Flavinylation of the precursor of mitochondrial dimethylglycine dehydrogenase by intact and solubilised mitochondria

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Abstract The flavinylation and the presequence processing of the mitochondrial matrix enzyme dimethylglycine dehydrogenase (Me_2GlyDH) were investigated with the reticulocyte lysate translated precursor (pMe_2GlyDH) added to solubilised mitoplasts of rat liver mitochondria. The flavinylation of pMe_2GlyDH was strictly dependent on the addition of mitochondrial protein(s), among which the mitochondrial flavinylation stimulating factor [Brizio C., et al. (2000) Eur. J. Biochem 267, 4346–4354], that actively promotes holo-Me_2GlyDH formation. The precursor processing, that accompanies the biogenesis of the enzyme, was not required to allow the flavinylation to proceed. The comparison of the time course of the flavinylation and the processing of pMe_2GlyDH demonstrated that the covalent attachment of the flavin moiety preceded the presequence processing by mitochondrial processing peptidase.

Key words: Dimethylglycine dehydrogenase; Flavinylation; Flavinylation stimulating factor; Mitochondrion; Precursor processing

1. Introduction

Mitochondria are the cellular site in which many flavoenzymes are located and where they act as dehydrogenases and oxidases in cooperation with the riboflavin-derived redox cofactors FMN and FAD (for a review see [1]). Although mitochondrial protein biogenesis has been intensively investigated over the last 20 years (for a review see [2]), the molecular mechanisms that secure and regulate the assembly of flavin cofactors with newly synthesised apo-flavoenzymes on their way into the organelle, remain relatively unknown.

We have recently clarified the mechanism(s) by which mitochondria obtain their flavin cofactors [3–5]. In both rat liver mitochondria (RLM) and *Saccharomyces cerevisiae* mitochondria, FAD synthesis occurs from riboflavin and/or FMN due to the existence of a riboflavin kinase (EC 2.7.1.26) and a FAD synthetase (EC 2.7.7.2). The latter has been characterised from RLM and localised in the mitochondrial matrix [5]. Consistently, riboflavin kinase, the product of the FMN1 gene in *S. cerevisiae*, has been found to be localised in the inner mitochondrial membrane [6].

FAD synthesis inside mitochondria may be related to the mitochondrial holo-flavoprotein formation occurring inside the organelles. Consisting evidences of intramitochondrial binding of FAD to apo-flavoproteins have been reported in studies carried out with medium chain acyl-CoA dehydrogenase (EC 1.3.99.3) and the flavoprotein subunit of succinate dehydrogenase (SDH) complex (EC 1.3.99.1) [7,8]. It has been also recently shown that FAD newly synthesised starting from FMN and ATP inside the mitochondrial matrix can be efficiently incorporated into the apo-flavoenzyme dimethylglycine dehydrogenase (Me_2GlyDH) (EC 1.5.99.2), a mitochondrial matrix enzyme involved in choline degradation. Moreover, the attachment of FAD to Me_2GlyDH via histidyl(*N*3)–(8α)FAD covalent linkage, is assisted by a mitochondrial flavinylation stimulating factor (mtFSF) localised in the matrix [9]. These findings corroborate the notion that cofactor binding occurs inside the organelles.

Like other mitochondrial flavoenzymes, Me_2GlyDH is synthesised in the cytosol as a precursor polypeptide, with an N-terminal extension which is removed on protein import inside the organelle by the mitochondrial processing peptidase (MPP) (EC 3.4.24.64) [10]. Indeed, we have partially purified the mtFSF as acting on the mature form of Me_2GlyDH (mMe_2GlyDH) synthesised in vitro [9]. The question about which the form of the protein (mature or precursor) is the in vivo substrate for flavinylation, that is to say whether once inside the organelle apo-protein flavinylation precedes or follows precursor processing, is still unsolved.

Here we show that the precursor form of Me_2GlyDH (pMe_2GlyDH) is a good substrate for mitochondria-assisted flavinylation reaction and that, in our model system, flavinylation of precursor precedes polypeptide processing by the MPP during the biogenesis of holo-Me_2GlyDH.

