The effects of polysaccharide, polyethylene glycol, and protein-crowding agents on the refolding of glucose-6-phosphate dehydrogenase (G6PDH) and protein disulfide isomerase have been examined. By increasing concentration during refolding, the reactivation yields of the two proteins decrease with the formation of soluble aggregates. In the presence of high concentrations of crowding agents the reactivation yields remain constant but with decreased refolding rates. The refolding of G6PDH changes from monophasic to biphasic first-order reactions in the presence of crowding agents, and the amplitude of the new slow phase increases with increasing concentrations of crowding agents. The molecular chaperone GroEL reverses the refolding kinetics of G6PDH from biphasic back to monophasic and accelerates the refolding process. Our results display the complexity and diversity of the effects of macromolecular crowding on both the thermodynamics and kinetics of protein folding.

In the past decades extensive studies on protein folding in vitro have provided a wealth of information for understanding how nascent polypeptides mature into functional proteins in cells and for surmounting difficulties in refolding inclusion bodies of many recombinant proteins. In vitro folding of proteins was performed usually at low concentrations to avoid aggregation during folding; however, in the cytoplasm of cells there are a large number of soluble and insoluble macromolecules at different concentrations, sometimes at very high concentrations depending on physiological and environmental conditions. It has been estimated that the concentration of macromolecules in cytoplasm could be in the range of 80–200 g/liter (1). Therefore all species of macromolecules occupy a large fraction of the total volume of a cell and such a media has been named “crowded” (2). One of the consequences of crowding is steric repulsion, which derived from the mutual impenetrability of macromolecules, and has been referred to more precisely as volume exclusion (3, 4). This is the most significant difference between the intracellular and artificial in vitro environment but has not been considered in the majority of biochemical experiments thus far to examine the effects of crowding on protein folding. van den Berg et al. (9) have studied the effect of crowding agents on the refolding of denatured lysozyme. Further kinetic analysis suggested that under conditions resembling the intracellular environment, the rates of many protein-folding processes are likely to be altered (10). For chaperone-assisted protein refolding, it has been reported that the release of nonnative polypeptide from GroEL was prevented in the presence of crowding agents (11), although contradictory results have also been reported that rhodanese can be released from GroEL in its nonnative form in the milieu of an intact cell (12). Recently it has been shown that macromolecular crowding modulates the oligomerization of GroEL during its refolding (13).

In this communication we have studied the effects of polysaccharide (Ficoll 70 and Dextran 70), polyethylene glycol 20000 (PEG),1 and the protein crowding agents bovine serum albumin (BSA), chicken egg ovalbumin, and lysozyme on the refolding of guanidine hydrochloride (GdnHCl)-denatured glucose-6-phosphate dehydrogenase (G6PDH) and protein disulfide isomerase (PDI) at 25 and 37 °C. The results show that the renaturation yields of the two proteins do not change in the presence of high concentrations (50–200 g/liter) of crowding agents; however, the refolding kinetics change markedly. The chaperone GroEL promotes the folding and reverses the effects of crowding agents on G6PDH folding.

EXPERIMENTAL PROCEDURES

Materials—G6PDH (type V, from baker yeast), BSA, ovalbumin, and GdnHCl were purchased from Sigma, PEG and chicken egg lysozyme were purchased from Roche Molecular Biochemicals, and Ficoll 70 and Dextran 70 were purchased from Amersham Pharmacia Biotech. PDI was prepared from bovine liver, and the thiol-protein oxidoreductase activity was assayed according to Lambert and Freedman (14). Escherichia coli GroEL was prepared according to Li and Wang (15). G6PDH was assayed according to Hansen and Gafni (16). In all experiments, 0.1 M potassium phosphate buffer (pH 7.5) containing 2.5 mM EDTA was used and referred to as phosphate buffer.

The concentrations of G6PDH and PDI were determined spectrophotometrically at 280 nm with the absorption coefficient (ε m M) of 1.15 for G6PDH (16) and 0.9 for PDI (17). The concentration of GroEL was measured by the method of Bradford (18) with BSA as a standard. G6PDH and PDI were taken as monomers and GroEL as a tetramer in the calculations of concentrations, because oligomeric G6PDH and PDI were presumably dissociated to monomers when fully denatured.

