Biogenesis of the Covalently Flavinylated Mitochondrial Enzyme Dimethylglycine Dehydrogenase*

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Rat dimethylglycine dehydrogenase (Me2GlyDH) was used as model protein to study the biogenesis of a covalently flavinylated mitochondrial enzyme. Here we show that: 1) enzymatically active holoenzyme correlated with trypsin resistance of the protein; 2) folding of the reticulocyte lysate-translated protein into the trypsin-resistant, holoenzyme form was a slow process that was stimulated by the presence of the flavin cofactor and was more efficient at 15 °C than at 30 °C; 3) the mitochondrial prerequisite reduced the extent but did not prevent holoenzyme formation; 4) covalent attachment of FAD to the Me2GlyDH apoenzyme proceeded spontaneously and did not require a mitochondrial protein factor; 5) in vitro only the precursor, but not the mature form, of the protein was imported into isolated rat liver mitochondria; in vivo, stably transfected HepG2 cells, both the precursor and the mature form were imported into the organelle; 6) holoenzyme formation in the cytoplasm did not prevent the translocation of the proteins into the mitochondria in vivo; and 7) lack of vitamin B6 in the tissue culture medium resulted in a reduced recovery of the precursor and the mature form of Me2GlyDH from cell mitochondria, suggesting a decreased efficiency of mitochondrial protein import.

Since the first description of an enzyme with covalently attached FAD (the flavoprotein subunit of succinate dehydrogenase) (1), an increasing number of flavinylated enzymes have been discovered (for a review, see Ref. 2). Several were added to the list recently: rat liver L-pipecolic acid oxidase (3), plant reticulbin oxidoreductase (4), streptomycetes mitomycin resistance protein (5), and penicillin vanillyl-alcohol oxidase (6). Using the bacterial 6-hydroxy-o-nicotine oxidase (EC 1.5.3.6; 6-HDNO)1 as a model enzyme, we showed that the covalent attachment of FAD to His-71 of the polypeptide via an FAD(N\textsuperscript{8})-(N\textsuperscript{3})histidyl linkage, the most common bond encountered in this group of flavoenzymes, takes place autocatalytically (7).

In eukaryotic cells, enzymes bearing this covalent modification are all compartmentalized: the fungal enzyme vanillyl-alcohol oxidase (7a) and mitochondrial matrix. This particular cellular location raises questions regarding the biogenesis of these enzymes. A holoenzyme synthetase within the cell compartment could be required for the flavinylation of the imported apoenzymes. Alternatively, attachment of FAD to the enzyme could proceed spontaneously during or following folding of the imported, mature form of the protein into its native conformation. It is generally assumed that import of a precursor protein into mitochondria requires its unfolding. Inside the organelle the precursor sequence is then removed by a special peptidase and the protein allowed to fold. One function of the prerequisite of mitochondrial proteins seems to consist in its interaction with cellular chaperones which keep the molecule in a loosely folded, import-competent conformation (for a review, see Ref. 9). Given this scenario of mitochondrial protein import, one may anticipate that spontaneous attachment of FAD to the precursor will not take place since autoflavinylation requires the folding of the protein into its native conformation (7). In addition, the covalently attached cofactor may block the import of the protein into its place of destination (9). Previous work performed with the bacterial enzyme 6-HDNO fused to the Me2GlyDH prescence showed that import of the fusion protein into rat liver mitochondria in vitro was not inhibited by the bound FAD (10). However, no detailed data were available on the biogenesis of an authentic mitochondrial flavinylated enzyme in eukaryotic cells. Here we present results obtained in vitro in the rabbit reticulocyte lysate (RL) and in vivo in stably transfected HepG2 cells on the flavinylation, cofactor-dependent folding and mitochondrial import of the mature and precursor form of rat liver mitochondrial Me2GlyDH.

MATERIALS AND METHODS

Chemicals—Cycloheximide, CCCP, digitonin, DMEM, FAD, FMN, phenylmethylsulfonyl fluoride, riboflavin, soybean trypsin inhibitor, and trypsin were purchased from Sigma (Deisenhofen, FRG). [S\textsuperscript{35}S]-Me-thionine-(S\textsuperscript{35}S) cysteine mix was from Amersham Corp. (Braunschweig, FRG); dimethylglycine, folate, genetin G418, and protease inhibitors were from Boehringer Mannheim (Mannheim, FRG); and RNase A was from Diagen (Hilden, FRG). All other chemicals were of highest purity available.

