Table II). The rate of transcription of IVD and ornithine transcarbamylase genes was unaffected by riboflavin deficiency.

Effects of FAD on in Vitro Translation—With the single exception of IVD, the severely decreased amounts of various acyl-CoA dehydrogenase and ETF proteins in the presence of greatly increased amounts of the respective mRNAs could be caused either by the inhibition of translation or post-translational processing, or by decreased stability of the protein products. In preliminary in vitro transcription/translation experiments, we observed that the amount of protein synthesized was variable depending on the batch of reticulocyte lysate used. The addition of 1 mM FAD into reticulocyte lysate generally caused an increase in the amount of protein synthesized, but the degree of increase varied depending on the base line activity. These preliminary results suggested that FAD enhances the translation activity and that a small, varying amount of FAD is present in reticulocyte lysate.

In order to accurately evaluate the in vitro effects of FAD, it was necessary to remove the small amount of endogenous FAD that was present in the rabbit reticulocyte lysate. For this purpose, we chose to use apoGOD. It had previously been shown that stable apoGOD could be prepared, and apoGOD binds FAD with high affinity, rapidly regenerating holo-GOD, and sequestering free FAD (28). First, we preincubated reticulocyte lysate with two different amounts of apoGOD for 1 h at 30 °C. In controls, holo-GOD (10 μM) or dHzO was used in a similar manner. Immediately after the pretreatment of lysate, in vitro transcription of mRNA was initiated by the addition of the in vitro transcribed mRNA, [3H]methionine, and the unlabeled minus-methionine amino acid mixture. The relative amount of each radioactive protein was determined by liquid scintillation. Among the two types of controls, the translation in the holo-GOD controls was slightly more active than in the dHzO controls (Fig. 3A). Particularly, pSCAD mRNA was translated 24% more actively with holo-GOD addition than with dHzO addition. The amounts of pSCAD, pIVD, and precursor ornithine transcarbamylase produced by the lysate in the presence of 10 μM apoGOD were 45–62% less than those synthesized by the lysate in the presence of holo-GOD. The translation activities of the lysate with the addition of 2 μM apoGOD were not as markedly decreased as those with the addition of 10 μM apoGOD (Fig. 3A). We tested the effect of apoGOD concentration up to 50 μM and confirmed that 10 μM of apoGOD was saturating for the sequestering of endogenous FAD in rabbit reticulocyte lysate.
Effects of FAD depletion on the stability of newly synthesized SCAD precursor. The amount of residual pSCADs 2 h present after the termination of translation in the presence of holo- and apoGOD was 47.8 and 68.4% of the amount of translated. This result indicates that the degradation of pSCAD was not accelerated by FAD depletion. Similar results were obtained when other acyl-CoA dehydrogenase precursors were tested.

Effects of FAD on mitochondrial import and processing of pSCAD. We prepared pSCAD in four different translation mixtures. In each experiment, reticulocyte lysate was first preincubated with DHO, holo-, or apoGOD. In the fourth group, 0.5 mM FAD was added to apoGOD (+) lysate at the beginning of the translation reaction. After in vitro translation, pSCAD was incubated with riboflavin-deficient or control mitochondria for 30 min, the reaction mixture was centrifuged, and the amount of mature (m) SCAD in the mitochondrial pellet was determined as described under "Experimental Procedures." In both deficient and control mitochondria, the rate of import/processing of the precursor was similar in all four types of experiments where the same mitochondria were used (Fig. 4).

We then determined more accurately the effects of FAD depletion in the translation mixture on the time course of the pSCAD processing in riboflavin-deficient mitochondria. As shown in Fig. 5, 90% of pSCAD, that was produced in the presence of holo-GOD, was imported by mitochondria and converted to mature SCAD within 30 min. The amount of mSCAD decreased only very gradually and over 70% still remained at 3 h. In contrast, 74 and 78% of pSCAD, which was produced in the presence of apoGOD was detected as mSCAD at 30–60 min, respectively. Thereafter, the amount of mSCAD decreased markedly faster than that produced from the precursor synthesized in the presence of holo-GOD. After 180 min of incubation, only 40% of the initial load of pSCAD produced in the FAD-depleted lysate remained as mSCAD in the mitochondria. This result confirmed that the rate of import/processing of the precursor translated in the presence of holo- and apoGOD was comparable. However, the resulting mSCAD was more stable in the former, suggesting that the small amount of endogenous riboflavin in the rabbit