1 The abbreviations used are: PEG, polyethylene glycol 20000; BSA, bovine serum albumin; GdnHCl, guanidine hydrochloride; G6PDH, glucose-6-phosphate dehydrogenase; PDI, protein disulfide isomerase.

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Denaturation and Reactivation of Proteins—

Denaturation of G6PDH was carried out by incubation of 12 or 24 μM enzyme with 4 mM GdnHCl at 25 °C for 24 h (16). PDI at 140 or 280 μM was fully denatured by treatment with 6.4 mM GdnHCl at 25 °C for 24 h (19). The refolding of denatured G6PDH or PDI was initiated by quick dilution at least 15-fold in phosphate buffer containing different concentrations of crowding agents and/or chaperone GroEL at 25 or 37 °C. In addition to PEG and polysaccharides (Ficoll 70 and Dextran 70) as background species, BSA, ovalbumin, and lysozyme were used as protein-crowding agents. The presence of the above crowding agents from 50 to 200 g/liter was to mimic the intracellular crowded environment in vitro. GroEL to G6PDH molar ratio of 2 has been determined to help the refolding of the substrate to a maximal level (16).

The refolding of G6PDH or PDI in the absence of crowding agents takes about 1 h to reach maximal activity recovery, which does not change in 24 h. The activity recovery was thus determined in 24 h after dilution, and the reactivation yield was expressed as a percentage of the activity of native enzyme, which was incubated at 25 or 37 °C for at least 24 h and showed full activity. The aggregation during refolding was monitored by 90° light scattering at 488 nm continuously in a Shimadzu RF5301PC spectrofluorometer. The residual denaturants in the refolding system had been examined and determined to have no effect on the activities of the enzymes and the chaperone activity of GroEL.

Analysis of Refolding Products of G6PDH and PDI—

Denatured G6PDH (12 μM) and denatured PDI (140 μM) was refolded by dilution to 0.36 and 7 μM, respectively, at 25 °C. The protein concentrations in the supernatants in refolding products after centrifugation at 16,000 rpm for 10 min were measured by spectrophotometry at 280 nm. Then the supernatants in the refolding products were analyzed in 24 h using a Bio-Rad SEC 250–5 column in a Bio-Rad BioLogic System with a flow rate of 0.5 ml/min.

Determination of Kinetic Constants of Protein Folding—

The time courses of the reactivation of G6PDH and PDI in the absence or presence of crowding agents and/or GroEL/MgATP were determined at 25 and 37 °C. The data were fitted well to exponential equations for first-order reactions (Y = Y_{max} (1 - e^{-kt}) in monophasic and Y = Y_{max1} (1 - e^{-kt}) + Y_{max2} (1 - e^{-kt}) in biphasic kinetics) using Microcal Origin 5.0 software.

RESULTS

Concentration-dependent Refolding of G6PDH and PDI—

As shown in Fig. 1A, the reactivation yield of G6PDH decreases from 40 to 19% at 25 °C and 14.0 to 2% at 37 °C with the concentration of denatured protein increasing from 0.12 to 1.44 μM. The reactivation yield of PDI decreases from 65 to 40% at 25 °C and 54 to 27% at 37 °C when the concentration increases from 2.3 to 11.7 μM (Fig. 1B). The concentration dependence of the refolding of the two proteins at 37 °C seems more significant than that at 25 °C. Concurrent with the marked decrease in the reactivation yield, aggregation during refolding increases significantly with increasing protein concentrations at both of 25 and 37 °C (Fig. 1). Because the reactivation yields at 25 °C are always higher than those at 37 °C at all protein concentrations examined, most of the experiments were carried out at 25 °C. G6PDH (0.36 μM) and PDI (7 μM) were used to examine the effect of crowding agents on the refolding for experimental convenience.

