In vivo and *in vitro* folding of a recombinant metalloenzyme, phosphomannose isomerase

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Phosphomannose isomerase (PMI) catalyses the interconversion of mannose 6-phosphate and fructose 6-phosphate in prokaryotic and eukaryotic cells. The enzyme is a metalloenzyme which contains 1 mol of zinc per mol of enzyme. Heterologous expression of the cDNA coding for the *Candida albicans* enzyme in the prokaryotic host *Escherichia coli* results in an expression level of up to 30% of total *E. coli* protein. Ten percent of recombinant PMI is expressed in the soluble fraction and 90% in inclusion bodies. Inclusion of a high level of zinc in the fermentation medium resulted in a fourfold increase in soluble protein. Co-expression of the bacterial chaperones, GroES and GroEL, resulted in a proportional twofold increase in soluble PMI while causing an overall decrease in the PMI expression level. Folding denatured PMI *in vitro* required reductant and zinc ions. The yield of renatured protein was increased by folding in the presence of GroEL and DnaK in an ATP-independent manner. The refolding yield of denatured soluble enzyme from a guanidine solution was threefold higher than that of folding monomerized inclusion body protein solubilized in guanidine hydrochloride. This suggests that a proportion of recombinant protein expressed in *E. coli* inclusion bodies may be irreversibly denatured.

INTRODUCTION

Phosphomannose isomerase (PMI) (EC 5.3.1.8) catalyses the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate in prokaryotic and eukaryotic organisms. It was first identified by Gracy and Noltmann [1] in yeast and shown to be a 50 kDa metalloprotein containing an essential zinc ion. *Candida albicans* PMI belongs to the Class I type of three structurally unrelated proteins identified with PMI activity [2].

PMI has been shown to be essential for the survival of yeasts since temperature-sensitive (Ts) mutants in the *pmi* gene locus are lethal [3,4]. We were therefore interested in studying the enzyme's mechanism in more detail in a search to find inhibitors of the enzyme for therapeutic use. In order to obtain sufficient amounts of protein for structure–function studies and to determine the three-dimensional structure of the enzyme by X-ray crystallography, we have expressed the *C. albicans* enzyme recombinantly in *Escherichia coli* [5]. The enzyme is expressed at very high levels under the control of the heat-inducible p*L* promoter, reaching up to 30% of the total protein. However, 90% is expressed in the inclusion body fraction. We therefore attempted to increase the fraction of soluble protein produced. Since the protein has an absolute requirement for zinc ions, we have investigated the effect of supplying a high concentration of this metal ion in the culture medium.

The bacterial molecular chaperones have been shown to play a role in preventing aggregation of host proteins, or directing them towards the proteolytic degradation pathway, when the organism is subjected to conditions of stress such as heat shock (for reviews see [6,7]). Two reports have suggested that co-expression of target proteins with molecular chaperones resulted in increased production of soluble protein. An increase in solubility was obtained for recombinant mammalian procollagenase by simultaneously overexpressing the GroES and GroEL proteins *in vivo* [8]. A second *E. coli* molecular chaperone, DnaK, was shown to significantly reduce the inclusion body formation during the heterologous expression of human growth hormone in *E. coli* [9]. The assistance of molecular chaperones in folding proteins *in vitro* has been studied extensively for several proteins [10–12]. We have therefore investigated the effect of overexpressing GroES and GroEL *in vivo* and the effect of these chaperones on the folding of PMI *in vitro*.

MATERIALS AND METHODS

Materials

Unless otherwise stated all chemicals and enzymes were purchased from Sigma and all chromatographic media were from Pharmacia.

Protein expression

The cloning of the cDNA coding for *C. albicans* PMI and its heterologous expression in *E. coli* strain B are described elsewhere [5]. The plasmids coding for the molecular chaperones GroEL, GroES and DnaK were a kind gift from Professor C. Georgopoulos. pTPG9 drives expression of DnaK under control of the *Tac* promoter (induced by 0.5 mM isopropylthiogalactoside). pOF39 drives expression of GroES and GroEL under their own promoter which is induced upon heat shock. Both plasmids are selectable by ampicillin resistance. Single

Abbreviations used: PMI, phosphomannose isomerase; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; TFA, trifluoroacetic acid; ICP, inductively coupled plasma.