2. Materials and methods

All reagents and enzymes were from Sigma. Digitonin was from Merck, [^{35}S]methionine-[^{35}S]cysteine mix was from Amersham, the rabbit reticulocyte lysate (RL) in vitro transcription-translation system was from Promega.

Mitochondria were isolated from the liver of male Wistar rats (150–200 g) and purified as reported in [11]. Mitochondrial preparations

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Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; Me_2GlyDH, dimethylglycine dehydrogenase; mMe_2GlyDH, mature form of Me_2GlyDH; MPP, mitochondrial processing peptidase; mtFSF, mitochondrial flavinylation stimulating factor; pMe_2GlyDH, precursor form of Me_2GlyDH; RL, reticulocyte lysate; RLM, rat liver mitochondria; SDH, succinate dehydrogenase

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showing a respiratory control index lower than three were discarded. Mitoplasts were obtained from purified RLM according to [11]. Mitochondrial protein was determined according to Waddel and Hill [12].

[^{35}S]Met-labelled pMe$_2$GlyDH protein was synthesised in vitro in the coupled transcription-translation rabbit RL system starting from the pSPT19-pMe$_2$GlyDH plasmid, according to [10].

The flavinylation of the [^{35}S]Met-labelled RL translated pMe$_2$-GlyDH was assayed by trypsin digestion, followed by SDS–PAGE and autoradiography, according to [9,10].

The import of the [^{35}S]Met-labelled RL translated pMe$_2$GlyDH in isolated mitochondria was assayed by treating the mitochondrial samples with trypsin, followed by re-isolation of mitochondria and SDS–PAGE analysis, performed essentially as in [10].

The densitometric quantification of the labelled protein bands on the autoradiograms was performed with an Image Master DTS (Pharmacia).

3. Results

The biogenesis of holo-Me$_2$GlyDH starting from the apo form of its precursor was studied here in a model system consisting of the in vitro synthesised protein added to solubilised mitoplasts in the presence of FAD, under the experimental conditions optimised before, when flavinylation was started from the mature form of the protein [9].

A typical experiment is shown in Fig. 1: the flavinylation of [^{35}S]Met-labelled pMe$_2$GlyDH synthesised in the transcription-translation rabbit RL system was determined by assaying the trypsin resistance of the protein, which was correlated with the flavinylated form [9,10]. The densitometric analysis of the autoradiograms in Fig. 1A is reported as a histogram in Fig. 1B.

In agreement with previous results [10], in the absence of mitoplasts, the RL translated protein was completely trypsin-sensitive after 60 min incubation with FAD, that is to say that the flavinylation of pMe$_2$GlyDH was negligible. The addition of solubilised mitoplasts caused the appearance of two trypsin-resistant, i.e. flavinylated, protein bands. All together, they represent about 50% of the total amount of RL translated protein, as measured in the trypsin-untreated sample. Indeed, the upper band in the trypsin-treated sample (Fig. 1A, lane 4) migrated at the same position of pMe$_2$GlyDH: it is named