Refolding Products of G6PDH and PDI—

Little aggregation is detected by light scattering during the refolding of G6PDH (0.36 μM) and PDI (7 μM) at either 25 or 37 °C, although the reactivation yields of the two enzymes are far from complete. By the gel filtration analysis shown in Fig. 2, native G6PDH appears as a dimer with an apparent molecular mass of 135 kDa, and PDI appears mainly as a dimer of 120 kDa with a small part as a tetramer of 255 kDa. The supernatant in refolding product of G6PDH contains components eluted in the void volume with an apparent molecular mass >1000 kDa. The ratio of high molecular mass product to the product eluted at the position of the native enzyme is 51:49. Oligomers of >1000 kDa also appear in the supernatant in refolding product of PDI with a ratio to the refolded molecules of 37:63. The above results indicate that the oligomeric folding products formed during refolding may be very heterogeneous, and the molecular sizes of most oligomers are not large enough to be detected by the simple light scattering method. Because the soluble proteins in the supernatant in the refolding products of G6PDH and PDI are 81 and 94% of all proteins detected by the absorbance at 280 nm, respectively, it is to be noted that the proportion of the peaks at the elution positions of native proteins in the total refolding products, 40% (49% × 81%) for G6PDH and 59% (63% × 95%) for PDI, are pretty close to the reactivation yields (squares) in Fig. 1A and B, respectively. Therefore, it can be assumed that the oligomeric refolding products, although most are soluble, are inactive and responsible for the decreased activity recovery.

Effect of Macromolecular Crowding Agents on the Reactivation of G6PDH and PDI—

BSA, ovalbumin, lysozyme, and PEG, but not Ficoll 70 and Dextran 70, were selected as crowding agents for G6PDH refolding, because these are noninhibitory on G6PDH activity after incubation at 200 g/liter with native G6PDH at 25 and 37 °C for 24 h. As shown in Fig. 3A, the reactivation yields of G6PDH in the presence of the crowding agents up to 200 g/liter do not change at either 25 and 37 °C. Ficoll 70, Dextran 70, and PEG were chosen as crowding agents for PDI refolding because they do not inhibit the activity of PDI, and they don’t interfere with the PDI assay. BSA, ovalbumin, and lysozyme could not be used, because the disulfide bonds in those proteins at high concentrations strongly interfere with the activity assay of PDI. Similar to the reactivation of G6PDH, the crowding agents at increasing concentra-
Effects of Macromolecular Crowding on Protein Folding

FIG. 2. Chromatographic analysis of the supernatants in refolding products of G6PDH and PDI. The supernatants in refolding products of G6PDH and PDI in 24 h after dilution were analyzed by using a Bio-Rad SEC 250–5 column at 25 °C. The readings of A at 280 nm (OD) have been normalized to make the same y axis scale in all the panels. A, the supernatant in refolding product of G6PDH; B, native G6PDH; C, the supernatant in refolding product of PDI; D, native PDI; E, molecular mass marker; peak 1, bovine thyroglobulin, 670 kDa; peak 2, bovine gamma globulin, 158 kDa; peak 3, chicken ovalbumin, 44 kDa; peak 4, horse myoglobin, 17 kDa; peak 5, vitamin B-12, 1.35 kDa.

Effect of Macromolecular Crowding Agents on Refolding Kinetics of G6PDH and PDI—The time courses of G6PDH refolding at 25 (Fig. 4) and 37 °C (Fig. 5) in the absence of crowding agents are fitted well to monophasic first-order reaction kinetics with the kinetic parameters shown in Table I. The presence of BSA does not change the first-order nature of the kinetics but slows the folding rate. With BSA concentration increased to 200 g/liter, the refolding becomes 3-fold slower compared with refolding in dilute solution (Figs. 4A and 5 and Table I). However, the presence of ovalbumin (Figs. 4B and 5), lysozyme (Fig. 4C), or PEG (Fig. 4D) radically alters the refolding kinetics of G6PDH to biphasic first-order reactions with the first as fast phase and the second as slow phase (Table I). At 25 °C with the concentration of ovalbumin and lysozyme increased to 200 g/liter, the amplitude of the fast phase, \( Y_{\text{max},1} \), reduced to \(-60\%\) with a decrease of rate constants, \( k_1 \), to \(-3\)-fold; the rate constants of the slow phase, \( k_2 \), decreased only slightly. The effects of PEG are much more marked with the \( Y_{\text{max},1} \) value decreased to less than \(10\%\) and the \( k_1 \) value decreased to only \(5\%\) when the concentration increases to 200 g/liter; however, the \( k_2 \) values change only slightly.