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transformants were made in *E. coli* B to produce the purified chaperones for *in vitro* folding experiments. Dual transformants were made by transforming the *C. albicans* PMI-expressing strain either with pTPG9 or pOF39. Fermentation conditions were otherwise identical to those published [13]. Standard fermentation medium contained 6 μM ZnO, whereas for high zinc conditions, $ZnCl_2$ was added to the fermentation medium to a final concentration of 1 mM.

Protein purification

All purifications were carried out at 4 °C unless otherwise stated. Recombinant *E. coli* B cell pellets were suspended in three times the volume of the cell wet weight in breakage buffer consisting of 50 mM Tris/HCl, pH 8.0, containing 0.1 mM PMSF, 1 mM benzamidine hydrochloride and 1 mM EDTA. The cells were broken by three passages through a French pressure cell with 30 s sonication on ice after each passage. The resulting solution was centrifuged at 10000 ***g*** for 60 min.

PMI was purified from the soluble fraction as previously described [5] from 30 g of *E. coli* cells grown under normal fermentation conditions and 40 g of cells grown under a high zinc ion concentration. PMI was isolated from the inclusion body fraction by dissolution in a 0.1 M Tris/HCl buffer, pH 7.5, containing 6 M guanidine hydrochloride and 1 mM dithiothreitol (DTT), at a concentration of 10–20 mg/ml. To ensure monomerization, the solution was heated for 1 h at 50 °C, and after cooling to room temperature, 100 ml aliquots were gel-filtered on a Sephadex S-300 column (5 cm diam. × 100 cm) equilibrated in the same buffer. The fractions containing the PMI protein were dialysed three times against 1 % acetic acid and lyophilized. A sample (5 mg) was dissolved in 0.1 % trifluoroacetic acid (TFA) and further purified by reverse-phase HPLC on a VarioPrep NUCLEOSIL 300-7 C8 column (10 mm internal diam. × 250 mm) (Macherey-Nagel) at a flow rate of 4 ml/min using a Beckman Gold system. Protein was eluted with a gradient of buffer A (0.1 % TFA/water, v/v) and buffer B (acetonitrile/water/TFA, 90:10:0.1, by vol.) using a gradient of 15–75 % buffer B over 40 min. The PMI-containing fraction was lyophilized.

GroES and GroEL were purified essentially according to [14] from 44 g of *E. coli* cells.

DnaK was purified from 15 g of *E. coli* cells. The soluble fraction was adjusted to 35 % (w/v) ammonium sulphate, and centrifuged for 60 min at 10000 ***g***. The pellet was dissolved in 50 mM Tris/HCl, pH 7.2, and applied to a Sephacryl S-200 HR column (5 cm diam. × 100 cm) equilibrated in the same buffer. Fractions containing the DnaK protein were pooled and adjusted to 50 mM NaCl and 12 mM 2-mercaptoethanol. The solution was applied to a HiLoad Sepharose-Q (26/10) column equilibrated in the same buffer. Protein was eluted by a 0 to 0.32 M NaCl gradient. The DnaK-containing fractions were dialysed against 50 mM Tris/HCl buffer, pH 7.2, and concentrated by loading the solution on to the HiLoad Sepharose-Q column described above. Protein was eluted with 0.4 M NaCl in the same buffer. The concentrated DnaK-containing fractions were dialysed against 50 mM Tris/HCl and stored at −20 °C.