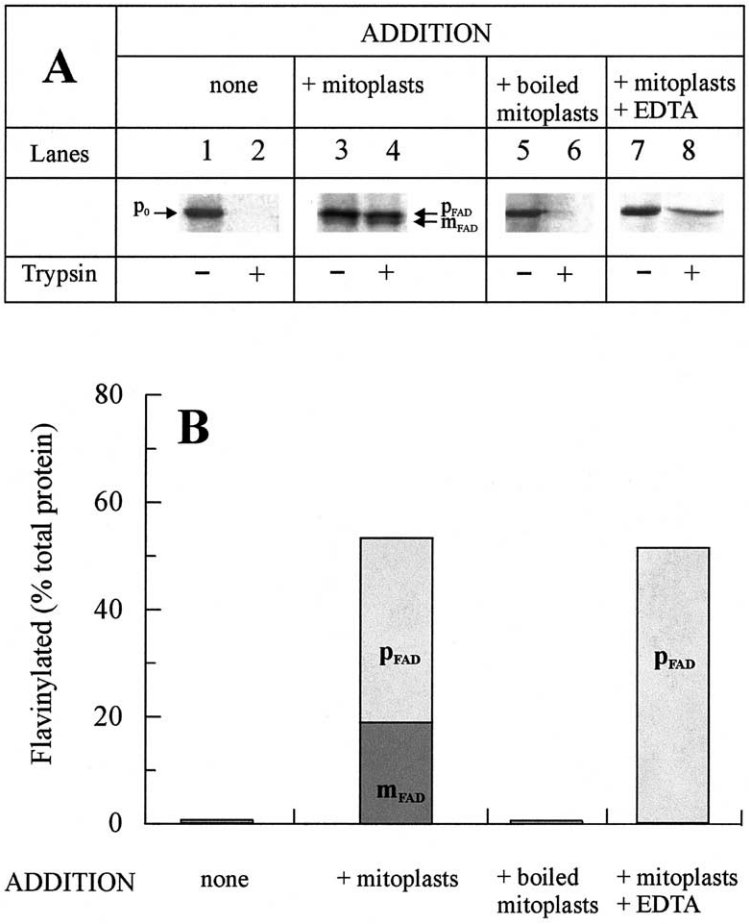
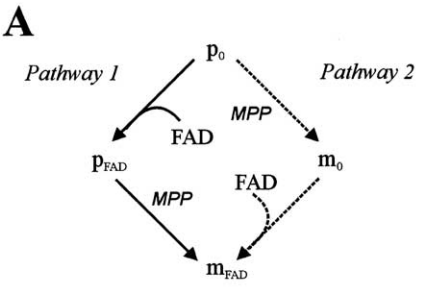


Fig. 1. Flavinylation of pMe$_2$GlyDH: effect of the addition of solubilised mitoplasts. A: 5 μl of [^{35}S]Met-labelled pMe$_2$GlyDH RL translation product was incubated in duplicate at 37°C in 45 μl of a medium consisting of 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 0.02 μg/μl oligomycin and 5 μM FAD, with the following additions: lanes 1 and 2, without additions; lanes 3 and 4, 0.4 mg of mitoplasts solubilised with Triton X-100 (0.5%); lanes 5 and 6, 0.4 mg solubilised mitoplasts boiled for 5 min before the addition; lanes 7 and 8, 0.4 mg solubilised mitoplasts and 1 mM EDTA. Following 60 min incubation one of the duplicate samples was trypsin treated. The samples were analysed by SDS–PAGE and the dried gels autoradiographed. The arrows indicate the unflavinylated pMe$_2$GlyDH, designed as p_0, the flavinylated pMe$_2$GlyDH, designed as p_{FAD}, and the flavinylated mMe$_2$GlyDH, designed as m_{FAD}. The upper band in the trypsin-untreated sample corresponds to both the unflavinylated and the flavinylated pMe$_2$GlyDH, i.e. p_0+p_{FAD}; the lower band in the trypsin-untreated sample corresponds to both the unflavinylated and the flavinylated mMe$_2$GlyDH, i.e. m_0+m_{FAD}. B: Densitometric analysis of the autoradiograms shown in A. The *y*-axis represents the amount of flavinylated protein forms, expressed as % of the total amount of RL translated protein present in the trypsin-untreated samples.



B	a						b			
Incubation Time	60 min		180 min		300 min		60 min		180 min	
Lanes	1	2	3	4	5	6	7	8	9	10
Trypsin	−	+	−	+	−	+	−	+	−	+
Temperature	37°C						30°C			

Fig. 2. Flavinylation and processing of pMe_2GlyDH in solubilised mitoplasts. A: Proposed pathways in the biogenesis of holo-Me_2GlyDH. B: The flavinylation assay was performed for the indicated incubation times at 37°C (a) or 30°C (b) in the presence of solubilised mitoplasts (0.4 mg protein), and sample analysis was carried out as described in Fig. 1.

here p_{FAD} and it represents 34% of the total amount of RL translated protein. The lower band in the trypsin-treated sample (Fig. 1A, lane 4) corresponds to the holo-mature form of Me_2GlyDH: it is named here m_{FAD} and it represents 19% of the total amount of RL translated protein. Thus, following the addition of solubilised mitoplasts, both flavinylation and the processing of the precursor take place, generating the holo-Me_2GlyDH.