Plasmid Constructs—Plasmid pCD-Me2GlyDH (11) was the starting DNA for subsequent cloning. pSPT19-M Me2GlyDH and pSPT19-pMe2GlyDH were constructed starting from pCD-Me2GlyDH which contains the Me2GlyDH cDNA sequence in the vector pCD (12). The sequence 5'-GAATCCCATCTATG-3' comprising an EcoRI restriction site (GAATCC) and an eukaryotic ribosomal binding site (CCACC) (13) was introduced in front of the Me2GlyDH start codon (ATG) with the aid of a mutagenic oligonucleotide. This allowed excision of the cDNA from pCD-pMe2GlyDH by digestion with the restriction enzymes EcoRI and

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1 The abbreviations used are: 6-HDNO, 6-hydroxy-o-nicotine oxidase; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DMEM, Dulbecco’s modified Eagle’s medium with 4500 mg glucose; FCS, fetal calf serum; Me2GlyDH, dimethylglycine dehydrogenase; MOPS, 3-N-morpholineproline sulfonic acid; MPP, mitochondrial processing peptidase; RL, reticulocyte lysate; PAGE, polyacylamide gel electrophoresis.
pSiI and insertion of the 2925-bp DNA fragment thus generated into pSPT19 (Boehringer Mannheim) digested with the restriction enzymes EcoRI and Smal. The ligated recombinant DNA was named pSPT19-mMe2GlyDH. pMe2GlyDH transcripts could be synthesized in vitro starting from the SP6 polymerase promoter situated on the plasmid with the aid of SP6 RNA polymerase (Boehringer Mannheim). Synthesis of transcripts was coupled with protein translation of Me2GlyDH in the rabbit RRL system kit obtained from Promega (Madison, WI). For the in vitro expression of the mature form of Me2GlyDH (mMe2GlyDH) the sequence 5'-GAATCCACCATG-3' was introduced into the Me2GlyDH cDNA contained in pc-pMe2GlyDH in such a way that the ATG codon replaced codon Ala-24 (11). Subcloning of the EcoRI/Smal DNA fragment of pMe2GlyDH into pSPT19 gave plasmid pSPT19-mMe2GlyDH. Transcription-translation of the plasmid carrying the cDNA generated a protein corresponding to the mature form of Me2GlyDH shortened by two serine residues at the amino-terminal end. The COOH-terminal deletions of the pMe2GlyDH designated ΔSFU and ΔXHO were achieved by digesting plasmid pSPT19-pMe2GlyDH with the restriction enzymes SfuI, HindIII and XhoI, SalI, respectively, and removing the excised Me2GlyDH DNA fragment. The plasmid vector carrying the deleted cDNA was then blunt ended with the Klenow fragment of DNA polymerase I and religated (14).

For expression of the Me2GlyDH proteins in eukaryotic cell lines, plasmids pSPT19-pMe2GlyDH and pSPT19-mMe2GlyDH were linearized by digestion with EcoRI, the restriction site filled in with the Klenow fragment of DNA polymerase I, followed by digestion with the restriction enzyme Xbal. The DNA fragment corresponding to the Me2GlyDH cDNA was isolated and ligated into the eukaryotic expression vector pRC-CMV (Promega) digested with HindIII, blunt ended with the Klenow fragment of DNA polymerase I, followed by digestion with XbaI. Plasmids were maintained in E. coli strain JM 109 and plasmid DNA was isolated from bacterial cells by a DNA isolation kit (Diagen, Hilden, FRG).

In Vitro Transcription-Translation—Coupled transcription-translation in the rabbit RLL in the presence of [35S]Met was done according to the supplier’s instructions (Promega).

Folding and Holoenzyme Formation of Me2GlyDH in the RL—[35S]GlyDH protein was synthesized in the coupled transcription-translation rabbit RRL system for 30 min at 30 °C. Translation was stopped by the addition of 80 μg/ml RNase A and 100 μg/ml cycloheximide. Folding of the translated proteins was then monitored at 15 and 30 °C in the absence or presence of 10 μM FAD, 10 μM folate, and 10 μM dimethylglycine, added separately or in combination to the incubation mixtures. Holoenzyme formation was tested by digestion of the translation assay for 15 min at 0°C. Digestion was stopped by the addition of 4 mg/ml soya bean trypsin inhibitor.