Analytical methods

SDS/PAGE was carried out on 10–15 % Phast gradient gels (Pharmacia) and on 8–16 % acrylamide mini-gels (Novex) according to the manufacturers' instructions, and the proteins visualized by staining with Coomassie Brilliant Blue, R250. The relative amounts of the recombinant proteins were determined by densitometric scanning of the acrylamide gels. Total protein was determined using the Bio-Rad protein assay with BSA as a standard. Purified proteins were quantified by the absorption coefficients at 280 nm of $A^{0.1\,\%}_{1\,cm} = 0.53$ for PMI, $A^{0.1\,\%}_{1\,cm} = 0.16$ for GroEL, $A^{0.1\,\%}_{1\,cm} = 0.123$ for GroES and $A^{0.1\,\%}_{1\,cm} = 0.21$ for DnaK, calculated from the amino acid sequences. The concentration of the final preparations were determined by amino acid analysis. Amino acid analysis was carried out by incubating the samples in 6 M HCl at 112 °C for 22 h, and the amino acid composition was determined using a Beckman 6000 amino acid analysis system with ninhydrin detection. Norleucine was added as an internal standard and phenol (1 mg/ml) as an antioxidant. Electrospray ionization mass spectroscopy (ESI-MS) was carried out on a Sciex API-III spectrometer. Samples (5 pmol) of protein were dissolved in aq. 50 % (v/v) methanol containing 2 % acetic acid. The solution was infused at a rate of 2 μl/min into the ionspray needle, which was maintained at 5000 V. The orifice voltage was 120 V. Spectra were co-added by repetitive scanning until an acceptable signal/noise ratio was obtained (usually 3–4 min). The zinc content of the protein was determined by inductively coupled plasma (ICP) atomic absorption spectroscopy.

PMI enzyme assay

The activity of PMI was measured at 37 °C using a coupled assay [15]. One unit is defined as the amount of enzyme producing 1 μmol of NADPH/min at 37 °C.

Renaturation of PMI

Native PMI was denatured by adding guanidine hydrochloride to a concentration of 6 M to the purified recombinant enzyme solution. Unless stated otherwise, the enzyme solution was diluted 100-fold into a 0.1 M Tris/HCl buffer, pH 8.0, containing 10^{-2} M DTT and 10^{-4} M $ZnCl_2$. The final concentration of PMI ranged between 1.5×10^{-5} and 7×10^{-5} M. The optimal concentration of DTT was determined by varying the concentration from 10^{-4} to 5×10^{-2} M in the renaturation buffer, at a constant concentration of 10^{-4} M $ZnCl_2$. The optimal concentration of Zn^{2+} was similarly determined by varying the concentration of $ZnCl_2$ from 10^{-8} to 2×10^{-2} M at a constant DTT concentration of 10^{-2} M. To test the effect of the chaperone proteins on folding *in vitro*, they were included in the renaturation buffer at the following molar ratios to PMI: GroEL (14-mer), 2:1; GroES (7-mer), 2:1; and DnaK, 60:1. To test the effect of BSA, it was included in the renaturation buffer at a 20-fold molar excess. The inclusion body PMI purified by reverse-phase HPLC was dissolved in 0.1 M Tris/HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride or 6 M urea prior to renaturation.

Aliquots (10 ml) of the renatured solutions were assayed for activity as described above.

PMI–GroEL association

A solution of denatured PMI (2 mg/ml) in 4 M guanidine hydrochloride was rapidly diluted 10-fold into renaturing buffer, and 1 ml immediately loaded on to a Superdex-200 (16/60) column equilibrated in 0.1 M Tris/HCl, pH 8.0. To investigate whether the PMI protein associated with the molecular chaperone while folding, both native PMI and the denatured solution were diluted as above into renaturing buffer containing a twofold molar excess of the GroEL 14-mer. The elution volume of native PMI was determined in the absence of chaperone. The fractions were assayed for PMI activity.

RESULTS

PMI expression

The expression of recombinant PMI in the soluble fraction of the *E. coli* cells was monitored by enzyme activity and by SDS/PAGE. SDS/PAGE analysis of the cell extracts of fermentations run under 6 μM and 1 mM $ZnCl_2$ (Figure 1) showed that under high zinc conditions the amount of PMI in the soluble fraction was increased (Figure 1, lane 8) compared with the amount expressed under normal fermentation conditions (lane 4). The overall expression level of the PMI protein was 30 % in both cases. Under normal fermentation conditions, in which the concentration of Zn^{2+} is 6 μM, the level of PMI activity was 11.5 units/mg of protein. However, when Zn^{2+} was included at a concentration of 1 mM, the PMI activity in the soluble fraction of the *E. coli* cell extract was 35 units/mg. The yields of purifed PMI from fermentations run under low and high Zn^{2+} conditions were 1.37 mg/g of *E. coli* cells and 5.4 mg/g of *E. coli* cells respectively (Table 1).