In order to test the effects of exogenous FAD on translation efficiency, reticulocyte lysate was first preincubated with 10 μM apoGOD for 1 h at 30°C. The translation was started by adding in vitro transcribed mRNA, [35S]methionine, the unlabeled Met-minus amino acid mixture, and increasing concentrations of FAD up to 1.0 mM. The results for pSCAD and precursor ornithine transcarbamylase are shown in Fig. 3B. The FAD addition stimulated the translation of pSCAD and precursor ornithine transcarbamylase mRNAs to similar degrees. For both proteins, the maximal activity was observed with 0.5 mM FAD, with the values approximately 2.5-fold over the basal translation activity. A similar degree of enhancement was also observed in IVD synthesis (data not shown). As has been well recognized, in vitro transcription/translation of CDNA generally produces not only the entire molecule initiated from the initiation ATG codon, but also smaller fragments of the intended protein, which are produced via the translation that is initiated from internal ATG codons. In the present experiment, it is important to note that the intensity of signals of the smaller fragment bands, initiated from internal ATG codons, was also increased by addition of FAD into the translation mixture.

Total cell free protein synthesis, using rat liver mRNA as a template, was stimulated approximately 50% by the addition of 0.5 mM FAD. The amount of albumin synthesized, as measured by immunoprecipitation (20), was increased more than 2-fold by the addition of 0.5 mM FAD.
prepared mitochondria from riboflavin-deficient rats at pSCADs, produced via presence of holo-GOD(+) or apoGOD(+), were incubated with freshly of reticulocyte lysate was taken up by the mitochondria, either independently or through binding to pSCAD.

The Effects of FAD Depletion on the Stability of the Mature SCAD, Medium Chain Acyl-CoA Dehydrogenase, IVD, and α-ETF in Mitochondria—In order to evaluate the effects of exogenous FAD on the stability of mature acyl-CoA dehydrogenases inside the riboflavin-deficient mitochondria, we carried out pulse chase-experiments as described under “Experimental Procedures.” Both mature SCAD and IVD, which were produced from the precursor translated in the presence of apoGOD, degraded faster than the enzymes produced from the precursor translated in the presence of holo-GOD (Fig. 6A). Approximately 80% of mSCAD and mIVD, that was translated in the holo-GOD(+)-lysate, survived after 4 h of incubation at 37 °C, whereas only less than 45% of mSCAD and mIVD, that was translated in the apoGOD(+)-lysate, remained intact. Mature medium chain acyl-CoA dehydrogenase and α-ETF, which were produced from the precursor translated in the presence of apoGOD, similarly degraded faster than the enzymes produced from the precursor translated in the presence of holo-GOD. However, they were somewhat different from SCAD and IVD in that the mature enzyme produced from the precursor which was translated in the holo-GOD(+)-lysate also decayed considerably, and the difference in the degradation rate between FAD-depleted and FAD-replete mature protein was not as great as in the case of SCAD and IVD. (Fig. 6B). In order to examine whether or not the decays of the mature enzymes in the holo-GOD(+)lysate group were due to a low endogenous FAD in the particular batch of the reticulocyte lysate, we preincubated the reticulocyte lysate in the presence of holo-GOD and 0.5 mM FAD. The decay of mature enzymes in this group was not different from that of the holo-GOD(+)lysate group, indicating that mature medium chain acyl-CoA dehydrogenase and ETF are not as stable as mature SCAD and IVD in FAD-replete conditions.

In order to test whether the precipitous loss of mature acyl-CoA dehydrogenases in riboflavin-deficient mitochondria was caused by the instability of apocysl-CoA dehydrogenases in the absence of FAD or by the loss of structural integrity of the deficient mitochondria after prolonged incubation, we performed similar experiments with precursor ornithine transcarbamylase, which does not contain FAD. Precursor ornithine transcarbamylase preparations, that were produced by in vitro transcription/translation under riboflavin-depleted and replete conditions, were incubated with riboflavin-deficient mitochondria. Two mature ornithine transcarbamylase preparations which were processed from these precursor ornithine transcarbamylase preparations survived equally well

with 70% of both remaining after 4 h of incubation (data not shown). These data indicate that the structural integrity of the deficient mitochondria is intact, and confirms that the loss of mature acyl-CoA dehydrogenases in the deficient mi-
Riboflavin-dependent Expression of Acyl-CoA Dehydrogenases

