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Plant glyoxysomal but not mitochondrial malate dehydrogenase can fold without chaperone assistance

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Abstract

Glyoxysomal (gMDH) and mitochondrial malate dehydrogenase (mMDH) from watermelon are synthesized as higher molecular weight precursor proteins. By overexpressing the precursor forms as well as the mature subunits with a histidine arm at the carboxy-terminus, it has been possible to purify relatively large amounts especially of the glyoxysomal precursor protein for studies of their refolding capacities after denaturation with guanidinium hydrochloride, heat or low pH. Glyoxysomal MDH and its precursor is capable of its spontaneous folding over a wide range of temperature conditions. Refolding can be enhanced by inclusion of BSA and ATP as stabilisers in the folding buffer. The N-terminal transit peptide of gMDH facilitates folding, but does not function as an intramolecular chaperon. Chemically denatured mitochondrial MDH requires chaperones for refolding. GroEL/GroES/ATP increase the yield and rate of watermelon mMDH folding dramatically while GroEL and Mg-ATP alone are not sufficient to provide folding assistance similar to the results with hydrophobic mammalian mMDH. The watermelon glyoxysomal MDH interacts with GroEL-like hydrophilic mammalian cytoplasmic MDH, a binding which has to be released by Mg-ATP before spontaneous folding can ensue. Interestingly, watermelon mMDH exhibited a much higher heat stability than gMDH or mammalian mMDH in the presence of BSA/ATP as well as GroEL/GroES/ATP. The differences between glyoxysomal and chaperone-assisted mitochondrial folding patterns are discussed.

Keywords: Glyoxysome; Mitochondrion; Isoenzyme; Chaperone; Folding; (*Citrullus vulgaris*)

1. Introduction

Glyoxysomal and mitochondrial malate dehydrogenase isoenzymes (L-malate-NAD-oxidoreductase, MDH, EC 1.1.1.37) in germinating watermelon seedlings (*Citrullus vulgaris*, Schrad.) are synthesized as higher molecular weight precursor proteins [1]. The glyoxysomal MDH precursor (pre-gMDH) is targeted into its organelle with an essential 37 amino acids long N-terminal transit peptide containing the peroxisomal targeting signal RL/I-X_5-HL (PTS2) [2,3]. A 27 amino acids long presequence with the characteristics of a positively charged amphiphilic α-helix directs the mitochondrial isoenzyme precursor (pre-mMDH) into the matrix of its organelle [4,5].

Glyoxysomal MDH from watermelon [2] and soybean [6] share with pumpkin glyoxysomal citrate synthase [7] and with microbody 3-ketoacyl-CoA thiolases from cucumber [8], mango [9] and rat [10,11] the special feature that they are synthesized with an N-terminal *cleavable* transit peptide containing the PTS2 targeting signal. On the other hand microbody enzymes from lower eucaryotes such as yeast are not synthesized as higher molecular weight precursor proteins and they do not necessarily use the same peroxisomal targeting signal as higher plants and mammals. Thus peroxisomal MDH [12] and citrate synthase [13–15] from *Saccharomyces cerevisiae* are targeted with the tripeptide Ser-Lys-Leu at the carboxy-terminus of the mature protein as a signal (PTS1). Peroxisomal 3-ketoacyl-CoA thiolase from *S. cerevisiae* [16,17], *Candida tropicalis* [11] and *Yarrowia lipolytica* [11] are targeted by a *non-cleavable* PTS2-signal located in the amino-terminal

Abbreviations: MDH, malate dehydrogenase; gMDH, glyoxysomal MDH; mMDH, mitochondrial MDH; PTS, peroxisomal targeting sequence; cMDH, cytoplasmic MDH; MDH-U, unfolded MDH; MDH-N, native MDH; GdnHCl, guanidinium hydrochloride.

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part of the mature protein. A cleavable targeting signal does not seem to be necessary for PTS2 mediated microbody import and this raises the question if the presequence of gMDH carries other functions such as a prosequence inhibiting enzymatic activity or a chaperone function comparable to that of the propeptide of carboxypeptidase Y from *S. cerevisiae* [18].

It has been shown previously that the presequence of gMDH of watermelon does not inhibit enzymatic activity [3], while a possible chaperone function is analysed in this paper. Specifically it is investigated, if the pre-gMDH after denaturation in 6 M guanidinium hydrochloride is renatured efficiently after dilution of the denaturant and if this is dependent on the presence of the presequence. In spite of high conservation of the cofactor binding domains and the strict conservation of the catalytic pocket of pig heart cytoplasmic (cMDH) and mitochondrial MDH (mMDH), these two enzymes respond very differently to chaperones during folding. The yield and rate of folding of cMDH is unaffected while that of mMDH is markedly increased in the presence of GroEL, GroES and Mg-ATP [19,20]. We therefore inquire if the plant mitochondrial MDH behaves with respect to folding like the pig heart mMDH and whether the gMDH is assisted in refolding by the GroEL-GroES-chaperone system or not. Comparison of the watermelon gMDH and mMDH primary structures reveals besides strict conservation of catalytic pocket and cofactor binding domain 65% overall identity and 76% similarity of amino acid residues [1].

To date it has been difficult to prepare large quantities of pre-mitochondrial proteins from animal cells. Expression in *Escherichia coli* (*E. coli*) apparently is not successful because of inherent proteolytic processing. We overexpressed watermelon pre-gMDH, gMDH, pre-mMDH, mMDH and the hybrid protein pre-g::mMDH with the glyoxysomal presequence fused to the mature mitochondrial subunit in *E. coli*. An affinity tag consisting of six histidine residues was added at the carboxy terminus for purification. It has been possible to purify relatively large amounts especially of malate dehydrogenase forms containing the glyoxysomal presequence. The folding behaviour of these proteins was investigated.