The expression levels of the GroES and GroEL proteins under control of their own promoter is approximately 50 % of total cell protein upon temperature induction (results not shown). Similarly PMI is expressed at very high levels, attaining 30 % of total protein after 4 h induction. When the two sets of proteins were concomitantly overexpressed and induced by a temperature shift to 42 °C, the PMI expression level dropped to 15 %, representing a twofold reduction in the expression level of the recombinant enzyme. However, the specific activity of the soluble enzyme was the same as found with normal fermentation conditions containing 6 μM $ZnCl_2$. Therefore the presence of high concentrations of chaperone protein prevents a significant amount of recombinant enzyme from being channelled into inclusion bodies.

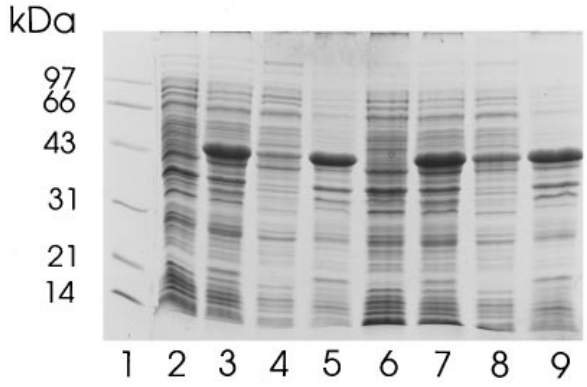

Figure 1 SDS/PAGE analysis of the expression levels of recombinant *C. albicans* PMI in *E. coli*

Lane 1, molecular mass markers; lanes 2–5, fermentations with 6 μM Zn ion in the medium; lanes 6–9, fermentations with 1 mM Zn ion in the medium; lanes 2 and 6, prior to induction; lanes 3 and 7, total cell extracts; lanes 4 and 8, soluble fraction; lanes 5 and 9, inclusion body pellet.

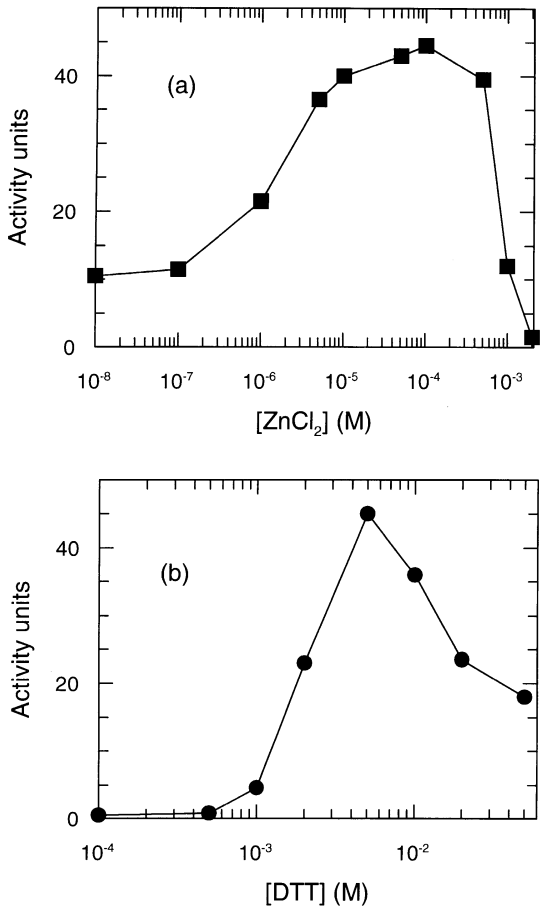

Figure 2 Determination of the optimal concentrations of DTT and zinc ions for the *in vitro* renaturation of PMI

(**a**) The denatured solution of PMI in 6 M guanidine hydrochloride was diluted 100-fold into 0.1 M Tris/HCl, pH 8.0, containing 10^{-2} M DTT and various concentrations of $ZnCl_2$ (■). (**b**) The renaturation was carried out with 10^{-4} M $ZnCl_2$ and various concentrations of DTT (●). The enzyme solutions were incubated at room temperature for 30 min, and the activity measured as described in the text.