The refolding of PDI remains to follow monophasic first-order reaction kinetics in the presence of 200 g/liter crowding agents at both 25 (Fig. 6A) and 37 °C (Fig. 6B) with the rate constants decreased to 60 (25 °C) and 41% (37 °C) for Ficoll 70 and to 46% (25 °C) for Dextran 70 (Fig. 6, insets). The rate constants for the refolding of G6PDH and PDI are higher at 37 °C than at 25 °C in either the absence or presence of crowding agents (Table I).

Effect of Chaperone GroEL on the Refolding of G6PDH under Crowding Conditions—Compared with Figs. 4 and 5, the presence of GroEL/MgATP dramatically increases the refolding yields of G6PDH from 44 to 87% (2-fold) at 25 °C and from 17 to 41% (2.4-fold) at 37 °C even in the presence of 200 g/liter BSA, ovalbumin, or lysozyme, which only reduce the rate constants 20–50% at both temperatures (Fig. 7 and Table II). More distinctively GroEL reverses the folding kinetics of G6PDH under crowding conditions from biphasic to monophasic first-order reactions and largely shortens the folding time from 2 to 1 h in the presence of 200 g/liter BSA and from almost 24 to...
Data are expressed as mean ± S.D. (n = 3–5).

Effects of Macromolecular Crowding on Protein Folding

FIG. 5. Time courses of G6PDH refolding under crowding conditions at 37 °C. Denatured G6PDH was refolded at 0.36 μM in the absence (■) or presence of 200 g/liter BSA (●) or ovalbumin (▲). The data are expressed as mean ± S.D. (n = 3–5).

only 2 h with 200 g/liter ovalbumin and lysozyme. The above suggests that crowding agents do not affect the GroEL-assisted refolding yields of G6PDH but do slow the folding rate; on the other hand GroEL functions to prevent the effect of crowding agents on G6PDH refolding kinetics. In contrast to the absence of GroEL/MgATP, the refolding rate constants at 37 °C always become lower than the respective values at 25 °C (Table II).

DISCUSSION

According to the crowding theory, macromolecules, by excluding volume to one another, reduce the configurational entropy and hence increase the free energy of a solution and the chemical potential of each species of macrosolute present in the solution (2). This results in the destabilization of either reactants or products and affects equilibrium toward the most favored state of the system, which excludes the least volume to the predominant background species. Association reactions are therefore highly favored under crowding conditions with the association constants, which could be several orders of magnitude larger than those measured in dilute solutions (3, 4). In this regard van den Berg et al. (9) have reported that high concentrations of BSA, ovalbumin, Ficoll 70, and Dextran 70 almost abolish the correct refolding of reduced and denatured lysozyme because of the enhanced aggregation and increase the refolding rate (10). They have claimed that their results are consistent with the prediction of excluded volume theory (9). The observations we report in the present study on the refolding of G6PDH and PDI in the presence of crowding agents have shown a different picture.

G6PDH from yeast (20) and PDI from bovine liver are both homodimeric molecules under the conditions of this study (only a small part of PDI exists as a homotetramer). The reactivation yields of G6PDH and PDI, depending on the protein concentration and temperature, are far from complete under the conditions used. The higher the protein concentration and the temperature, the lower the reactivation yields and the stronger the aggregation. Little large-scale aggregates during the refolding of G6PDH at 0.36 μM and PDI at 7 μM can be detected by light scattering measurements or collected by centrifugation at 16,000 rpm at 25 °C, and “soluble” aggregates of >1000 kDa characterized by gel filtration analysis form and correspond to wrongly folded and inactive molecules. The above indicates that similar to that for other proteins, which form aggregates or even precipitates during folding, aggregation during the folding of G6PDH or PDI, although mainly soluble, is also the principle competitor with correct folding and is responsible for the incomplete reactivation.

Considering the association of two subunits is necessary for the reactivation of denatured G6PDH or PDI in monomeric form, the refolding kinetics are shown below in Scheme 1.

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Considering the association of two subunits is necessary for the reactivation of denatured G6PDH or PDI in monomeric form, the reactivation kinetics are shown below in Scheme 1.

In step I (the correct folding pathway), two single unfolded or partially refolded subunits of G6PDH or PDI (U) associate to become a dimeric folding intermediate (I). Step II depends on the conformational change from I to a transient state (I*) to the native state N. In step III, aggregation from U to aggregate (U*) competes with the correct folding of steps I and II.