In order to analyse the influence of the glyoxysomal presequence on the stability of the precursor protein relative to the mature enzyme, the protocol for the carboxypeptidase Y analysis [18] was adapted, since it could be shown with this enzyme that the proenzyme is less stable than mature carboxypeptidase towards denaturation and this metastable state is thought to promote reversibility in the folding pathway. For the analysis of the folding behaviour of the different watermelon MDH proteins we took advantage of the reversible protein denaturation in vitro allowing measurement of refolding and association [21] and used regained enzymatic activity as the most sensitive criterion for correct folding. Chaperone-assisted refolding was determined with GroEL and GroES from *E. coli* as the best known prototype of this expanding class of proteins.

In this first comparison of the folding behaviour of higher plant glyoxysomal and mitochondrial malate dehydrogenases significant differences between these organelle-bound isoenzymes were identified. Glyoxysomal MDH is capable of spontaneous folding without chaperone assistance, while refolding of chemically denatured mitochondrial MDH is substantially facilitated by the presence of GroEL/GroES/ATP.

2. Materials and methods

2.1. Overexpression of cDNAs encoding pre-gMDH, gMDH, pre-g::mMDH, pre-mMDH and mMDH

The cDNA for pre-gMDH [2], pre-mMDH [4] and pre-g::mMDH [3] were cloned into the *E. coli*-expression vector pQE60 (Quiagen) between the restriction sites *Nco*I and *Bgl*II; the *Nco*I-site also provided the start codon. The necessary restriction sites at the 5'-end and 3'-end of the cDNA sequence were added by PCR. The mature subunits without presequence, gMDH and mMDH, including the *Nco*I- and *Bgl*II-site were prepared by PCR and cloned into the same vector. The sense primer for pre-gMDH was 5'-AGCCATGGGGACAGCCGATTCCGGATGTT-3' and for pre-mMDH was 5'-AGCCATGGGAAAGGC-CTCAATTCTCAGATCCGTT-3'. In both cases the GGA-codon for Gly was introduced between the start codon ATG and the following CAG for Gln or AAG for Lys, respective to restore the *Nco*I site. The sense primer for gMDH was 5'-AGCCATGGCTAAAGGCGGAGCTC-CCGGG-3', the sense primer for mMDH was 5'-AGC-CATGGCGACCGAATCGGTGCCGGAAAGGAAA-3', thus providing a start methionine in front of the mature subunit sequence. The antisense primer for pre-gMDH/gMDH was 5'-GCAGATCTGCTTCT-GATGAAGGAAACTCC-3', the antisense primer for pre-mMDH/mMDH was 5'-GCAGATCTATTTGCATT-TGCAAACTGGAT-3'. For the production of the hybrid-clone pre-g::mMDH, the pre-gMDH-sense primer was used in combination with the mMDH-antisense primer.

The pQE60-vector is based on the expression vector pDS/RBII [22,23] and adds an affinity tag consisting of six consecutive histidine residues at the C-terminus. Expression was carried out in the *E. coli* host M15 (Quiagen) for pre-gMDH or in the 'superhost' SG13009 [24] for the other MDH-species in 2 × TY-medium (16 g bactotryptone, 10 g yeast extract, 5 g NaCl in 1 l). In each case, protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The optimal expression time was tested on small scale cultures (20 ml). Optimal growth time after induction was 8 h for pre-gMDH and 4 h for the other MDH-species. *E. coli* cells were lysed with 3 ml lysis buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl,

100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 2 μg/ml leupeptin) per gram wet weights. Both the precursor and mature MDHs were found in the soluble fraction of the lysed *E. coli* and not in inclusion bodies. Purification of MDH was carried out according to manufacturers' recommendations. MDH from 800 ml culture were purified on a Ni^{2+}-affinity column (4 ml volume). MDH was eluted from the Ni^{2+}-affinity resin with a 30-ml pH 6.0 to pH 4.0 gradient.

2.2. Analysis and activity of the expressed proteins

The purity and identity of the expressed proteins were verified by SDS-PAGE and by Western blot analysis followed by immunodetection with anti-gMDH and anti-mMDH antibodies. Malate dehydrogenase activity was assayed in 100 mM sodium phosphate, pH 7.8, 0.2 (w/v) BSA, 188 μM NADH and 700 μM oxaloacetic acid by measuring the change in absorption at 340 nm. Protein estimation was carried out according to Bradford (Bio-Rad). The N-terminus of the purified proteins was sequenced by automated amino acid sequence analysis with an Applied Biosystems gas-phase sequencer model 470A, equipped with an on-line PTH-amino acid detector, model 120A.

2.3. Unfolding and refolding studies

Denaturation and refolding was carried out according to methods described in Refs. [21,25]. Chemically unfolded MDH (MDH-U) was made either by denaturation with guanidinium-hydrochloride (GdnHCl) or at pH 2.3 using the following reaction mixtures: 3.9 μl MDH, 3.0 μl 200 mM DTT and 55.5 μl 8 M GdnHCl in 50 mM Tris-HCl pH 8.0 (7.11 M final concentration) or 0.2 M glycin, pH 2.3 (0.18 M glycin, pH 2.3, final concentration), respectively. The final protein concentration was 50 μg/ml for watermelon MDH and 300 μg/ml for pig heart MDH (Boehringer-Mannheim, Germany). Denaturation was carried out for 1 h on ice (guanidinium-hydrochloride) or for 1 h at 15°C (pH 2.3), respectively. As a control native MDH (MDH-N) was treated under the same conditions, except that only buffer (50 mM Tris-HCl, pH 8.0) instead of denaturing agent was added. Renaturation was started by 50-fold dilution into folding buffer: 50 mM Tris-HCl, pH 7.6, 10 mM KCl, 4 mM DTT and 7 mM $MgCl_2$. In some experiments the folding buffer also contained 0.05% BSA with 1.0 mM ATP or 0.4 μg/μl GroEL (0.4×10^{-6} M of the 14-mer) with 0.03 μg/μl GroES (0.4×10^{-6} M of the 7-mer) (both obtained from Boehringer Mannheim, Germany) and 1 mM ATP. Refolding after chemical denaturation was carried out at 0°C, 15°C or 36°C. At appropriate time intervals MDH-activity was measured on an aliquot of the reaction mixture. One hundred percent enzymatic activity is defined as that displayed by an equal amount of MDH that had not been denatured before dilution but was otherwise treated identically. Temperature-induced unfolding was investigated as follows: MDH-N (0.96 μg/μl for watermelon MDH; 0.3 μg/μl for pig heart MDH) was diluted 50-fold into folding buffer (see above) containing either BSA/ATP or GroEL/GroES/ATP and incubated at 46°C or 48°C. At appropriate time intervals MDH activity was measured in an aliquot. One hundred percent activity is defined as the activity displayed in the assay before starting the heat treatment.