Table 1 Influence of zinc ion concentration in the fermentation medium and the simultaneous overexpression of GroES and GroEL on the partition of soluble and inclusion body recombinant PMI

The results shown for the effect of high zinc ion concentration represent one of three fermentations. PMI was not purified from the concomitant overexpression of the *E. coli* molecular GroES and GroEL chaperones. Abbreviation: nd, not determined.

Fermentation condition	PMI activity in the soluble fraction (units/mg of protein)	*E. coli* cell paste (g)	PMI expression (% total protein)	Purified PMI (mg)	Yield (mg of PMI/g of *E. coli* cells)
6 μM ZnO	11.5	30.4	29.8	41.6	1.37
1 μM $ZnCl_2$	35.0	40.0	30.5	216.2	5.41
+ GroESL	7.8	nd	15.0	nd	nd

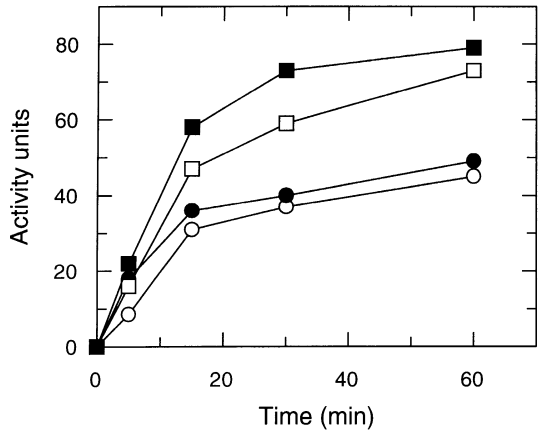

Figure 3 The effect of the bacterial chaperones GroEL and DnaK on the *in vitro* renaturation of PMI

Denatured PMI in 6 M guanidine hydrochloride was diluted 100-fold into 0.1 M Tris/HCl, pH 8.0, containing 10^{-2} M DTT and 10^{-4} M $ZnCl_2$ (○). A 60-fold molar excess of DnaK (■), a 2-fold molar excess of GroEL (□) or a 20-fold molar excess of BSA (●) was added to the renaturation buffer prior to the addition of the denatured enzyme.

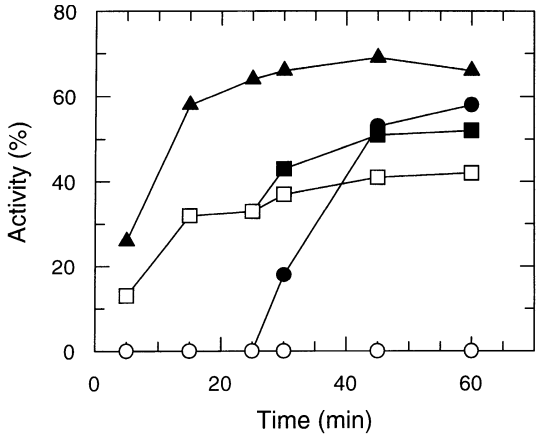

Figure 4 The effect of the sequential addition of chaperone or the co-factors on renaturation of PMI

The denatured enzyme in 6 M guanidine hydrochloride was diluted 100-fold into the renaturation buffer, and incubated for 25 min at 23 °C. 0.1 M Tris/HCl, pH 8.0, containing 10^{-2} M DTT and 10^{-4} M $ZnCl_2$ (□) followed by the addition of a twofold molar excess of the GroEL 14-mer (■); 0.1 M Tris/HCl, pH 8.0, containing a twofold molar excess of the GroEL 14-mer (○) followed by the addition of 10^{-2} M DTT and 10^{-4} M $ZnCl_2$ (●); 0.1 M Tris/HCl, pH 8.0, containing 10^{-2} M DTT, 10^{-4} M $ZnCl_2$ and a twofold molar excess of the GroEL 14-mer (▲).