Crowding has been suggested to affect mainly the folding of those proteins that are prone to aggregation (9) but not to create this tendency de novo (5). Different from lysozyme, a monomeric protein that is strongly prone to aggregation, G6PDH and PDI are both dimeric molecules with a weaker tendency to aggregate. No change of the refolding yields of G6PDH and PDI in a crowding environment with 200 g/liter crowding agents may result from the combination of the two opposite effects of crowding; to enhance the self-association of monomeric subunits for the assembly of active dimeric molecules and to increase the unproductive aggregation, although not strongly. The balance between the above two pathways is kept in crowded conditions. In this regard it has been reported that crowding modulates the oligomerization of GroEL in two different ways: it increases the tendency of self-association of monomers at equilibrium and modifies the relative rates of the two competing self-association reactions, namely the productive assembly to native GroEL and unproductive aggregation (13). Although crowding agents strongly increase the aggregation of lysozyme, it almost abolishes its refolding. The consequential effects of crowding on the folding of a multisubunit protein depend on the properties of aggregation and association of the particular protein, and the effects differ between them.

As shown in Scheme 1, step I is a second-order reaction and depends on the encounter rate of two U monomers. Step II is a first-order reaction depending on conformational change. It has been reported that the reactivation kinetics of G6PDH at concentrations higher than 0.217 μM become a first-order reaction, and the conformational change from an inactive dimeric folding intermediate to native form is a rate-determining step (21). The effects of crowding on diffusion and conformational change oppose each other with respect to reaction rates (5). In theory, the maximal reaction rate possible for any bimolecular reaction has been suggested to be diffusion-controlled rather than transition state-limited when the concentration of crowding agents becomes high enough (3–5). But it is found that the refolding rate of G6PDH and PDI show the feature of a first-order reaction either in dilute or crowded solution, indicating that conformational change but not association is the rate-determining step. At the same time, crowding decreases the refolding rate of oligomeric G6PDH and PDI but increases the refolding rate of monomeric reduced lysozyme (9). In accordance to the excluded volume theory, we propose the following folding pathway in the presence of crowding agents to explain the experimental observations (Scheme 1). The folding of I to N might involve an unfolding process, which results in less compact I*, and the conformational change during the unfolding is the rate-determining step, which is unfavorable with the decreased refolding rates under crowding conditions (2). It has...
Effects of Macromolecular Crowding on Protein Folding

TABLE I

Kinetic parameters of G6PDH refolding in the presence of crowding agents

| Crowding agent | Y_{max,1} | k_{1} | Y_{max,2} | k_{2} |
|----------------|----------|------|----------|------|
| 25 °C          | 0        | 45.3 ± 0.8 | 133.5 ± 12.2 | 10^{-3} min^{-1} |
| BSA            | 100      | 44.7 ± 0.5 | 76.9 ± 4.2 | 10^{-3} min^{-1} |
| Ovalbumin      | 200      | 43.9 ± 0.7 | 43.2 ± 2.4 | 10^{-3} min^{-1} |
| lysozyme      | 100      | 32.7 ± 1.0 | 127.6 ± 11.1 | 10^{-3} min^{-1} |
| PEG           | 200      | 29.2 ± 1.0 | 50.1 ± 3.1 | 10^{-3} min^{-1} |
| 37 °C          | 0        | 16.7 ± 0.2 | 151.5 ± 8.4 | 10^{-3} min^{-1} |
| BSA            | 200      | 16.1 ± 0.7 | 64.4 ± 1.3 | 10^{-3} min^{-1} |
| Ovalbumin      | 200      | 10.1 ± 0.1 | 85.2 ± 3.0 | 10^{-3} min^{-1} |