The boiling of detergent-solubilised mitoplasts, prior to the addition to the flavinylation assay, resulted in a complete loss of flavinylated proteins, confirming that a mitochondrial protein component(s) is involved in flavinylation of Me_2GlyDH. The treatment of solubilised mitoplasts with EDTA (1 mM) to inhibit MPP [13] left the RL translated precursor completely unprocessed, but still efficiently flavinylated; in fact, about 50% of the total amount of RL translated protein was present as p_{FAD}. Thus, precursor processing is not strictly required to allow the flavinylation to proceed.

In a control experiment, carried out to compare the efficiency of flavinylation starting from pMe_2GlyDH with that obtained starting from the mature form of the protein, the flavinylation of the [^{35}S]Met-labelled RL translated mMe_2GlyDH was assayed in the absence or presence of solubilised mitoplasts, under the same experimental conditions as described in Fig. 1 (not shown). In agreement with previous results [9], the addition of mitoplasts stimulated flavinylation of mMe_2GlyDH from 8% of the total amount of RL translated protein (as measured in the RL system) to 40%.

These findings clearly show that, under the conditions in which no flavinylation of precursor in RL is observed, pMe_2GlyDH is a good substrate for mitoplast-assisted flavinylation reaction, even better than the RL translated mMe_2GlyDH.

Since during mitoplast-assisted flavinylation of pMe_2GlyDH a fraction of the protein was processed by the MPP, the question arises as to how the holo-Me_2GlyDH (m_{FAD}) was generated. As outlined in the scheme in Fig. 2A, m_{FAD} could be generated via two alternative pathways. In pathway 1, m_{FAD} is produced by the processing of the flavinylated precursor (p_{FAD}), that is to say that the flavinylation precedes processing. In pathway 2, m_{FAD} is produced by the flavinylation of the mature form of the protein (m_0), previously generated by the processing of the unflavinylated precursor (p_0) synthesised in the RL system.

In the attempt to solve this question, the flavinylation and the processing starting from [^{35}S]Met-labelled pMe_2GlyDH were determined at various incubation times in the presence of solubilised mitoplasts (Fig. 2B). Examining the protein bands corresponding to p_{FAD} and m_{FAD} in Fig. 2B, a clear difference was evident in the relative amount of the two flavinylated protein forms present in the trypsin-treated samples at each time. At 37°C (panel a), the p_{FAD} band was the major flavinylated form present at 60 min and the relative amount of m_{FAD} increased with time. At 30°C (panel b), the total amount of the flavinylated forms was lower as compared to 37°C, due to a significant decrease in the amount of p_{FAD}.

The kinetic analysis of p_{FAD}, m_0 and m_{FAD} formation was performed by gel scanning densitometry of the bands in Fig. 2B and it is presented in Fig. 3. The total amounts of the flavinylated forms of the protein, i.e. $p_{FAD}+m_{FAD}$ (●) present in the trypsin-treated samples were reported in Fig. 3A as % of the total amount of RL translated protein. The amount of either p_{FAD} (■) or m_{FAD} (□) was also separately reported in Fig. 3A as % of the total amount of RL translated protein. Due to the presence of solubilised mitoplasts, flavinylation, starting from p_0, rapidly increased with time, being 100% after 180 min incubation at 37°C (●, solid line). The appearance of p_{FAD} (■, solid line) was clearly much faster as compared to the appearance of m_{FAD} (□, solid line), p_{FAD} representing already 80% of total translated protein at 180 min. At longer incubation times, when protein flavinylation was already complete, a decrease in the amount of p_{FAD} was observed and it was parallel to the increase in the amount of m_{FAD}, in agreement with the processing of p_{FAD} to give m_{FAD} catalysed by MPP.