In Vitro Mitochondrial Protein Import—Rat liver mitochondria were prepared as described in Conboy et al. (15). Mitochondrial import assays were performed by incubating 4 μl of [35S]Met-labeled RL Me2GlyDH translation product with freshly isolated rat liver mitochondria at a final concentration of 50 mg/ml as described by Stolz et al. (10). After incubation, the isolated mitochondria were incubated before performing the import assays with either 12 μM FAD, 12 μM FMN, or 12 μM riboflavin at 27 °C for 5 min. Separation of mitochondria into a soluble and a membrane fraction and densitometric quantification of labeled protein bands on autoradiograms was performed as described in Stolz et al. (10).

Transfection of Cell Lines—Cells of the hepatoblastoma cell line HepG2 were grown at 37 °C as monolayer under 5% CO2 in DMEM supplemented with 10% fetal calf serum (FCS). They were transfected with plasmid DNA pRC-CMV-pMe2GlyDH and pRC-CMV-mMe2GlyDH by the calcium phosphate procedure (16). After 72 h, selection for transfected cells was performed by incubation of the medium with 85 μg/ml G418. Stolz et al. (10) have shown that this concentration allows selection of the majority of the cells in the culture. After selection, the isolated viable cells were propagated in a medium containing 10% FCS and 500 μg/ml genicidin G418. Genticin-resistant clones were picked and expanded in the same medium. After initial selection, the extent of translational expression of the new transfected sequences within individual clones was determined by Western blotting (see below). Transfectants with the highest level of expression were used for subsequent studies.

Pulse-Chase Experiments—Cells were grown to 90% confluency in 10-cm plastic dishes (approximately 5 × 104 to 10 × 104 cells per dish) and fed 12 h before labeling with fresh DMEM containing 10% FCS. Cells were incubated with [35S]methionine and [35S]cysteine for 12 h with or without 10 μM CCCP. After incubation with the labeling medium for 2 h, cells were washed twice with medium without methionine and cysteine and labeled for 1 h with [35S]methionine and [35S]cysteine. CCCP was added, and incubation was continued at 37 °C. When CCCP was used, it was added to the medium at a final concentration of 100 μM, 30 min before the addition of the labeling medium and was included at the same concentration during the labeling of the cells. Monolayers were labeled for 1 h in the presence or absence of 100 μM CCCP, the medium aspirated, the monolayers washed twice with phosphate-buffered saline, and methionine-containing DMEM plus 10% FCS and with or without 10 μM CCCP added.

Preparation of Cell Fractions—Release of the cytosolic and mitochondrial fraction was performed essentially as described in Janski and Cornell (17). The cytosolic fraction was held at 4 °C until immunoprecipitation or Western blotting was performed. The pellet containing the mitochondrial fraction was washed twice with 200 μl of fractionation medium (20 mM MOPS, 3 mM EDTA, pH 7.0) without digitonin and resuspended in 100 μl of mitochondrial homogenization buffer (220 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH, pH 7.6). The mitochondrial proteins were released by freezing the pellet three or four times in liquid nitrogen. Lactate dehydrogenase (EC 1.1.1.27) and citrate synthase (EC 4.1.3.7) activity was determined in each cell fraction according to Vassault (18) and Shepherd and Garland (19), respectively.

Immunoprecipitation and SDS-PAGE—Cell monolayers were washed with phosphate-buffered saline and then incubated 30 min at 4 °C with low salt lysis buffer (1% Triton X-100, 50 mM Tris, 10 mM EDTA, pH 8.0). The lysed cells were centrifuged for 3 min at 12,000 × g. Protein content was determined by the method of Bradford (20).

For immunoprecipitation the medium supplemented the isolated mitochondria were incubated before the addition of 100 μg Protein A-Sepharose/ml for 1 h at 4 °C with 10 μl anti-Me2GlyDH antisera in 300 μl of Triton buffer (1% Triton, 300 mM NaCl, 5 mM EDTA, 20 μl Triton, pH 7.5). To the centrifuged and washed pellet 200 μg of protein of either cell lysate or cytosolic or mitochondrial fraction and 300 μl of Triton buffer were added, and the mixture was incubated for 1 h at 4 °C. Following centrifugation and washing four times with Triton buffer, the immunocomplexes were analyzed by SDS-PAGE according to Laemmli (21). The 7.5% gels were fixed, dried, and autoradiographed.