Folding of PMI

No activity was observed on dilution of PMI denatured in a guanidine solution into Tris/HCl buffer. However, enzyme activity could be restored if reducing agents such as DTT or 2-mercaptoethanol were included in the buffer. Subsequently the yield of activity was found to be significantly enhanced by the inclusion of $ZnCl_2$. The optimal concentration of the reductant DTT was found to be 5×10^{-3} M (Figure 2b) and that of the zinc metal ion, 1×10^{-4} M (Figure 2a). Dilution of the denatured native PMI enzyme into the renaturing buffer containing these two inorganic co-factors restored 30–40 % of the specific activity of the purified recombinant enzyme.

The *E. coli* molecular chaperones GroEL and DnaK were able to increase the amount of active enzyme renatured *in vitro* twofold over the basal level obtained in the renaturing buffer containing DTT and $ZnCl_2$ (Figure 3). The addition of GroES to the renaturation buffer containing GroEL had no effect. Moreover, the chaperone assistance of GroEL appeared to be energy independent, since addition of ATP at concentrations up to 5 mM did not enhance the renaturation yield. The effect of the chaperones appeared, however, to be specific. The addition of a non-related protein BSA, at a 20-fold molar excess over the PMI protein, resulted in no increase in regain of activity (Figure 3).

We have observed a notable difference in the renaturation yields of the native PMI enzyme which is denatured by 6 M guanidine hydrochloride, and the PMI which is purified from inclusion bodies in 6 M guanidine hydrochloride (Table 2). The renaturation was carried out using the conditions determined to be optimal for renaturation, and including a twofold excess of GroEL 14-mer. It was found that 60–80 % of the activity can be restored when renaturing the soluble purified recombinant enzyme which has been denatured. However, when renaturing the inclusion body protein, the maximum specific activity recovered was only 30 % of the specific activity of the purified protein. The inclusion body material was shown to be PMI by N-terminal sequencing and its concentration verified by amino acid analysis.

The specific effect of the molecular chaperone GroEL was further investigated by the sequential dilution of the denatured enzyme into the renaturing buffer for 25 min, prior to the addition of the chaperone, or conversely, by incubating the renaturing enzyme with the chaperone for 25 min prior to the addition of DTT and $ZnCl_2$. As shown in Figure 4, incubation of the enzyme in the renaturing buffer containing DTT and Zn results in 42 % recovery of activity over 60 min. Incubation in renaturing buffer containing GroEL results in the recovery of 66 %. If the enzyme is renatured in the presence of GroEL without the reductant and the metal ion, no activity is observed until these two factors are added, whereupon 58 % is restored. Furthermore, the recovery of activity in renaturing buffer is improved if GroEL is added to the solution after the first 25 min when the 37 % of enzyme activity is recovered, to 52 % over the following 35 min.

Lastly, since the effect of GroEL was independent of its co-chaperone GroES and ATP, we wished to demonstrate an interaction between the PMI and the GroEL 14-mer. We therefore subjected the renaturation solutions to size-exclusion

Table 2 Comparison of the renaturation yields of denatured PMI and inclusion body PMI

The renaturations were carried out with 10^{-4} M $ZnCl_2$ and 5×10^{-3} M DTT and a twofold excess of GroEL 14-mer as described in the text. The determinations were performed in triplicate. The results shown are the averages of three experiments.