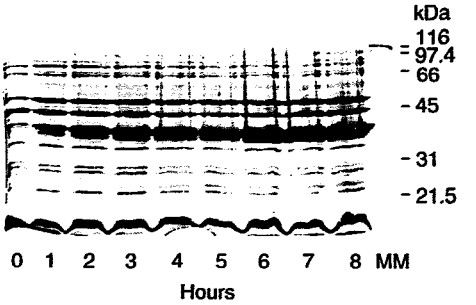

Fig. 1. Increasing expression of pre-gMDH from 1 to 8 h in 1 ml aliquots from growing culture of *E. coli* transformed with expression vector pQE 60 containing the DNA insert encoding watermelon pre-gMDH. The proteins of the bacterial pellet were separated by 12.5% SDS-PAGE and stained with Coomassie brilliant blue. MM, molecular weight marker.

3. Results

3.1. Expression and purification of malate dehydrogenases

Pre-gMDH, gMDH, pre-g::mMDH, pre-mMDH and mMDH from watermelon were overexpressed in *E. coli* using the high level expression vector pQE60 (Quiagen), which adds six consecutive histidine residues at the carboxy-terminus for purification on a Ni^{2+}-containing resin. Fig. 1 illustrates the expression of watermelon pre-gMDH with increasing time after induction. The expression pattern of the other MDH species was similar. Fig. 2a shows the purified MDH species when analysed on a SDS-polyacrylamide gel: gMDH from watermelon and mMDH from pig heart migrate as expected with a mass of 33 kDa, whereas mMDH from watermelon, in spite of its similar polypeptide length, migrates with a mass of 38 kDa. This anomalous migration behaviour is also observed upon immunoprecipitation of mMDH from a crude watermelon protein extract [26]. The migration behaviour of the higher molecular weight precursors pre-gMDH, pre-g::mMDH and pre-mMDH is determined by the migration characteristics of the mature protein [5]. Western blot analysis and decoration with anti-gMDH- or anti-mMDH-antibodies demonstrated that the expressed and purified proteins indeed were malate dehydrogenases (Fig. 2b). The yield of puri-

fied MDH from 800 ml *E. coli* culture was approximately 5 mg. All purified MDH species were enzymatically active, possibly due to the chaperones and ATP present in the *E. coli* lysate. The specific activities were as follows: pre-gMDH 2.450 U/mg protein, pre-g::mMDH 2.370 U/mg protein, gMDH 4.780 U/mg protein, pre-mMDH 1.450 U/mg protein and mMDH 2.150 U/mg protein. In comparison the isoenzymes purified from a crude watermelon protein extract exhibit an activity of 9000 U/mg protein for gMDH and 5.500 U/mg protein for mMDH [27]. This suggests that some of the recombinant enzyme molecules were misfolded and inactive. It has to be noted that both recombinant gMDH and gMDH purified from watermelon cotyledons had higher specific activity than the two mMDH enzymes. The amino terminus of the purified MDHs was sequenced for confirmation and especially to ensure that the presequences had not been degraded. Authentic amino termini were found for both, mature gMDH and mMDH. The sequences were Ala-Lys-Gly-Gly-Ala-Pro-Gly-Phe-Lys-Val- and Ala-Thr-Glu-Ser-Val-Pro-Gly-Arg-Lys-Val-respectively [1], the start methionine being removed by *E. coli*. Sequence analysis of the purified precursor forms yielded several amino termini. The pre-gMDH preparation as well as the pre-g::mMDH preparation contained 90% of the sequence Gly-Gln-Pro-Ile-Pro-Asp-Val-Asn-Gln-Arg- (i.e., start methionine removed) and 10% of the sequence Ile-Ser-Ala-His-Leu-His-Pro-Pro-Lys-Ser- (i.e., 13 amino acids of the 37 amino acids long presequence were missing due to cleavage after the second Arg). This implies, that the pre-gMDH- and the pre-g::mMDH-preparation contained 90% full-length precursor, while 10% were slightly truncated by a trypsin-like protease. The pre-mMDH preparation exhibited three sequences: 15% had the N-terminal sequence Gly-Lys-Ala-Ser-Ile-Leu-Arg-Ser-Val-Arg- (i.e., only the start methionine had been removed), 15% had the sequence Ser-Val-Arg-Ser-Ala-Val-Ser-Arg-Ser-Ser- (i.e., 7 amino acids had been eliminated) and 70% had the sequence Ser-Ala-Val-Ser-Arg-Ser-Ser-Ser-Ser-Asn- (i.e., 10 amino acids were missing from the 27 residues long presequence). This means that the preparation contained only 15% full-length precursor, while the remaining 85% were cleaved by a trypsin-like protease after arginine residues. The transit peptides, especially the mitochondrial one, seem to be more sensitive towards proteolytic attack than the mature subunit. We can conclude that the precursor proteins are combined to an enzymatically active homodimer with the transit peptides floating on the side and therefore being unprotected even in the presence of appropriate trypsin inhibitors. Studies with pre-mMDH have to allow for the circumstance that only 15% of the molecules contain a full-length transit peptide, and this limits the conclusions that can be drawn from the 'presence' of the mitochondrial presequence.