Western Blotting—Total cell lysate and cytosolic or mitochondrial fractions were resuspended in sample buffer, and proteins were separated on 7.5% polyacrylamide gels by SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane (Optitran-BA-S 85 Schleicher & Schuell, Dassel, Germany) using a trans-blot semidry electrophoretic transfer cell (Bio-Rad, München, Germany). The Me2GlyDH protein was visualized with the aid of specific antisera and a chemiluminescence Western blotting kit as described by the supplier (Boehringer Mannheim).

Non-denaturing Gel Electrophoresis—Nondenaturing polyacrylamide gel electrophoresis was conducted according to Davis (22) using a 2.5% stacking gel, pH 6.7, and a 7% separating gel, pH 8.9. Gels were stained for dehydrogenase activity by incubation in a solution containing 0.2 μM dimethylglycine, 0.1 μM potassium phosphate, pH 7.2, 0.67% phenazine methosulfate, and 0.5 mM nitro blue tetrazolium at room temperature. Unreacted tetrazolium dye was then removed by diffusion into water.

RESULTS

Activity Staining and Trypsin Resistance of Me2GlyDH Holoenzyme—Expressed in Rat Liver Hepatocytes and Stably Transfected HepG2 Cells—When the cytosolic and the mitochondrial fractions of rat liver were analyzed by Western blotting for the presence of Me2GlyDH, a Me2GlyDH antibody-reactive band was detectable only in the mitochondrial fraction but not in the cytosolic fraction (Fig. 1, Panel A, lanes 1 and 2). Trypsin treatment revealed that this band was protease resistant (Fig. 1, Panel A, lane 3). This mitochondrially located, trypsin-resistant, mature form of Me2GlyDH represented enzymatically active holoenzyme as demonstrated by activity staining on nondenaturing polyacrylamide gels (Fig. 1, Panel A, lanes 4 and 5).

It exhibited a certain microheterogeneity indicated by the presence of several enzymatically active bands. This heterogeneity of Me2GlyDH has been observed before (23).

Human hepatoblastoma HepG2 cells, which do not express Me2GlyDH (results not shown), were stably transfected with pRC-CMV-pMe2GlyDH and pRC-CMV-mMe2GlyDH, encoding the precursor and mature forms of the protein, respectively. Analysis of the intracellular distribution of the Me2GlyDH proteins on Western blots showed that both the precursor as
me GlyDH) in the mitochondria (Fig. 1, lanes 2 and 3) of isolated rat liver mitochondria were trypsin digested (lane 3), analyzed on 7.5% SDS-PAGE, Western blotted, and decorated with antibodies to Me2GlyDH. The mitochondrial fraction (lane 4) was separated after digestion with 0.2 mg/ml trypsin (lane 5) under non-denaturing conditions and activity stained for Me2GlyDH. Panels B and C, HepG2 cells transfected with pRC-CMV-pMe2GlyDH and pRC-CMV-mMe2GlyDH were fractionated in cytosol (lane 1) and mitochondria (lanes 2 and 3) and analyzed by activity staining (lanes 4 and 5) in the same way as in Panel A.

well as the mature form were imported into mitochondria (Fig. 1, Panel B and C). The imported protein showed trypsin resistance (Fig. 1, Panels B and C, lanes 3), and exhibited on non-denaturing gels enzymatic activity and the same microheterogeneity as the Me2GlyDH protein isolated from rat liver mitochondria (Fig. 1, Panel B and C, lanes 4 and 5).

Translation and Folding of Me2GlyDH into Holoenzyme in the RL—The stably transfected HepG2 cells showed that trypsin resistance of Me2GlyDH correlated with the enzymatically active holoenzyme form. This observation allowed us to test in the RL translation system the conditions required for folding of the precursor and mature protein into the trypsin-resistant conformation. The percentage of trypsin resistance was determined by comparison of the intensity of the [35S]Met-labeled Me2GlyDH band on the autoradiogram of the nondigested sample with that of the trypsin-digested sample. Fig. 2, Panel A, presents the temperature dependence of Me2GlyDH holoenzyme formation. Trypsin digestion of the precursor protein removed most of the presequence of pMe2GlyDH (10), at a site closely preceding the MPP processing site (compare lanes 1 and 2). Formation of the trypsin-resistant form of the protein was more efficient at 15°C than at 30°C (compare lanes 2 and 4). Similar results were obtained with the mature form of the protein (Fig. 2, Panel A, mMe2GlyDH). However, the yield of trypsin-resistant protein after 5 h incubation at 15°C was higher than in the case of the precursor form (30% for mMe2GlyDH and 12% for pMe2GlyDH on average from three independent folding experiments).