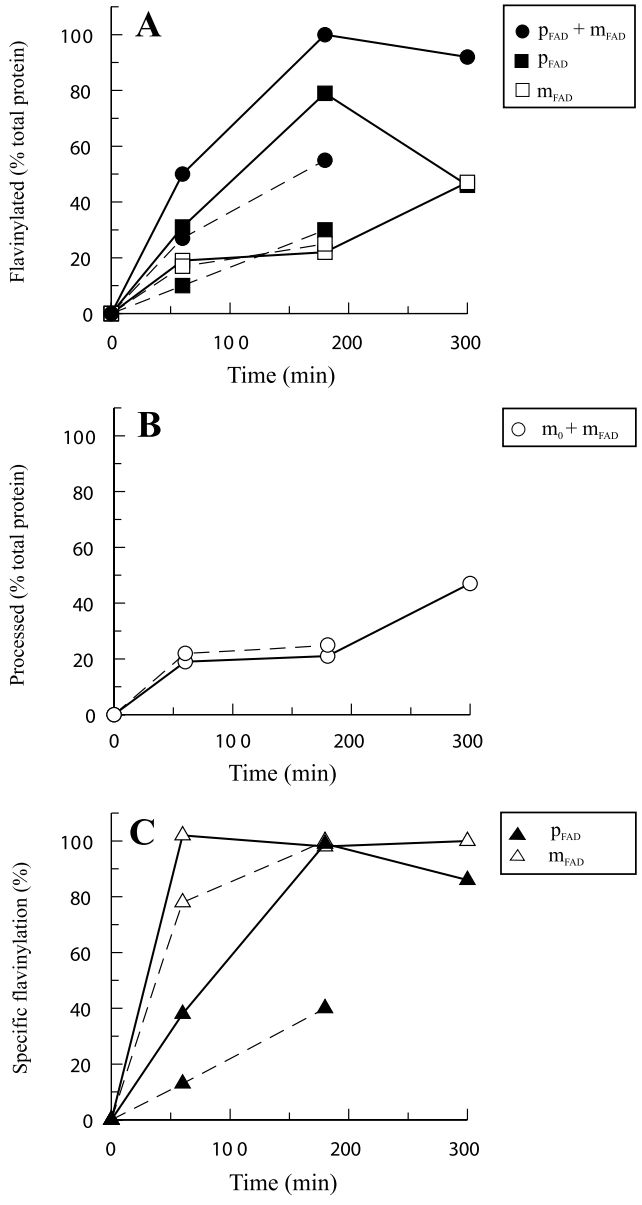


Fig. 3. Time course of flavinylation and processing of pMe_2GlyDH in solubilised mitoplasts. The amounts of p_0, p_{FAD}, m_0 and m_{FAD} were determined by densitometric analysis of the autoradiograms in Fig. 2B, and reported as a function of the incubation time. A: Shown is the amount of flavinylated protein forms, i.e. $p_{FAD}+m_{FAD}$ (●), p_{FAD} (■) and m_{FAD} (□), respectively expressed as % of the total amount of RL translated protein. B: Shown is the amount of mMe_2GlyDH, i.e. m_0+m_{FAD}, expressed as % of the total amount of RL translated protein. C: Shown are the specific flavinylation of pMe_2GlyDH (▲), expressed as % of the amount of p_{FAD} out of the amount of p_0+p_{FAD}, and the specific flavinylation of mMe_2GlyDH (△), expressed as % of the amount of m_{FAD} out of the amount of m_0+m_{FAD}. Solid lines and dashed lines represent data obtained at 37°C and 30°C, respectively.

This sequence of events strongly suggests the prevailing occurrence of pathway 1 in the process of holoenzyme formation. The limiting step of the global pathway, as measured at 37°C, should be the processing of p_{FAD}.

In line with this finding, further observation derives from the data in Fig. 3B, in which the total amount of the mature form of the protein, i.e. m_0+m_{FAD} (which can be measured in the trypsin-untreated samples of Fig. 2B), is expressed as a function of time. At 37°C, the appearance of m_0+m_{FAD} (Fig. 3B, solid line) proceeded clearly much slower than the appearance of $p_{FAD}+m_{FAD}$ (Fig. 3A, ●, solid line). Moreover, comparing the amount of m_0+m_{FAD} (Fig. 3B, solid line) with that of m_{FAD} (Fig. 3A, □, solid line), it seems that they are almost coincident. This means that m_0, i.e. the unflavinylated mature form of the protein, was virtually absent at each time.