The Site of FAD Attachment in SCAD Biosynthesis—The above experiments suggested that the FAD, that was present in the incubation media for the mitochondrial import experiment, was utilized in the formation of the mature proteins. However, it is not clear whether FAD in the incubation media was imported into mitochondria independently and then bound to the mature apoprotein inside the mitochondria, or imported into mitochondria as a complex with the precursor and was subsequently processed to the mature holoprotein. In order to eliminate the effects of FAD present in the incubation media, two kinds of pSCAD preparations were made. The first preparation was translated in the presence of endogenous FAD in the reticulocyte lysate, and the translation mixture was further incubated with 10 μM apoGOD for 60 min in order to eliminate free FAD [FAD(+)]-pSCAD. The second preparation was translated using the reticulocyte lysate that had been preincubated with 10 μM apoGOD for 60 min [FAD(-)-pSCAD]. Each of the FAD(+) -pSCAD and FAD(-)-pSCAD translation mixtures was incubated with normal and riboflavin-deficient mitochondria. As shown in Fig. 7, two types of mature SCADs, one derived from FAD(+) -pSCAD and the other derived from FAD(-)-pSCAD, survived equally well for 4 h in normal mitochondria. In riboflavin-deficient mitochondria, in contrast, the amounts of both mature proteins markedly decreased with time. These results indicate that incorporation of FAD ligand occurs inside the mitochondria after mature SCAD is produced. Furthermore, these results indicate that FAD is taken up by the mitochondria in its free form independent of pSCAD.

FIG. 7. Experiment exploring the site for FAD binding to apoprotein. In vitro translation of pSCAD mRNA was performed in the presence and absence of 10 μM apoGOD for 1 h and designated as FAD(−) and FAD(+) -precursors, respectively. FAD(+) -precursor was further incubated with 10 μM apoGOD for 1 h in order to remove FAD in the translation media. FAD(−) was incubated for 1 h without further addition. Each translation product was incubated with freshly isolated mitochondria from control or riboflavin-deficient rats for 30 min at 27°C. The stability of mature SCAD was examined as described in Fig. 6. Values were obtained by measuring the radioactivity of each band in SDS-PAGE analysis. The amount of mature SCAD was expressed as a percentage of the amount of mature SCAD that was present inside the mitochondria after trypsin digestion but just prior to shifting the temperature to 37°C (0 min). Symbols are: C—C, FAD(+) -pSCAD in control mitochondria; ———Δ, FAD(−) -pSCAD in control mitochondria; •—•, FAD(+) -pSCAD in riboflavin-deficient mitochondria; and ▲—▲, FAD(−) -pSCAD in riboflavin-deficient mitochondria.

DISCUSSION

The marked decrease in the amounts of various acyl-CoA dehydrogenase and ETF proteins and the presence of a normal amount and activity of ornithine transcarbamylase, a pyridoxal enzyme, in the mitochondria of riboflavin-deficient rat livers indicates that the decrease of various acyl-CoA dehydrogenase proteins was caused by the loss of FAD, the essential prosthetic group. The decrease in the acyl-CoA dehydrogenase activities in riboflavin-deficient rats, measured after the in vitro addition of FAD, was more severe than the decrease in the amount of respective enzyme protein, suggesting that a considerable portion of the residual enzyme proteins may have already degenerated.

Our data indicate that the amounts of various acyl-CoA dehydrogenases and ETF in rat mitochondria are subject to FAD-related control at least at three sites. First, the expression of the three acyl-CoA dehydrogenase (pSCAD, precursor medium chain acyl-CoA dehydrogenase, and precursor long chain acyl-CoA dehydrogenase) and pa-ETF genes, that are involved in the fatty acid oxidation, appears to be commonly regulated by a FAD-related feedback mechanism, producing a markedly increased amount of respective mRNA in FAD deficiency. Of particular interest in this regard is the previous observation by Brady et al. (36) that although it is not a flavoprotein, the amount of carnitine palmitoyltransferase mRNA in riboflavin-deficient rat liver was increased two to three times over the control level. Carnitine palmitoyltransferase initiates mitochondrial fatty acid oxidation by converting extramitochondrial acyl-CoA to acyl-carnitine, which transverses the mitochondrial membrane. Unlike acyl-CoA dehydrogenases and ETF, however, the carnitine palmitoyltransferase activity was also increased two to three times over the control (36). Thus, the data by us and by Brady's group together suggest that the expression of the genes involved in the fatty acid oxidation appear to be coordinately regulated by a common, FAD-related mechanism. At present, the factor that stimulates transcription of these genes has yet to be identified.

The peculiar lack of increase in the amount of IVD mRNA indicated that the expression of the IVD is regulated separately from that of the enzymes related to fatty acid oxidation. As suggested from the enzyme induction study by Freeman et al. (37), it is possible that the expression of the IVD gene is regulated coordinately with other enzymes involved in the leucine oxidation pathway by a factor that is unrelated to FAD. These data suggest that some of the regulatory components of the IVD gene are different from those of other acyl-CoA dehydrogenase genes.