The RL contains low levels of endogenous FAD (24), which may not be sufficient for Me2GlyDH holoenzyme formation. The second cofactor of the enzyme, H4PteGlu5, is unstable; but the protein also binds folic acid. We analyzed the folding kinetics of the RL translated mature and precursor Me2GlyDH in the presence of externally added FAD, folic acid and the Me2GlyDH substrate, dimethylglycine (Fig. 2, Panels B and C). After 20 h, the highest yield of the native, trypsin-resistant holoenzyme form was obtained when both cofactors were present; the stimulating effect of the cofactors being strongest during the initial phase of folding (2.5 h). After 20 h, assays without external additions reached the same level of trypsin-resistant Me2GlyDH as that obtained in the presence of FAD only (Fig. 2, Panel B, ○ and ■). The presence of the substrate dimethylglycine did not change the kinetics of folding (Fig. 2, Panel B, ■). Thus, the efficiency of folding of mMe2GlyDH was dependent on the cofactor concentrations in the assays.

The yield of trypsin-resistant pMe2GlyDH was lower when compared to that obtained with mMe2GlyDH. Within the first 2.5 h folding was more efficient in the presence of cofactors and substrate (Fig. 2, Panel C, □ and △) than without additions (Fig. 2, Panel C, ○). The final yield of trypsin-resistant protein varied between 20 and 40%. Apparently the presence hampered the folding of the protein into the trypsin-resistant conformation. Binding of RL chaperons to the presequence could be responsible for the slowed and less efficient folding of the precursor. We analyzed the folding of the two proteins in the presence of added ATP without noting any effect on the efficiency of folding of the mature protein (not shown). However, folding of the precursor protein into the trypsin-resistant form was stimulated in the presence of ATP (33% trypsin resistance after 5 h as compared to 20% in the absence of ATP).

The experiments presented thus far did not exclude the possibility that a holoenzyme syntheptase present in the mitochondrial matrix may stimulate folding of the mature protein into its trypsin-resistant conformation and thus FAD attachment. Addition of various concentrations of mitochondrial protein extract (10, 25, and 50 µg per assay) to the folding reactions had, however, no effect (Fig. 2, Panel D, shows results at 25 µg of mitochondrial protein).

In Vitro Translation, Mitochondrial Import, and Trypsin Resistance of Wild Type and Carboxyl-terminally Deleted Me2GlyDH Proteins—Incubation of the [35S]Met-labeled pMe2GlyDH translation product with isolated rat liver mitochondria resulted in the import of the protein. The mature form of Me2GlyDH, however, was not imported (results not shown). Incubation of mitochondria with flavins prior to import increased the yield of trypsin-resistant Me2GlyDH holoenzyme in the matrix, the highest increase being observed with FAD (70% trypsin resistance with as compared to 45% without preincubation of mitochondria with FAD).

Me2GlyDH contains a FAD and a H4PteGlu5 binding domain. This conclusion is inferred from the primary sequence of the protein, which shows a typically dinucleotide binding motif (Gly49XXGly51) situated at the NH2-terminal part of the protein (11) and a positively charged sequence rich in Lys residues (Lys740-XX-Lys-XXX-Lys-XXX-Lys-XX-Lys-XX-Lys-ArgArg764) at the COOH-terminal part of the protein. Based on the comparison with the folate polyglutamate cofactor binding site of other proteins (25), this sequence may represent the binding site of the H2PteGlu5 cofactor of Me2GlyDH. In addition, recent sequence comparison of bacterial sarcosine oxidase with Me2GlyDH revealed a significant amino acid sequence similarity among the enzymes in the FAD-binding domain (26). These considerations prompted us to examine whether the NH2-terminal FAD-domain of the protein folds by itself, in an FAD-dependent manner, into a trypsin-
resistant conformation. Two COOH-terminal deletions were studied, the first reduces the 95-kDa pMe₂GlyDH to a 68-kDa protein (∆SFU) and the second to a 52-kDa protein (∆XHO). When these deleted proteins were translated in the RL, they were in a trypsin-sensitive form. They were imported into rat liver mitochondria (Fig. 3, Panel A), but remained trypsin-sensitive inside the mitochondrial matrix (Fig. 3, Panel B, lanes 1 and 2). Preincubation of mitochondria with various flavins previous to import, did not change the trypsin sensitivity of the imported deletion proteins (Fig. 3, Panel B, lanes 3–8). These results suggest that the flavin domain does not fold independently into a trypsin-resistant conformation, but that folding and therefore FAD attachment requires the entire polypeptide chain.