This conclusion was better pointed out by the re-plotting of the data shown in Fig. 3C, in which the specific fraction of flavinylated precursor (▲) and the specific fraction of flavinylated mature form (△) were separately reported as a function of time. The time course performed at 37°C (solid lines), clearly shows that almost all of the mMe_2GlyDH generated in the assay was in its m_{FAD} form at each incubation time. Conversely, at 60 min about 60% of the precursor was in its p_0 form, i.e. unflavinylated and unprocessed. This strengthens the proposal that the processing of p_0, namely the first step in pathway 2, is unlikely. Therefore, in our model system, the m_{FAD} seemed to be generated from pathway 1.

To better substantiate this point, supposing that flavinylation and processing were differently influenced by temperature, we made a kinetic analysis of the process of holoenzyme formation at 30°C (Fig. 2B, b), and report it in Fig. 3 as dashed lines. The appearance of the flavinylated forms of the protein ($p_{FAD}+m_{FAD}$) at 30°C was slower than that measured at 37°C (Fig. 3A, ●, compare dashed line with solid line). Indeed, the RL translated protein was only 55% flavinylated after 180 min incubation at 30°C, whereas at 37°C the maximal flavinylation was already obtained. On the contrary, the total amount of the mature form of the protein (m_0+m_{FAD}) was roughly the same at 30°C and 37°C after 60 and 180 min of incubation (Fig. 3B, compare dashed line with solid line). Therefore the flavinylation rate turned out to be more sensitive to temperature than processing. The amount of p_{FAD} at 30°C was sensibly lower than that detected at 37°C (Fig. 3A, ■, compare dashed line with solid line), with most of the precursor remaining unflavinylated (Fig. 3C, ▲, dashed line). On the other hand, still no significant formation of m_0 was observed. In fact, almost all of the mMe_2GlyDH generated in the assay was found again in its m_{FAD} form (Fig. 3C, △, dashed line).

Similar kinetics of flavinylation with respect to the processing was found also in intact mitochondria (Fig. 4). Freshly isolated mitochondria were incubated at 30°C with [^{35}S]Met-labelled RL translated pMe_2GlyDH and the import of the protein was demonstrated by the protection of intramitochondrially located proteins from externally added trypsin, essentially as described in [10]. The densitometric analysis of the autoradiograms in Fig. 4A is reported in Fig. 4B as a histogram. The RL translated pMe_2GlyDH, which remained unflavinylated in the absence of mitochondria (Fig. 4A, lanes 1, 2), was efficiently imported and processed by MPP, when incubated with mitochondria for 15 min. At this time, most of the total imported protein was represented by the mature form of the protein and about 20% of the protein imported remained unprocessed. Increase of the incubation time with mitochondria to 30 min induced a significant increase in the amount of the imported protein. The increase was essentially due to an increase in the amount of precursor, which represents at this time about 40% of the total imported protein. Uncoupling of mitochondria by carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) prevented the appearance