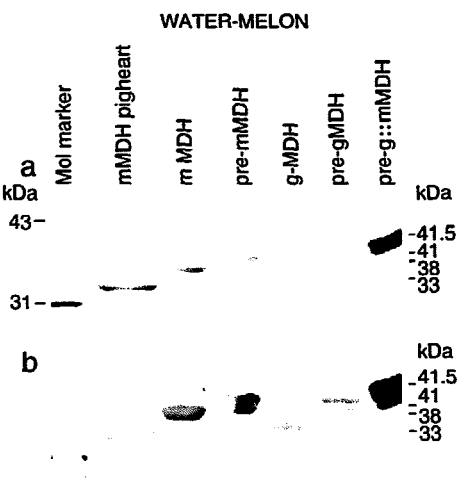

Fig. 2. Overexpressed and purified watermelon MDH species were separated by 12.5% SDS-PAGE, staining with Coomassie brilliant blue (Fig. 2a) and identified by decoration of Western blots with anti-gMDH antibodies (Fig. 2b). The anti-gMDH antibodies exhibit cross-reaction with mMDH, especially when high amounts of protein are loaded (5 μg protein per lane).

3.2. Glyoxysomal malate dehydrogenase is capable of spontaneous folding, which is not inhibited but facilitated by the N-terminal transit peptide

Pre-gMDH and gMDH were denatured with 7.11 M guanidinium hydrochloride (GdnHCl) on ice for 1 h. No enzymatic activity was detectable at this time. Refolding was assayed in folding buffer at 0°C and 15°C (Fig. 3A). Refolding of gMDH was spontaneous, the enzyme solution regaining 18% of its activity after 30 min at 0°C and 85% of its activity after 30 min at 15°C. The N-terminal presequence did not inhibit this refolding ability, but rather enhanced it slightly: pre-gMDH regained 24% activity after 30 min at 0°C and 100% activity after 30 min at 15°C. In the presence of BSA/ATP refolding was even faster in agreement with the stabilising effect of these compounds on folding intermediates of NADH-dependent enzymes [28,29] (data not shown). The influence of the chaperone system GroEL/GroES/ATP was assayed at 36°C and compared with the effect of adding BSA/ATP to the folding buffer (Fig. 3B). In the presence of BSA/ATP gMDH regained 75% activity after 30 min and pre-gMDH attained 100% activity after 10 min. Spontaneous folding was so fast that it started immediately after 50-fold dilution of the denaturant in the photometer-cuvette during measurement of the enzymatic activity which caused a recovery at 'time point zero' of 10–20%. After 30 min the pre-gMDH preparation gave a recovery value for MDH activity of 130%. This remarkable increase in activity might either be due to repair of some of the misfolded

recombinant enzyme or an improved dimer formation with the folding buffer used. The presence of GroEL/GroES/ATP instead of BSA/ATP during folding accelerated the recovery of MDH activity marginally (Fig. 3B). Denaturation of gMDH and pre-gMDH at pH 2.3 for 1 h at 15°C and refolding in folding buffer at 0°C and 15°C and at 36°C in the presence of BSA/ATP or GroEL/Gro ES/ATP gave a similar picture (data not shown). We conclude, that pre-gMDH and gMDH have a remarkable capability of spontaneous folding, which can be enhanced by stabilisers and chaperone assistance. Presence of the transit peptide in the pre-gMDH facilitated the spontaneous and assisted folding ability of gMDH.

Since folding of gMDH at 36°C after chemical denaturation takes place without chaperone requirement, we tried to find conditions under which chaperone assistance is necessary. gMDH and pre-gMDH were incubated at 46°C (Fig. 5A) and 48°C (Fig. 5C) for heat-induced unfolding, and loss of enzymatic activity was determined. During incubation at 46°C in folding buffer in the presence of BSA/ATP 40% activity of pre-gMDH and 55% activity of gMDH, respectively, were lost within the first 5 min. After that time the proteins remained remarkably stable exhibiting after 30 min still 45% (pre-gMDH) and 24% (gMDH) of the original activity. After incubation in folding buffer without BSA/ATP for 30 min pre-gMDH and gMDH exhibited only 15% of the original activity. Addition of BSA *or* ATP improved the heat stability, but not as