In Vivo Expression of pMe₂GlyDH and mMe₂GlyDH in Stably Transfected HepG2 Cells—HepG2 cells stably transfected with pRC-CMV-pMe₂GlyDH and pRC-CMV-mMe₂GlyDH were employed in pulse-chase assays to analyze the distribution of the labeled proteins between cytoplasmic and mitochondrial fractions (Fig. 4). After a 0-min chase the Me₂GlyDH precursor as well as the mature form were recovered in the cytoplasmic fraction (Fig. 4, Panels A and B, lanes 1 and 2). Surprisingly, after a 15-min chase both proteins were predominantly found in the mitochondrial matrix (Fig. 4, Panels A and B, lanes 3 and 4).

**Fig. 3.** Import of the COOH-terminally deleted pMe₂GlyDH/∆SFU and pMe₂GlyDH/∆XHO proteins into rat liver mitochondria. Panel A, [35S]Met-labeled pMe₂GlyDH/∆SFU and pMe₂GlyDH/∆XHO proteins were synthesized in the rabbit RL and translation assays were analyzed by SDS-PAGE without (lane 1) or following trypsin digestion (lane 2). Aliquots of the translation assays were incubated with isolated rat liver mitochondria (lane 3) and import estimated by trypsin digestion (lane 4). Panel B, mitochondria were preincubated prior to import without flavins (lanes 1 and 2), with 12 μM FAD (lanes 3 and 4), with 12 μM FMN (lanes 5 and 6) or with 12 μM riboflavin (lanes 7 and 8). Following import the mitochondria were reisolated and fractionated, and the soluble fraction were either not treated (lanes 1, 3, 5, 7) or treated with trypsin (lanes 2, 4, 6, 8). The proteins were analyzed by SDS-PAGE and autoradiography.

out at 30 °C in the absence of cofactors (○), in the presence of 10 μM FAD (●), in the presence of 10 μM FAD and 10 μM folate (□), or in the presence of 10 μM FAD, 10 μM folate, and 10 μM dimethylglycine (■). After 30 min cycloheximide and RNase A were added, and incubation was continued at 15 °C. At the indicated time points equal aliquots were taken and submitted to trypsin digestion and analyzed by SDS-PAGE. Shown is the percentage of trypsin-resistant mMe₂GlyDH protein present after protease treatment as compared to the amount of mMe₂GlyDH protein in undigested control assays. Panel C, as in Panel B, but performed with pMe₂GlyDH. Panel D, coupled transcription-translation of pMe₂GlyDH and mMe₂GlyDH was stopped after 30 min, 2 μl of the assays were trypsin-digested; incubation of the remaining assays was continued at 15 °C in the presence or absence of 25 μg of mitochondrial matrix extract. Aliquots were taken, trypsin-digested, and analyzed as in Panel B. The amount of trypsin-resistant Me₂GlyDH proteins was expressed as percentage of Me₂GlyDH present in parallel assays not treated with protease, at 0 h of incubation (black bars), without or with mitochondrial matrix or after 5 h of incubation (white bars) without or with mitochondrial matrix.
Mitochondrial import of Me2GlyDH in vivo. Panel A, pRC-CMV-pMe2GlyDH transfected HepG2 cells were labeled with [35S]Met in the presence of CCCP for 60 min and then chased for 15 min in the absence (lanes 2, 3, 4, and 5) or in the presence of the inhibitor (lanes 6, 7, 8, and 9). Cytosol (lanes 2, 3, 6, and 7) and mitochondria (lanes 4, 5, 8, and 9) were isolated, trypsin-digested, immunoprecipitated, separated on 7.5% polyacrylamide gel by SDS-PAGE, and autoradiographed. Lane 1 shows the in vitro translation product of pMe2GlyDH. Panel B, same experiment as in Panel A performed with HepG2 cells stably transfected with the mature form of Me2GlyDH.

4), indicating that in vivo also the mature Me2GlyDH, lacking the mitochondrial presequence, was imported into mitochondria.