The second stage, at which the synthesis of various acyl-CoA dehydrogenases and α-ETF is regulated, is the translation step. The enhancement by FAD of efficiency of acyl-CoA dehydrogenase-mRNA translation, as well as that of ornithine transcarbamylase and serum albumin mRNAs, indicates that this is a general effect on the translation system per se. As a result, the synthesis of all proteins is decreased in riboflavin deficiency. The decreased protein synthesis may be the cause of the growth retardation in riboflavin-deficient rats. As suggested from the enzyme induction study by Frerman et al. (36), it is possible that the expression of the IVD gene is regulated coordinately with other enzymes involved in the leucine oxidation pathway by a factor that is unrelated to FAD. These data suggest that some of the regulatory components of the IVD gene are different from those of other acyl-CoA dehydrogenase genes.

The peculiar lack of increase in the amount of IVD mRNA indicated that the expression of the IVD is regulated separately from that of the enzymes related to fatty acid oxidation. As suggested from the enzyme induction study by Freeman et al. (37), it is possible that the expression of the IVD gene is regulated coordinately with other enzymes involved in the leucine oxidation pathway by a factor that is unrelated to FAD. These data suggest that some of the regulatory components of the IVD gene are different from those of other acyl-CoA dehydrogenase genes.

The peculiar lack of increase in the amount of IVD mRNA indicated that the expression of the IVD is regulated separately from that of the enzymes related to fatty acid oxidation. As suggested from the enzyme induction study by Freeman et al. (37), it is possible that the expression of the IVD gene is regulated coordinately with other enzymes involved in the leucine oxidation pathway by a factor that is unrelated to FAD. These data suggest that some of the regulatory components of the IVD gene are different from those of other acyl-CoA dehydrogenase genes.
in pyridoxal-deficient rats. The degradation of NAD-dependent enzymes, such as lactic dehydrogenase and glutamic dehydrogenase in the gut of niacin-deficient rats, was also accelerated by the appearance of specific proteinases (39).

ETF contains 1 mol of FAD/dimer. Based on the experiments in which ETF-dimer was cross-linked to an FAD-analog or ETF-Coenzyme-Q oxidoreductase, it has been proposed that FAD is bound to β-ETF (22, 23). The accelerated degradation of both subunits, as observed here, indicates that apoβ-ETF is unstable. It has been known from the studies of genetic α- and β-ETF deficiencies in man that both α- and β-ETFs become labile when they do not form the dimer (21, 40).

Among the three regulatory mechanisms mentioned above, obviously the main factor causing the deficiency of various acyl-CoA dehydrogenases and α-ETF in riboflavin-deficient rats was the instability of apoproteins in the mitochondria. The marked increase of the mRNAs for fatty acid-related enzymes did not fully compensate for the loss of respective proteins that is caused by the two other mechanisms. However, the increased mRNA does appear to have alleviated the loss of proteins to some degree. This can be seen in the difference in the amount of SCAD and IVD proteins in riboflavin-deficient rats. The degree of inhibition of translation and the degree of instability of the two proteins were comparable, but the loss of IVD was considerably more severe than that of SCAD. This difference is probably related to the fact that the amount of SCAD mRNA was greatly increased whereas that of IVD mRNA remained unchanged.

The site of FAD attachment, whether it occurs on the precursor in the cytosol or on the mature form in the mitochon-dria, was previously unknown. The lack of any effects of FAD on the stability of precursors and on the processes of mitochondrial import/processing as shown here is consistent with previous observations by others of other mitochondrial enzymes, such as serine:pyruvate aminotransferase and aspartate aminotransferase containing non-covalently bound prosthetic groups. These previous studies suggested that the prosthetic group is not required for mitochondrial import of the precursor (41, 42). The evidence by us and others suggests that complete recognition of the precursors of mitochondrial proteins is closely correlated with a loosely folded structure (43). Import of some precursor proteins into isolated mitochondria is blocked by ligands that induce a stable structure (44, 45). The lack of hindrance to mitochondrial import of acyl-CoA dehydrogenase and α-ETF precursors in the presence of FAD in our experiment suggests that acyl-CoA dehydrogenase precursors do not form tight complexes with FAD in the cytosol. These considerations support the notion that loosely folded acyl-CoA dehydrogenase precursors are imported into mitochondria in their apo form and then processed to the mature subunit to which FAD binds. Furthermore, our observation that mature SCAD was much more stable in normal mitochondria than in riboflavin-deficient mitochondria, regardless of whether translation was carried out in an FAD-depleted or FAD replete environment, indicates that FAD becomes ligated to the mature enzyme inside the mitochondria.

Acknowledgments—Ornithine transcarbamylase cDNA and the antibody to this enzyme were the gifts from Drs. Wayne Fenton and Frantisek Kalousek of this department. We thank Deborah Raymond for reading this manuscript.

REFERENCES