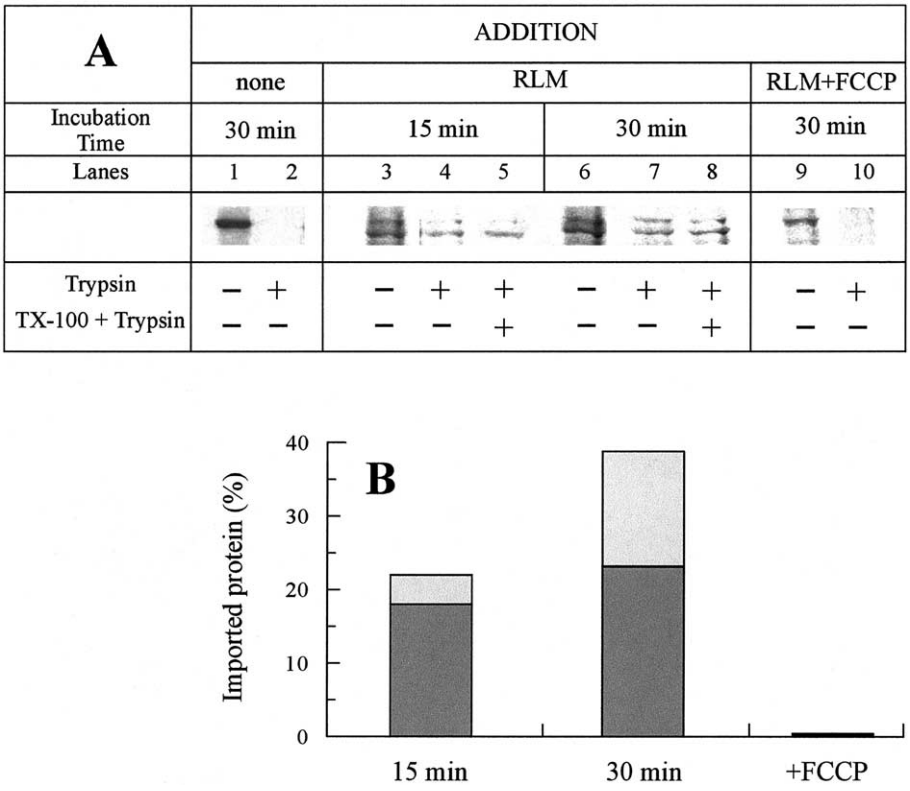


Fig. 4. Import of pMe_2GlyDH into isolated RLM. 5 μl of [^{35}S]Met-labelled pMe_2GlyDH RL translation product was incubated at 30°C in the absence or presence of freshly isolated RLM (8 mg/ml) in 35 μl of standard medium consisting of 2 mM HEPES, pH 7.4, 220 mM mannitol, 70 mM sucrose. Where indicated 1.25 μM FCCP was added to the incubation medium. Following 15 or 30 min incubation, the samples were either not treated or treated with trypsin, to assess the import of the protein, and then mitochondria were re-isolated by centrifugation. Where indicated, the mitochondrial pellets were lysed by 0.5% Triton X-100 and further treated with trypsin, to estimate flavinylation of the imported proteins. The proteins were analysed by SDS–PAGE and autoradiography. B: Densitometric analysis of the autoradiograms shown in A. The *y*-axis represents the amount of imported protein forms measured in the trypsin-treated samples (lanes 4, 7, 10) and expressed as % of the total amount of protein present in the trypsin-untreated samples (lanes 3, 6, 9). pMe_2GlyDH and mMe_2GlyDH are represented by the light grey bars and the dark grey bars, respectively.

of any imported protein band. As in the case of the experiment performed in the solubilised model system at 30°C, no m_0 was found inside mitochondria. The mature form of the protein imported into the organelle was found, in fact, at each time completely flavinylated, while the precursor partially flavinylated, as judged by the trypsin resistance of the proteins that were found in the Triton X-100 lysates of mitochondria re-isolated after the import.

4. Discussion

The aim of this work was to gain further insight into the mechanism by which the biogenesis of a mitochondrial covalently flavinylated enzyme, that is the matrix located Me_2-GlyDH, takes place.

We have previously demonstrated that an efficient and complete flavinylation of the mMe_2GlyDH strictly requires a protein factor localised in the mitochondrial matrix, the mtFSF [9]. The mitochondrial matrix actively participates in holo-Me_2GlyDH biogenesis also by providing the flavin cofactor [5,9]. Considering that Me_2GlyDH is synthesised in the cytosol as a precursor polypeptide, we asked whether mitochondrial component(s) are able to promote holoenzyme formation starting from the precursor.

The experimental data presented here clearly show that, in the presence of solubilised mitoplasts, concomitant with processing due to MPP a rapid and efficient flavinylation of the pMe_2GlyDH takes place.