TABLE II

Kinetic parameters of GroEL-assisted refolding of G6PDH in the presence of crowding agents

| Crowding agent (200 g/liter) | 25 °C | 37 °C |
|-----------------------------|-------|-------|
|                             | Y_{max} | k | Y_{max} | k |
| 0                           | 86.9 ± 1.4 | 97.2 ± 6.5 | 40.6 ± 0.6 | 63.9 ± 3.3 |
| BSA                         | 88.7 ± 3.1 | 53.1 ± 5.8 | 41.0 ± 0.2 | 53.6 ± 4.6 |
| Ovalbumin                   | 87.6 ± 0.7 | 45.0 ± 1.1 | ND          | ND          |
| lysozyme                    | 87.6 ± 0.7 | 45.0 ± 1.1 | ND          | ND          |

been found that in the refolding of bovine pancreatic trypsin inhibitor two key kinetic intermediates with disulfides of (30–51, 5–38) and (30–51, 5–14) unfolded to a substantial extent to become one with (30–51, 5–55) as an overall rate-limiting step just before attaining the fully native conformation with disulfides of (30–51, 5–55, 14–38) (22). The GroEL-assisted refolding of rhodanese was also slower in the presence of Xenopus oocyte extract but with a similar refolding yield compared with that in the absence of the extract (12). In fact, it has been assumed that the effect of crowding on reaction is complex and depends on the nature of the reaction, the species, and the concentrations of crowding agents (5).

The most remarkable feature of the effect of crowding agents (except BSA) on the refolding of G6PDH is to change the refolding kinetics from monophasic to biphasic of the first-order reaction. With increasing the concentration of crowding agents, the amplitude of the slow phase increases and that of the fast phase decreases but with decreased rate constants for both phases. We therefore further propose for G6PDH refolding that the presence of crowding agent results in a new folding pathway with I'_2, more compact than I_2, and the unfolding barrier from I'_2 through I_2'' to N_2 would be higher than that from I_2.

FIG. 6. Time courses of PDI refolding under crowding conditions. Denatured PDI was refolded at 7 μM at 25 (A) or 37 °C (B) in the absence (■) and presence of 200 g/liter Ficoll 70 (□) or Dextran 70 (▲). The insets show the rate constants of PDI refolding. The data are expressed as mean ± S.D. (n = 3–5).

FIG. 7. Time courses of GroEL-assisted refolding of G6PDH under crowding conditions. Denatured G6PDH was refolded at 0.36 μM at 25 (A) or 37 °C (B) containing 0.72 μM GroEL and 5 mM MgATP in the absence (■) and presence of 200 g/liter BSA (□), ovalbumin (▲), or lysozyme (▲). The data are expressed as mean ± S.D. (n = 3–5).
Thus with increasing concentrations of crowding agents, G6PDH folding intermediates would populate in the more compact state $I_2$ so as to increase the amplitude of the slow phase. BSA is an exceptional crowding agent and at high concentrations keeps the refolding kinetics of G6PDH monophasic. It has been noticed that BSA affected the refolding of denatured and reduced lysozyme, the refolding rate of which was increased significantly under crowding conditions compared with the much slower rate of this process compared with the much slower denatured and oxidized lysozyme was suggested to be caused by the high rate of this process compared with the much slower rate in crowded cells for some proteins, and molecular chaperones can counteract the effects of crowding. Compared with the refolding of lysozyme under crowding conditions, it seems that the excluded volume effect on protein folding is very complex and diverse and depends on the nature of proteins, interactions, and crowding agents.

The absence of large effects of crowding on the refolding of denatured and oxidized lysozyme was suggested to be caused by the high rate of this process compared with the much slower refolding of denatured and reduced lysozyme, the refolding rate of which was increased significantly under crowing conditions (9). It was therefore suggested that proteins might fold more quickly in vivo as a result of crowding, consequently making them less susceptible to aggregation (9). In fact, the refolding yield of reduced lysozyme in the presence of crowding agents decreased because of aggregation even though the refolding rate was increased (9). The refolding yields of G6PDH and PDI do not change but have slower folding rates under crowding conditions. It seems that effects of crowing on the reaction rate, folding yield, and aggregation of different proteins could be different.

One of the strategies of living cells to combat the effects of crowding is to employ molecular chaperones as evolutionary products (23). GroEL/MgATP dramatically enhances the refolding yield of G6PDH, and crowding is not able to interfere with the assisted effect of chaperone GroEL but only decreases the folding rate, because the dissociation of the GroEL-G6PDH complex would be prevented by the crowding agents so as to retard substrate folding. BSA and Ficoll decrease the PDI-assisted refolding of lysozyme (9). The effects of macromolecular crowding on chaperone-assisted refolding of different proteins also seem different. The most prominent effect of GroEL/MgATP on the refolding of G6PDH under crowding conditions observed in the present study is that GroEL “reverses” the refolding kinetics from biphasic to monophasic with the elimination of the slow phase produced by crowding agents and thus largely accelerates the refolding process compared with the spontaneous refolding under crowding conditions. Obviously, chaperones counteract the effects of crowding and function to increase the folding efficiency of proteins within cells.