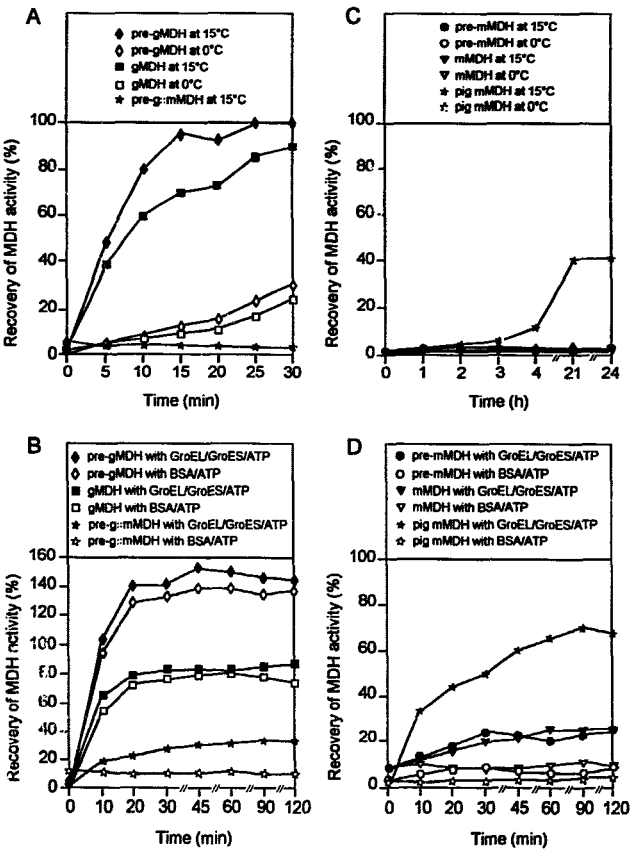

Fig. 3. Folding of MDH after denaturation with 7.11 M guanidinium-hydrochloride (1 h at 0°C). Folding of the polypeptide was started by 50-fold dilution into 50 mM Tris-HCl (pH 7.6), 7 mM $MgCl_2$, 10 mM KCl and 1 mM DTT. One hundred percent activity is defined as the activity displayed by an equal amount of MDH that had not been denatured before dilution but was otherwise treated identically. (A) Folding of pre-gMDH, gMDH and pre-g::mMDH in the absence of BSA or chaperones at 0°C or 15°C. (B) Folding of pre-gMDH, gMDH and pre-g::mMDH in the presence of BSA/ATP or in the presence of GroEL/GroES/ATP at 36°C. (C) Folding of watermelon pre-mMDH and mMDH and pig mMDH in the absence of BSA or chaperones at 0°C or 15°C. (D) Folding of watermelon pre-mMDH and mMDH and pig mMDH in the presence of BSA/ATP or in the presence of GroEL/GroES/ATP at 36°C.

efficient as the simultaneous addition of BSA and ATP (data not shown). Incubation of pre-gMDH and gMDH at 46°C in the presence of GroEL/GroES instead of BSA had opposite effects on the heat stability: pre-gMDH seemed to be destabilized while gMDH became relatively more stable (Fig. 5A). We conclude from these data that both pre-gMDH and gMDH are significantly stabilized by BSA and ATP. They do not necessarily aggregate at 46°C in the absence of chaperones. On the other hand, pre-gMDH has a slightly higher stability compared to gMDH at 46°C; chaperone-assistance is not yet necessary and pre-gMDH has to get rid of GroEL/GroES before it can fold spontaneously; as a consequence pre-gMDH seems to be less heat-stable in the presence of chaperones instead of BSA. To prove this hypothesis we incubated pre-gMDH and gMDH at 48°C under the same conditions (Fig. 5C). At this temperature both pre-gMDH and gMDH were inactive after 20 min and were perhaps somewhat more heat-tolerant in the presence of chaperones instead of BSA.

3.3. Mitochondrial malate dehydrogenase from watermelon requires chaperone assistance for folding similar but not equal to pig heart mMDH

Pre-mMDH, pre-g::mMDH and mMDH from watermelon were chemically denatured and refolding was assayed at 0°C and 15°C in folding buffer without BSA/ATP. As a control, mammalian mMDH was examined, which is known to refold efficiently only in the presence of GroEL/GroES/ATP [25,30]. After denaturation with GdnHCl watermelon mMDH and pre-mMDH exhibited no spontaneous refolding within 24 h (Fig. 3C). This is in marked contrast to pre-gMDH and gMDH (Fig. 3A). Pre-g::mMDH behaved like pre-mMDH and mMDH and exhibited no spontaneous folding at 15°C (Fig. 3A). Mammalian mMDH regains no measurable activity at 0°C within 24 h, whereas at 15°C it had attained 12% activity after 4 h and 40% after 24 h (Fig. 3C) in agreement with previous observations [25]. Denaturation with GdnHCl and refolding in the presence of BSA/ATP of mammalian mMDH at 36°C results in irreversible aggregation, whereas refolding in the presence of GroEL/GroES/ATP leads to 70% activity within 2 h (Fig. 3D). Mitochondrial mMDH from watermelon regains only 25% activity in the presence of chaperones within 2 h; the pre-mMDH preparation behaves similarly. Refolding in the presence of BSA/ATP remains below 10%. The hybrid pre-g::mMDH behaved like mMDH regaining at 36°C 30% activity within 2 h in the presence of GroEL/GroES/ATP (Fig. 3B). Clearly, watermelon mMDH is accessible to chaperone-assisted folding after denaturation with GdnHCl, but the chaperones are not as efficient as with mammalian mMDH. A different picture is obtained when denaturation is effected with low pH. Interestingly in this case, watermelon mMDH refolded spontaneously after denaturation at pH 2.3 more readily than mammalian mMDH at both 0°C and 15°C (Fig. 4A). Chaperone-assisted folding was even more pronounced when refolding took place at 36°C in the presence of GroEL/GroES/ATP (Fig. 4B): chaperone-assistance increased refolding of the enzymes in the pre-mMDH preparation within 30 min to 70%, that of mMDH from watermelon to around 40%, while only 10% of mammalian mMDH was refolded.

During heat-induced unfolding at 46°C in the presence of BSA/ATP, watermelon mMDH lost 30% activity within the first 5 min and remained from then on remarkably stable, exhibiting after 1 h at 46°C still 50% of its original activity; incubation at 46°C in the presence of chaperones

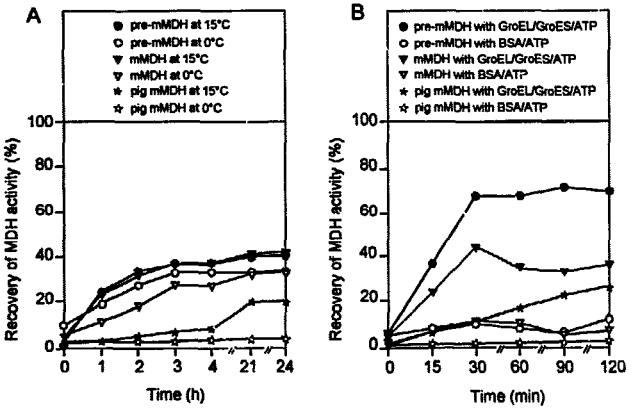

Fig. 4. Folding of mMDH after denaturation with 0.18 M glycine (pH 2.3) (1 h at 15°C). Start of folding and definition of 100% activity was as in Fig. 3. (A) Folding of watermelon pre-mMDH and mMDH and pig mMDH in the absence of BSA or chaperones at 0°C or 15°C. (B) Folding of watermelon pre-mMDH and mMDH and pig mMDH in the presence of BSA/ATP or in the presence of GroEL/GroES/ATP at 36°C.

instead of BSA did not increase the heat stability (Fig. 5B). Under these conditions mitochondrial MDH had a higher heat stability than glyoxysomal MDH (c.f., Fig. 5B versus 5A). The hybrid protein pre-g::mMDH had a heat stability similar to mMDH at 46°C (Fig. 5A), and was also more stable than pre-gMDH/gMDH at 48°C (Fig. 5C); addition of chaperones instead of BSA had no significant effect. Mammalian mMDH on the other hand lost within the first 5 min 90% activity in the presence of BSA/ATP, a denaturation which was slightly delayed by chaperones (Fig. 5B). Mammalian mMDH served as an important control, since it is known to require chaperones to prevent denaturation even at 36°C [19].