Mitochondrial component(s), i.e. mtFSF [9] promote flavinylation independently from presequence processing by MPP. In this respect, the flavinylation requirements of Me_2GlyDH seem to differ from those of the flavoprotein subunit of the yeast SDH complex, a mitochondrial enzyme which contains the same type of FAD attachment, that is the histidyl(*N*3)–(8α)FAD bond. Indeed, in this case, covalent flavinylation occurs after that the mitochondrial presequence has been removed in the matrix by MPP (for review see [14]).

We targeted here the question regarding the sequence of events between the flavinylation and the processing during biogenesis of holo-Me_2GlyDH. The kinetic analysis of the appearance of the flavinylated proteins and of the precursor processing to the mature form catalysed by solubilised mitoplasts at 37°C, indicates that the flavinylation of the precursor, in our model system, preceded the precursor processing. This means that the sequence of events leading to the generation of the holo-Me_2GlyDH is that described in Fig. 2A as pathway 1.

To address the question of how relevant is flavinylation for precursor processing, the latter should be measured in the absence of FAD. Indeed, the omission of externally added FAD from the flavinylation assay did not significantly reduce the flavinylation rate (not shown). This is due to the presence

of endogenous FAD in the mitoplast extracts sufficient for flavinylation of the RL translated pMe_2GlyDH, as previously demonstrated with the RL translated mMe_2GlyDH [9]. Unfortunately, the attempt to deplete endogenous mitochondrial FAD, for example by treating the mitoplast extracts with nucleotide pyrophosphatase, resulted in a complete loss of RL translated protein.

Thus, a different experimental strategy was used. Lowering the temperature from 37°C to 30°C, we obtained a condition in which flavinylation rate (but not processing rate) was sensibly lowered. As a consequence of temperature decrease, the rate of appearance of p_{FAD} was slowed down more than 50%. Although the relative amount of p_0 was consequently higher than at 37°C, still no formation of m_0 from p_0 was observed. In other words, when kinetic conditions were unfavourable for p_0 flavinylation, processing still occurred following p_{FAD} formation. This consideration, together with the finding that EDTA inhibition of MPP catalysed processing did not influence at all flavinylation, allows us to propose that holo-Me_2-GlyDH is formed via pathway 1.

To confirm this conclusion and to exclude that MPP efficiency in the model system used is impaired by solubilisation of mitoplasts, investigation of kinetics of the flavinylation and the processing of pMe_2GlyDH in intact mitochondria were performed. In agreement with previous results [10], the RL translated pMe_2GlyDH is efficiently imported and processed into mitochondria. Following import, the mature form of the protein was found at each time completely flavinylated, that is to say that still no formation of m_0 was observed, even when the unprocessed precursor increased with time inside the organelle.

The physiological significance of results presented here merits further discussion. We have previously demonstrated that covalent flavinylation of both the precursor and mMe_2GlyDH can slowly proceed in the RL system, in agreement with an autocatalytic mechanism [9,10]. We confirm here the previous results [10], demonstrating that autoflavinylation of precursor in the RL system is much less efficient than that of the mature form. Contrarily, the mitoplast-assisted flavinylation starting from pMe_2GlyDH is more efficient than that observed when starting from the RL translated mMe_2GlyDH.

Thus, the mitochondrial presequence influences, in an opposite way, autoflavinylation in the RL system and mitoplast-assisted flavinylation. This would indicate that the presequence promotes a Me_2GlyDH conformation and/or interaction with mitochondrial stimulatory factor(s), that in turn accelerates flavinylation.

Based on (i) the relevant implication of mitochondrial components in the holo-Me_2GlyDH biogenesis, (ii) the finding that autoflavinylation is slowed down by the mitochondrial presequence, (iii) the evidence that the mitochondrial presequence is not strictly required for import of Me_2GlyDH into mitochondria in vivo [10], we can hypothesise an additional functional role for the presequence of pMe_2GlyDH. It may prevent flavinylation from occurring outside mitochondria and it may become a 'sensor' of the correct localisation of the protein, once the precursor has been imported, thus allowing a preferential recognition of the apo-protein by specific mitochondrial component(s), which allows flavinylation, as mtFSF.

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