GroEL, as a heat shock protein, assists G6PDH refolding at 37 °C more efficiently but slower than at 25 °C, suggesting stronger binding between G6PDH and GroEL at a higher temperature (16). It has been reported that the binding between GroEL and denatured Cys-Ala double mutant β-lactamase increased by increasing the temperature in the measured range, and higher temperatures favored the GroEL-assisted reactivation as an endothermic reaction (24).

The effects of crowding agents on the refolding of G6PDH or PDI are similar at 25 and 37 °C showing weak temperature dependence. Actually, the refolding kinetics of lysozyme in the presence of macromolecular crowding was studied at 20 °C (10) but not at 37 °C for studies on refolding and aggregation (9).

The macromolecular crowding is important, but unfortunately it is a largely neglected aspect of the intracellular environment (5). We have investigated the refolding of G6PDH and PDI under crowding conditions and proposed that folding yields could be retained at the cost of the decrease of folding rates in crowded cells for some proteins, and molecular chaperones can counteract the effects of crowding. Compared with the refolding of lysozyme under crowding conditions, it seems that the excluded volume effect on protein folding is very complex and diverse and depends on the nature of proteins, interactions, and crowding agents.

REFERENCES
1. Swaminathan, R., Hwang, C. P., and Verkman, A. S. (1997) Biophys. J. 72, 1900–1907
2. Minton, A. P. (2000) Curr. Opin. Struct. Biol. 10, 34–39
3. Zimmerman, S. B., and Minton, A. P. (1993) Annu. Rev. Biophys. Biomol. Struct. 22, 27–65
4. Minton, A. P. (2001) J. Biol. Chem. 276, 10577–10580
5. Ellis, R. J. (2001) Curr. Opin. Struct. Biol. 11, 114–119
6. Minton, A. P. (1983) Mol. Cell. Biochem. 55, 119–149
7. Minton, A. P. (1992) Biophys. J. 63, 1090–1100
8. Minton, A. P., Colclasure, G. C., and Parker, J. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10504–10506
9. van den Berg, B., Ellis, R. J., and Dobson, C. M. (1999) EMBO J. 18, 6927–6933
10. van den Berg, B., Wain, R., Dobson, C. M., and Ellis, R. J. (2000) EMBO J. 19, 3870–3875
11. Martin, J., and Hartl, F.-U. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1107–1112
12. Burston, S. G., Weissman, J. S., Farr, G. W., Fenton, W. A., and Horwich, A. L. (1996) Nature 383, 96–99
13. Galán, A., Sot, B., Llorca, O., Carrascosa, J. L., Valpuesta, J. M., and Muga, A. (2001) J. Biol. Chem. 276, 957–964
14. Lambert, N., and Freedman, R. B. (1983) Biochem. J. 213, 225–234
15. Li, J., and Wang, C. C. (1999) J. Biol. Chem. 274, 10790–10794
16. Hansen, J. E., and Gafni, A. (1993) J. Biol. Chem. 268, 21632–21636
17. Hu, C.-H., and Wang, C.-C. (1988) Chin. Biochem. J. 4, 61–68
18. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
19. Morjana, N. A., McKeone B. J., and Gilbert, H. F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2107–2111
20. Yue, R. H., Noltmann, E. A., and Kuby, S. A. (1969) J. Biol. Chem. 244, 1533–1541
21. Plomer, J. J., and Gafni, A. (1993) Biochim. Biophys. Acta 1993, 89–96
22. Creighton, T. E. (1997) Biol. Chem. 378, 731–744
23. Ellis, R. J. (1997) Curr. Biol. 7, R531–R533
24. Gervasoni, P., Gehrig, P., and Plückthun, A. (1998) J. Mol. Biol. 275, 663–675

![Scheme 2](image-url)
Effects of Macromolecular Crowding on the Refolding of Glucose-6-phosphate Dehydrogenase and Protein Disulfide Isomerase

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