We conclude from our data, that watermelon mMDH does not possess the spontaneous folding ability that is characteristic for gMDH. Watermelon mMDH is susceptible to chaperone-assisted refolding similar to mammalian mMDH. However, the refolding kinetics after denaturation with GdnHCl or extreme pH differs for plant and mammalian mMDH. Surprisingly, watermelon mMDH displays a higher stability during heat-induced unfolding than glyoxysomal MDH or mammalian mMDH. The hybrid protein pre-g::mMDH behaves like mitochondrial MDH.

3.4. Watermelon gMDH and mMDH isoenzymes exhibit different affinities for GroEL

Substrate proteins differ in affinities to chaperones and their nucleotide complexes. Some proteins dissociate quickly and spontaneously from GroEL, others require only Mg-ATP and still others need both GroES and Mg-ATP [20]. Since gMDH refolds spontaneously without

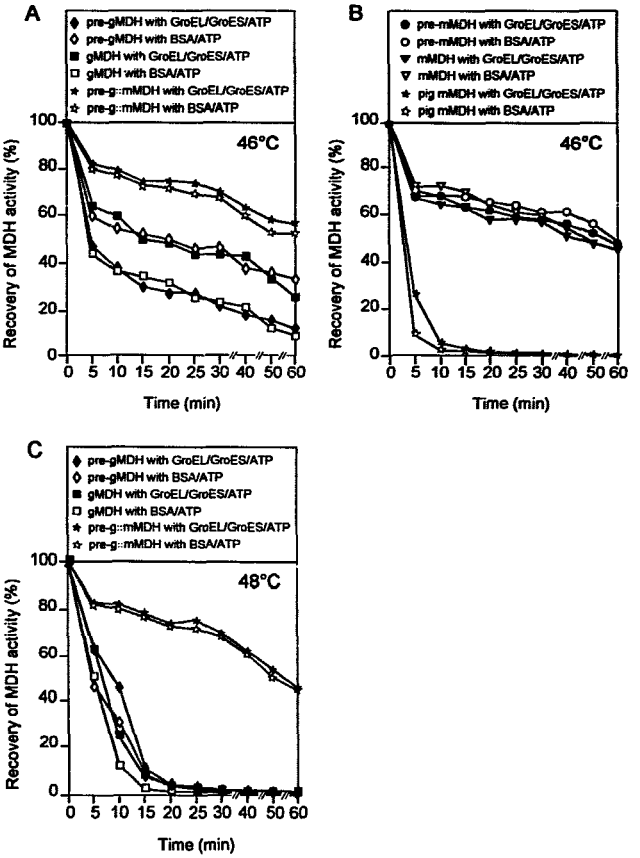

Fig. 5. Chaperone- or BSA-mediated stabilisation during heat-induced denaturation at 46°C (A and B) or 48°C (C). One hundred percent activity is defined as the activity displayed in the assay before starting the heat treatment. (A) and (C) Stabilisation of pre-gMDH, gMDH and pre-g::mMDH by BSA/ATP or by GroEL/GroES/ATP. (B) Stabilisation of watermelon pre-mMDH and mMDH and pig mMDH by BSA/ATP or by GroEL/GroES/ATP.

chaperone-assistance, we wanted to know if gMDH reacts with GroEL in the same or a different way to mMDH. Glyoxysomal MDH was denatured with GdnHCl (gMDH-U); refolding was followed at 36°C in folding buffer with either GroEL + GroES + ATP, or with GroEL + GroES alone for 30 min followed by addition of ATP. Alternatively, GroEL + ATP was first provided alone and GroES was added after 30 min. Folding buffer containing BSA + ATP served as a control. In parallel non-denatured gMDH (gMDH-N) was incubated in folding buffer containing BSA + ATP and used as undenatured control activity (Fig. 6A). In this experiment gMDH regained 90% activity after 30 min in folding buffer with GroEL + GroES + ATP, and 60% with BSA + ATP. Addition of GroEL + GroES to the denatured gMDH enzyme in the absence of ATP prevented folding almost entirely. Upon addition of ATP to the gMDH/chaperone complex the folding process ensued instantaneously: after 10 min 90% activity was regained. Similar results were obtained when the folding buffer contained only GroEL to begin with, and GroES + ATP were added after 30 min (data not shown). In buffer containing GroEL + ATP refolding proceeded slowly to yield 40% activity after 30 min. Subsequent addition of GroES did not accelerate refolding. These results demonstrate that denatured gMDH molecules react with chaperone molecules which prevent folding to active enzyme until ATP is provided to release the chaperones from the extended polypeptide chain. Addition of ATP without GroES supports dissociation of gMDH from GroEL to a limited extent and slower than in the presence of the complete system (GroEL + GroES + ATP). After the ATP-consuming dissociation of GroEL the glyoxysomal MDH probably folds spontaneously. Denatured watermelon mMDH behaves in a very different way (Fig. 6B). Spontaneous folding in the presence of BSA + ATP is not possible. In the presence of GroEL + GroES + ATP 25% refolding can be obtained after 60 min. Addition of GroEL + GroES without ATP inhibits folding, but keeps the mMDH in a folding competent form. As a consequence subsequent addition of ATP starts folding. In contrast to gMDH, addition of GroEL + ATP without GroES does not permit the folding of mMDH. In the absence of GroES mitochondrial MDH will be released by ATP from GroEL in an unfolded aggregated form, which cannot be rescued and refolded by subsequent addition of GroES.