	Specific activity (units/mg)	% r-PMI
r-PMI	168.4 ± 19.4	100
r-PMI, denatured in 6 M guanidine hydrochloride	118.5 ± 18.5	70.3
Inclusion body PMI, S-300 pool in 6 M guanidine hydrochloride	36.6 ± 15	21.7
Inclusion body PMI, HPLC-purified in 6 M guanidine hydrochloride	34.3 ± 13	20.4
Inclusion body PMI, HPLC-purified in 6 M urea	34.0 ± 9.1	20.2

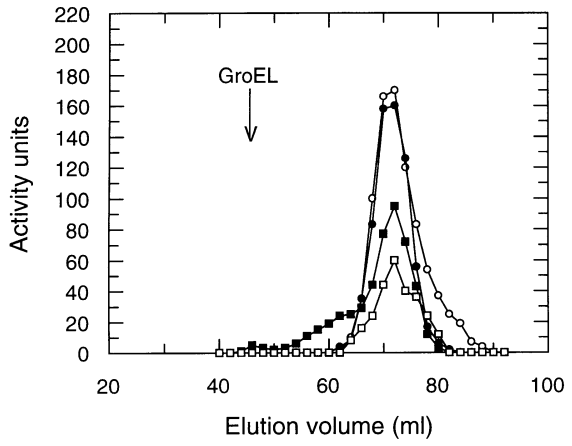

Figure 5 Size-exclusion chromatography of renaturation solutions of recombinant PMI

The PMI solutions were diluted 10-fold into the renaturing buffer as described in the text and 1 ml loaded on to a Superdex 75 (16/10) column equilibrated in 0.1 M Tris/HCl buffer, pH 8.0. The fractions were analysed for PMI activity. ○, Soluble purified recombinant PMI; ●, soluble purified recombinant PMI and GroEL; □, denatured PMI; ■, denatured PMI and GroEL. The elution volume of the GroEL 14-mer as determined by the absorbance at 280 nm is indicated.

chromatography (Figure 5). Native PMI eluted at a molecular mass corresponding to a spherical protein with a mass of 47 kDa, both in the presence and absence of GroEL. Activity from the denatured PMI in the absence of molecular chaperone was similarly only detected at the elution volume corresponding to 47 kDa. However, when the denatured PMI was renatured in the presence of GroEL, a small but significant amount of activity was clearly observed at elution volumes corresponding to masses much greater than 47 kDa. We attribute this apparently high-molecular-mass material to its association with GroEL during its renaturation.

DISCUSSION

In order to obtain large quantities of protein for structure–function studies, and in particular for X-ray crystallography, it is often desirable to obtain the maximal amount of a recombinant protein expressed in the soluble fraction of *E. coli*. The expression of recombinant *C. albicans* PMI in the heterologous *E. coli* expression system resulted in high levels of recombinant protein, but distributed in both the soluble and insoluble inclusion body fractions; we have therefore used this system to investigate whether we could increase the proportion of soluble protein.

PMI isolated from brewers' yeast was shown to contain 1 mol of zinc per mol of protein [1]. Recombinant PMI produced as the soluble, active enzyme incorporates zinc at the correct stoichiometry. ICP-atomic absorption spectroscopy of recombinant *C. albicans* PMI demonstrated the incorporation of 0.7 mol of zinc per mol of protein (J. J. Smith, A. J. Thomson, A. E. I. Proudfoot and T. N. C. Wells, unpublished work). Determination of the metal content of the inclusion body protein was not possible, since the analysis requires that the protein be in solution, which entails solubilization in a strong denaturant. ICP-atomic absorption spectroscopy of the native enzyme dissolved in guanidine hydrochloride showed that the denaturation had caused loss of the metal ion (results not shown), and ESI-MS of the soluble protein following reverse-phase HPLC gives the molecular mass of the apo-enzyme [17]. However, since the yield of soluble active enzyme was increased fourfold by the inclusion of a high concentration of zinc ions in the fermentation medium, we conclude that one of the factors driving the expression of recombinant PMI into inclusion bodies is the limitation in metal concentration to enable correct folding of the active enzyme.

Simultaneous overexpression of the *E. coli* molecular chaperones GroES and GroEL with recombinant PMI had two effects. First, the overall expression of the PMI protein decreased from 30% of total protein to 15%. Overexpression of chaperone proteins in *E. coli* recombinantly expressing a heterologous protein has been previously reported to cause an overall decrease in the expression level of the recombinant protein [18,19]. However, in both cases the proportion of soluble protein was increased. Since the specific activity of PMI in the soluble fraction, when co-overexpressed with GroES and GroEL, is not lower than when expressed alone, this effectively represents a twofold increase in recombinant soluble protein. A study on the heterologous expression of the metalloprotein Cu,Zn-superoxide dismutase in *E. coli* and simultaneous overexpression of the bacterial chaperones GroES and GroEL resulted in a twofold increase in enzyme activity [20]. In this study the authors suggest that the chaperones increase the stability of partially folded apo-protein, thereby favouring metal uptake by newly synthesized protein, which could also be applied to the PMI protein folding in the *E. coli* cell.