Cells were labeled with [35S]Met, and mitochondrial import was blocked by the respiratory chain inhibitor CCCP for 60 min in order to allow the formation of the holoenzyme in the cytoplasm. Following a chase of 15 min with cold methionine in the absence of the inhibitor, both the pMe2GlyDH as well as the mMe2GlyDH were translocated into the organelle and recovered from the mitochondrial matrix fraction in the protease-resistant form (Fig. 5, Panels A and B, compare lanes 2, 3, and 4, 5). When the chase was performed in the presence of CCCP, mitochondrial import was inhibited and the proteins were recovered mainly from the cytoplasmic fraction (Fig. 5, Panels A and B, compare lanes 6, 7, and 8, 9). The precursor and mature form of Me2GlyDH, which accumulated in the presence of CCCP in the cytoplasm, folded during the 60 min of CCCP treatment into the trypsin-resistant conformation (Fig. 5, Panels A and B, lane 7). These results indicated that formation of holoenzyme did not prevent the mitochondrial import of Me2GlyDH in vivo.

Processing of pMe2GlyDH in the Cytoplasm of HepG2 Cells—When pMe2GlyDH translated in the RL was incubated with the cytoplasmic fraction of HepG2 cells, the precursor protein was processed to a smaller molecular weight species. Initially we assumed that contamination of the cytosol with MPP originating from mitochondria broken during the isolation procedure was responsible for the observed effect. Closer inspection by SDS-PAGE of the molecular weight of the protein form processed from the cytosolic fraction of HepG2 cells and of the molecular weight of the mature protein generated by the MPP revealed that the processed form exhibited a molecular weight that was intermediate between the precursor form and the mature, MPP processed form of the enzyme (Fig. 6, Panel A). Analysis of the specificity of the Hepton cytoplasmic protease activity by protease inhibitors revealed an inhibition spectrum characteristic for thiol proteases (Fig. 6, Panel B). Since this molecular weight Me2GlyDH species did not accumulate in the cytoplasm of labeled HepG2 cells, we conclude that it was imported into mitochondria. We assume that the proteolytic processing removed part of the mitochondrial presequence.

Effect of Riboflavin Depletion on Me2GlyDH Biogenesis in HepG2 Cells—HepG2 cells stably transfected with pRC-CMV-pMe2GlyDH and pRC-CMV-mMe2GlyDH were grown in riboflavin-free cell culture medium supplemented with dialyzed FCS. Riboflavin deficiency became evident after 5 days by a decrease in cell growth. Fig. 7 shows that when riboflavin-deficient cells were pulse labeled with [35S]Met on the 5th day, the uptake of the labeled pMe2GlyDH into the mitochondria was decreased as compared to that from mitochondria of non-deprived cells (Fig. 7, Panel A, compare lanes 2 and 4). When the same experiment was performed with HepG2 cells stably transfected with pRC-CMV-mMe2GlyDH, cells kept under riboflavin deficiency accumulated [35S]Met-labeled mMe2GlyDH in the cytosol (Fig. 7, Panel B, compare lanes 1 and 3). Nevertheless, a certain level of mitochondrial import of both the precursor and the mature form could be observed (Fig. 7, Panel A and B, lanes 2 and 4). This observation may be explained by a less efficient mitochondrial Me2GlyDH import in riboflavin-deficient cells.

**DISCUSSION**

The analysis of the biogenesis of the covalent modification of Me2GlyDH demonstrated that, as shown for many proteins (27), the holoenzyme formation was stabilized by the incorporation of the cofactor and was trypsin-resistant. Significantly, folding of the RL-translated Me2GlyDH into the trypsin-resistant conformation proceeded more efficiently at 15°C than at 30°C and was independent of mitochondrial protein. The experimental data support the conclusion that Me2GlyDH holoenzyme formation in the RL in vitro takes place spontaneously in line with an autoflavinylation process demonstrated first for the bacterial enzyme 6-hydroxy-D-nicotine oxidase carrying the same histidine(N3)-(8)FAD linkage as Me2GlyDH (7). There exists now strong support for the notion that covalent FAD attachment progresses autocatalytically and depends on a conformation of the protein favorable for the interaction of the isoalloxazine ring with the reactive group of the enzyme.
HepG2 cells lacks two serine residues at the NH2 terminus. Further experiments have shown that the presence of these two amino acids significantly increases the protease resistance of the protein. The removal of these residues results in a decrease in protease sensitivity, indicating that folding and trypsin resistance are related to the presence of these amino acids.