4. Discussion

4.1. The functions of the higher plant glyoxysomal and mitochondrial presequences

Recombinant expressed precursor and mature forms of the glyoxysomal and mitochondrial watermelon MDH provide a unique opportunity to analyse the folding stability and refolding capacity of these two isoenzymes with a mature protein amino acid sequence similarity of 76%.

While the PTS2 signal in the glyoxysomal presequence is necessary and sufficient to target gMDH and mMDH into microbodies [3,5], it does not inhibit enzymatic activity. Likewise the mitochondrial transit peptide is necessary for targeting mMDH, but experiments to determine the activity of pre-mMDH have so far been inconclusive. Both mature and precursor glyoxysomal MDH have a remarkable capability of unassisted refolding after denaturation in 7.11 M GdnHCl (Fig. 3A,B) or low pH. Watermelon mitochondrial MDH on the other hand has only a limited capability of unassisted refolding after denaturation and

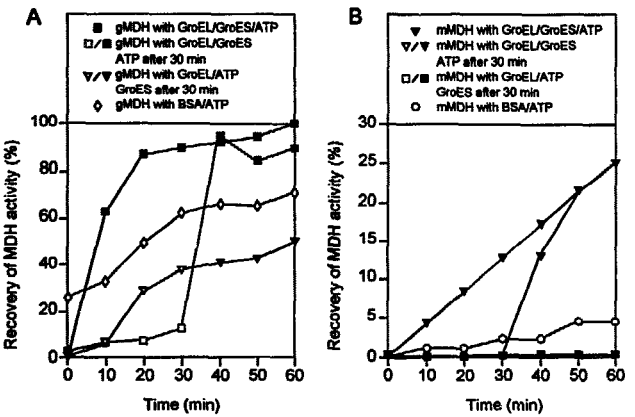

Fig. 6. Requirements for chaperone-mediated folding of gMDH (A) and watermelon mMDH (B) at 36°C. Denaturation was carried out with 7.11 M GdnHCl (1 h at 0°C). Refolding was started by 50-fold dilution into folding buffer (50 mM Tris-HCl, pH 7.6, 7 mM MgCl, 10 mM KCl and 1 mM DTT) containing either GroEL and GroES and ATP or GroEL and GroES with addition of ATP after 30 min or GroEL and ATP with addition of GroES after 30 min or BSA and ATP.

requires addition of chaperones to increase the folding efficiency (Fig. 4B and 6B). With the caveat of the limited amount of untruncated pre-mMDH that could be tested in a mixture with truncated pre-mMDH, no indication of an effect of the presequence on in vitro refolding was obtained. When the glyoxysomal presequence is attached to the mature mitochondrial MDH (pre-g::mMDH), the refolding behaviour is similar to that of mMDH and unlike that of gMDH (Fig. 3A,B). In watermelon it is thus the mature part of the enzyme protein that determines the capacity and characteristics of (re-)folding. While comparisons of the folding behaviour of plant with mammalian or yeast microbody enzymes are not yet possible, the results with the watermelon mMDH precursor can be compared to those reported for mammalian mitochondrial enzymes. Both the higher molecular weight precursor of mammalian aspartate aminotransferase and its mature form can be efficiently renatured after unfolding with GdnHCl and the aminoterminal transit peptide did not inhibit the refolding [31,32]. Likewise, the presequence in the mammalian mitochondrial ornithine transcarbamylase does not prevent folding. However, after denaturation only 18% of the initial enzymatic activity could be regained after several hours. The obtained precursor ornithine transcarbamylase had a lower specific activity than the mature subunit under similar conditions and the precursor protein trimer appeared less compact and stable than the trimer of the mature protein [33]. Addition of GroEL/GroES/ATP increased folding and assembly of ornithine transcarbamylase [34]. Unfortunately the refolding behaviour of pig pre-mMDH remains unknown. The glyoxysomal presequence had a pronounced effect when folding was carried out at 36°C (Fig. 3B). In the presence of BSA/ATP or GroEL/GroES/ATP, unfolded or misaggregated dimers present in the recombinant enzyme preparation could be normalised with the presequence attached but not in its absence (130% recovery!).

A large number of prokaryotic as well as eucaryotic proteins are produced with amino terminal propeptides. These amino-terminal extensions are in some cases essential for mediating proper folding of the proteins in question and are then termed intramolecular chaperones. Production in the form of a precursor is an obligatory step for the production of active subtilisin [35], a lytic protease [36] and carboxypeptidase Y [18]. Maturation of proteins which possess intramolecular chaperones involves folding, autoprocessing and degradation of the autoprocessed propeptide. Apart from being transiently attached via peptide linkage to the molecules they fold, other significant differences between molecular chaperones and intramolecular chaperones also exist [35]: (1) intramolecular chaperones are essential for folding of the protein they are attached to. Molecular chaperones on the other hand may act by facilitating folding, even if the protein can fold in the absence of a molecular chaperone with low efficiency. Glyoxysomal MDH is capable of spontaneous and fast folding without the amino terminal transit peptide. (2) A folded protein can also interact with its intramolecular chaperone after the completion of the folding process. It acts as a competitive inhibitor by, e.g., binding to the active site of the enzyme and prevents its enzymatic activity. Again pre-gMDH is enzymatically active; the presequence does not act as a competitive inhibitor. Based on both criteria, it is clear that the transit peptide of pre-gMDH does not represent an intramolecular chaperone.