In view of the fact that we were able to increase the proportion of soluble PMI *in vivo*, we then studied its folding *in vitro*. The absolute requirement of a reducing agent in order to regain any activity is surprising. Cys-150 has been shown to be in the proximity of the active site of PMI as the enzyme can be irreversibly inactivated by acetylation of this residue by iodoacetamide, an event which is substrate-protectable [21]. During the process of denaturation, modification of this, or another cysteine, probably by the formation of a disulphide bond, must in some way impede the correct folding of the enzyme, and prevent the apo-enzyme from acquiring the essential zinc ion. After reduction of the incorrect disulphide, metal uptake can occur, and thus the yield of renaturation can be significantly enhanced by the inclusion of zinc metal ions in the renaturation buffer. At high concentrations of either DTT or zinc ion, these two molecules become inhibitors. The inhibitory effect of chelating agents which remove the essential zinc ion was described in the pioneering work on PMI of Gracy and Noltmann [22]. These authors described the inhibition by micromolar concentrations of Zn^{2+}, and these observations have been further investigated in extensive kinetic studies on *Saccharomyces cerevisiae* PMI [15] which showed that PMI has a second binding site for Zn^{2+} as well as for other bivalent metals. Recombinant *C. albicans* PMI [5,18] and human PMI [2] are similarly inhibited by the bivalent zinc ion.

Since we observed a proportional increase in soluble protein *in vivo*, when the GroES and GroEL molecular chaperones were overexpressed, their effects on the renaturation of recombinant PMI *in vitro* was investigated, as well as another *E. coli* chaperone, DnaK. A twofold increase in regain of activity was observed when both GroEL and DnaK were included in the renaturation buffer. However, both were energy-independent, as ATP had no effect. These chaperones have been reported to have ATPase activity, which is necessary for their chaperone function [23,24]. In addition, GroEL normally functions in conjunction with its co-chaperone GroES, [11,25] but we were not able to observe any effect of the GroES heptamer. However, the increase in renaturation observed for the two chaperones appeared to be

specific. First, an unrelated protein, BSA, had no effect, even at a 20-fold molar excess, eliminating the possiblity that the chaperone effect observed was simply stabilization by increased protein concentration. Secondly, the sequential addition of the chaperone to the renaturation buffer demonstrated an effect of the chaperone protein itself. Lastly, an association between GroEL and the renaturing protein, but not the native enzyme, could be demonstrated by size-exclusion chromatography.

Finally, we have demonstrated a significant difference in the yield of renaturation between inclusion body PMI and that obtained for the renaturation of the enzyme purified from the soluble fraction, and subsequently denatured with guanidine hydrochloride. The inclusion body protein, even after isolation by reverse-phase HPLC, behaved as a heterogeneous sample on ESI-MS analysis, thereby excluding the identification of degradation or oxidation of critical residues. It is outside the scope of this work to determine the reasons for this difference in folding yield between inclusion body protein and the refolding of the denatured soluble enzyme. However, we suggest that this may be the first demonstration that inclusion body protein can form tangles, such as those recently described for DNA [26], that are impossible to renature, despite the complete monomerization of the inclusion body material.

In conclusion, we present data which indicate that the yield of the soluble form of a recombinant metalloprotein can be significantly increased by a high concentration of the essential metal ion in the fermentation medium. Although the concomitant overexpression of the *E. coli* chaperones GroES and GroEL appears to increase the amount of soluble protein, no increase in yield is obtained as their co-expression results in a lower level of expression of recombinant protein. They do, however, significantly increase the folding yield *in vitro*, in an energy-independent manner.

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