In agreement with reports on the folding of precursor molecules of mitochondrial enzymes and plastid enzymes, the presequence of Me2GlyDH slowed down the folding of the protein into a conformation similar to that of the mature form. The decreased folding efficiency of precursor proteins has been attributed to the interaction of chaperones present in the rabbit reticulocyte lysate (37). The observation that the efficiency of folding of pMe2GlyDH into the protease-resistant conformation was increased in the presence of ATP may be explained by this finding.

It is interesting to note that the results obtained in vitro, in stably transfected HepG2 cells, differed in several respects from those observed in the in vitro rabbit reticulocyte lysate system. In the HepG2 cell cytosol, the pMe2GlyDH and mMe2GlyDH proteins synthesized during the [35S]Met pulse rapidly became trypsin-resistant, indicating that folding in vivo needed less time than in the rabbit reticulocyte lysate system. Also noteworthy is the observation that HepG2 cells contain a cytosolic protease that seems to remove part of the NH2-terminal amino acid sequence of pMe2GlyDH since no change in molecular weight of the mMe2GlyDH expressed in HepG2 cells was observed. Although we have found no indication of this protease in hepatocytes, fibroblasts, or Cos 7 cells and it may therefore be a particular feature of this hepatoblastoma cell line, import of mitochondrial precursor proteins in HepG2 cells seemed not to be impaired. Factors present in the HepG2 cytosol but absent from the RL may mediate this import. This possibility is documented in the case of mMe2GlyDH which is imported into mitochondria in vivo but not in vitro. The mature form of the protein expressed in HepG2 cells lacks two serine residues at the NH2 terminus. Deletion of these two amino acids did not affect enzyme activity, the FAD binding domain, or trypsin resistance of the protein. We therefore conclude that this Me2GlyDH species behaves in all respects relevant to the aspects investigated in this work as the authentic mMe2GlyDH generated by the MPP. It is not yet clear what sequences within the mature protein may be responsible for the observed in vitro import. Translocation of the mMe2GlyDH accumulated in the cytoplasm of HepG2 cells following CCCP treatment apparently took place with the covalently bound flavin cofactor since the protein was in its protease-resistant form characteristic for the holoenzyme. This conclusion is in agreement with results obtained with the fusion protein consisting of the Me2GlyDH presequence and the bacterial enzyme 6-HDNO (10). Import of the mature form of mitochondrial enzymes has been shown previously (34, 38–40).

Mitochondria seem to be able to take up riboflavin and/or FMN and to synthesize FAD (41). Indeed, preincubation of mitochondria with flavins increased the amount of trypsin-resistant Me2GlyDH. This may indicate a suboptimal FAD supply for flavinylation reactions in the matrix.

Mitochondria isolated from rats kept on a riboflavin-deficient diet showed an increased protease sensitivity of acyl-CoA dehydrogenases and electron transfer flavoprotein as compared to mitochondria isolated from rats fed a diet with sufficient riboflavin (24). The Me2GlyDH proteins expressed during pulse-chase experiments in HepG2 cells grown in riboflavin-deficient medium did not exhibit a significant increase in protease sensitivity, but a decreased mitochondrial uptake. Since riboflavin deficiency may have multiple effects in the cell, it is difficult to make a specific correlation between riboflavin supply and import of flavoenzymes, but such a relationship cannot be excluded.

An amino acid sequence comparison of Me2GlyDH with bacterial and mammalian enzymes of C1-metabolism revealed similarities among these enzymes in both the FAD binding as well as the H$_2$PteGl$_5$u, binding domain (26). Me2GlyDH as well as the related sarcosine dehydrogenase of rat liver mitochondria (42) may have been generated by the fusion of two primordial genes (26). This hypothesis could imply that the two domains of Me2GlyDH represent independent folding units. However, the results obtained with Me2GlyDH deletion proteins suggest that the two domains do not fold independently from one another.

It may be interesting to note that the same type of covalent attachment of FAD to bacterial sarcosine oxidase and mammalian Me2GlyDH and sarcosine dehydrogenase was conserved during evolution. The same observation applies to the flavoprotein subunit of succinate dehydrogenase (43). The biochemical reason behind the conservation of this particular protein-cofactor interaction has not yet found an explanation (44). In mammals Me2GlyDH, sarcosine dehydrogenase, and succinate dehydrogenase are mitochondrially located. According to the theory of bacterial origin of mitochondria (see Ref. 9 for a discussion) one may speculate that these enzymes and their particular type of cofactor linkage were introduced into the ancestor of the eukaryotic cell by the bacterial endosymbiont.

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