4.2. Interaction of glyoxysomal and mitochondrial MDH with GroEL / GroES / ATP chaperones

After synthesis in the cytosol, most mitochondrial proteins must traverse the mitochondrial double membrane envelope at contact sites to reach their functional location. During this process, the proteins become unfolded prior to import with the aid of cytosolic chaperones and after crossing the two lipid bilayers they refold to the appropriate conformation with the assistance of mitochondrial molecular chaperones [37,38]. Spontaneous refolding of the mitochondrial precursor protein might be an undesirable property for polypeptide chains which have to traverse an envelope in extended form. Since the glyoxysomal MDH with or without the presequence can fold rapidly without chaperone assistance, the question arises, whether import into glyoxysomes requires unfolding of the polypeptide chain. In contrast to mitochondria and chloroplasts, microbodies have a single membrane which allows transit of proteins in folded and subunit assembled form using several targeting sequences and receptors [39,40] In such a case cytosolic chaperones might not be needed to keep the glyoxysomal proteins in an extended form prior to import. Although involvement of 70 kDa heat shock proteins in peroxisomal import has been demonstrated [41], they may not play the same role as in mitochondrial import.

Watermelon gMDH and mMDH react in a different manner with the chaperone system GroEL/GroES/ATP. Glyoxysomal MDH folds spontaneously over a wide range of conditions, whereas mMDH needs chaperone assistance for folding. We therefore investigated whether gMDH is able to react with the GroEL/GroES system. The mechanism by which correctly folded proteins are recovered from stable complexes with GroEL is not well understood. Certain target proteins require ATP and GroES, while others can dispense with the co-chaperoning. Schmidt and co-workers examined the chaperone-assisted folding of ribulose-1,5-bisphosphate carboxylase (Rubisco) from *Rhodospirillum rubrum*, pig heart citrate synthase and mitochondrial malate dehydrogenase, three proteins that at least under certain conditions require both chaperoning components for successful reactivation [42]. In all cases the need for GroES depended on the folding environment. Under non-permissive conditions, when unassisted spontaneous folding could not occur, reactivation to the native

state required the complete chaperone system (GroEL + GroES + Mg-ATP). Under permissive conditions, that allowed spontaneous folding, the presence of GroES was no longer mandatory. Upon the addition of ATP alone, all three target proteins could be released from GroEL in a form, that was capable of reaching the native state. In the permissive setting, GroES merely accelerated the rate of the ATP-dependent release process. In this respect watermelon mMDH is similar to mammalian mMDH [25] since it requires, after chemical denaturation for folding at 36°C, the complete system: in the presence of GroEL + ATP alone incompletely folded mMDH species are released from GroEL, which are prone to aggregation and cannot be rescued by later addition of GroES. Glyoxysomal MDH on the other hand is under the same conditions capable of spontaneous folding. Folding in the presence of GroEL + ATP is possible, but retarded by the slow ATP catalysed dissociation of GroEL, which in turn inhibits the spontaneous folding. In the presence of the complete system folding is faster then in the presence of BSA + ATP. Addition of GroEL + GroES alone suspends folding, which, however, is immediately resumed upon addition of ATP.

We conclude that glyoxysomal MDH is by all criteria a protein capable to react with chaperones. Heat-induced unfolding at 46°C creates permissive conditions for pre-gMDH, because folding in the presence of BSA + ATP was slightly faster than in the presence of chaperones. For gMDH 46°C leads to non-permissive conditions, since folding was improved in the presence of chaperones. Temperature modulation of folding conditions is limited to a narrow interval. Incubation of pre-gMDH and gMDH at 48°C provided non-permissive conditions for both proteins, since folding of both was improved in the presence of chaperones. *Escherichia coli* has mastered the feat of being able to grow over a span of 40°C Celsius degrees, from 8°C to 48°C [43]. Therefore 48°C might be the highest temperature at which folding assistance by GroEL/GroES can be expected.

Watermelon glyoxysomal MDH behaves similar to mammalian cytoplasmic MDH [20]. The hydrophilic and stable cytoplasmic MDH is bound by GroEL and released by Mg-ATP under conditions when the hydrophilic and unstable mMDH requires both Mg-ATP and GroES. Cytoplasmic (cAspAT) and mitochondrial aspartate aminotransferase (mAspAT) from mammals is another pair of isoenzymes, where homologous proteins exhibit different affinities for GroEL [44]. GroEL arrests the refolding of mAspAT throughout the temperature range of 0–45°C. Adding Mg-ATP released very little mAspAT from GroEL; but addition of GroES and Mg-ATP yielded significant refolded and active mAspAT. In the case of cAspAT GroEL slowed down refolding at 0°C, and arrested it completely at 30°C. Mg-ATP alone was sufficient to effect the release of cAspAT from GroEL at any temperature examined. The simplest explanation for this diversity of behaviour is that the release depends on two factors: (i) the binding affinity between the unfolded protein and the chaperone surface, and (ii) the free energy of folding (i.e., the stability) of the substrate protein. If the unfolded protein binds weakly and the folded state is very stable, then the substrate will be released easily. This implies that the hydrophobicity of the surface of the protein, which interacts with the chaperones or the solution, is one of the determining factors.

Hydrophobic interactions are low at a low temperature and high at a high temperature. We can assume that gMDH is not very hydrophobic. At high temperature hydrophobic interactions would not be strong enough for aggregation to take place with spontaneous folding as a result. Mammalian mMDH is stable at low temperature. Since it is more hydrophobic, strong interaction occurs at a high temperature and aggregation without chaperone-assistance is the result. Interestingly plant mMDH needs chaperone-assistance for folding after chemical denaturation similar to mammalian mMDH, but has a significantly better heat-stability at 46°C.

Comparison of watermelon mMDH with mammalian, yeast and *E. coli* MDH gives 55–60% overall sequence identicality of residues; on the other hand, the amino acid sequences of mammalian mMDH and cMDH show only about 23% sequence identicality, whereas a comparison of watermelon gMDH and mMDH gives 65% overall identicality [1]. Nevertheless glyoxysomal MDH is similar to cytoplasmic MDH and AspAT in that it exhibits a broader range of permissive conditions for chaperone unassisted folding than mitochondrial isoenzymes. A few amino acid changes are enough to modulate the hydrophobic surface of a protein and thereby alter its capacity to react with chaperones. The relative high sequence similarity of watermelon glyoxysomal and mitochondrial MDH may permit by domain swapping to identify protein regions of importance for chaperone interaction.

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