Denaturation of Phosphofructokinase-1 from *Saccharomyces cerevisiae* by Guanidinium Chloride and Reconstitution of the Unfolded Subunits to Their Catalytically Active Form[†,‡]

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ABSTRACT: Unfolding and refolding of heterooctameric phosphofructokinase-1 from *Saccharomyces cerevisiae* were investigated by application of kinetic, hydrodynamic, and spectroscopic methods and by use of guanidinium chloride (GdmCl) as denaturant. Inactivation of the enzyme starts at about 0.3 M GdmCl and undergoes a sharp unfolding transition in a narrow range of the denaturant concentration. The inactivation is accompanied by a dissociation of the enzyme into dimers (at 0.6 M GdmCl), which could be detected by changes of the circular dichroism and intrinsic fluorescence. Protein aggregates were observed from 0.7 to 1.5 M GdmCl that unfold at higher denaturant concentrations. Refolding of chemically denatured phosphofructokinase proceeds as a stepwise process via the generation of elements of secondary structure, the formation of assembly-competent monomers that associate to heterodimers and the assembly of dimers to heterotetramers and heterooctamers. The assembly reactions seem to be rate-limiting. Recovery of the enzyme activity (maximum 65%) competes with an nonproductive aggregation of the subunits. α-Cyclodextrin functions as an artificial chaperone by preventing aggregation of the subunits, whereas ATP is suggested to support the generation of heterodimers that are competent to a further assembly.

Folding and assembly of proteins are basic events in realizing the genetic message upon preceding translation. Both processes are determined by the sequence of the amino acids and are driven by small but significant differences in the Gibbs free energy in the course of the folding pathway (reviewed in ref *1*).

In vivo, folding of proteins was found to occur spontaneously, either without additional factors and/or, with respect to correctness and progress of the straightforward reaction, by diverse folding helpers that have been summarized as molecular chaperones (reviewed in refs *2* and *3*). Refolding of denatured polypeptides in vitro has been an important issue for both basic research and applied biotechnology, in particular for the production of those recombinant proteins that tend to aggregate in inclusion bodies when overexpressed in host cells (*4*).

The major advances in understanding protein folding come from the investigation of renaturation of small model proteins denatured by urea, guanidinium chloride (GdmCl),[1] pH, or heat.

Refolding of oligomeric proteins raises a number of additional problems, the most important of which are related to the specificity of association of subunits. This process turns out to be specific, so that recognition sites of subunits must be formed during the entire folding process. However, the folded structure of the isolated subunits may have only marginal stability in the absence of the other subunits, since their interaction sites are mostly hydrophobic in nature (*1*). Refolding pathways of a number of oligomeric enzymes, such as lactate dehydrogenase (*5*−*7*), aspartate aminotransferase (*8*), glutathione transferase (*9*, *10*), and β-galactosidase (*11*), have been studied in detail. The molecular masses of their respective monomers ranged from 30 to 50 kDa, with the exception of the monomer of galactosidase, exhibiting 116 kDa (*12*). In the case of lactate dehydrogenase, a first rate-limiting step was found to be the formation of dimers in a bimolecular reaction followed by an unimolecular folding step to dimers that are competent for the tetramer formation (*7*).

Extended studies on refolding of larger heterooligomeric proteins have not been reported yet. The assembly pathway of different kinds of subunits to a biologically active structure

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[1] Abbreviations: CD, circular dichroism; DTT, dithiothreitol; Fru 6-P, fructose 6-phosphate; GdmCl, guanidinium chloride; GDH, glycerolphosphate dehydrogenase; PBS, phosphate buffered saline (50 mM sodium phosphate, pH 7.5, and 155 mM sodium chloride); PBST, phosphate buffered saline containing 0.05% Tween 20; Pfk-1, phosphofructokinase-1 (EC 2.7.1.11); PMSF, phenylmethanesulfonyl fluoride; SEC, size- exclusion chromatography; TIM, triosephoshate isomerase; IU, international unit of enzyme activity (1 μmol/min at 25 °C).

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appears to be more complex. Therefore, the investigation of such a process may lead to an increase of our knowledge on the mechanism of assembly and the significance of protein−protein interactions in oligomeric structures.

Since phosphofructokinase-1 (Pfk-1) from the yeast *Saccharomyces cerevisiae* is a well-known example of a large heterooctameric protein, we were interested in studying the kinetics of unfolding and the pathway of refolding and reconstitution.

Pfk-1 catalyzes the formation of fructose 1,6-bisphosphate from fructose 6-phosphate (Fru 6-P) and MgATP and plays a key role in regulating the glycolytic flux (*13*−*15*). It is well characterized in terms of kinetic and molecular parameters (*16*, *17*). The two types of subunits, α and *β*, exhibiting calculated masses of 108 and 105 kDa, respectively (*18*), assemble to heterotetramers. Two of them combine to a closed octamer (*19*, *20*), possessing a mass of about 835 kDa. The octameric form of Pfk-1 was found to be stable in the protein concentration range used in this study. A significant dissociation of the native enzyme was observed at first below 1 *μ*g/mL (*26*).

Both subunits, encoded by the genes *PFK1* and *PFK2*, are structurally similar (*18*). In single-deletion mutants of *Saccharomyces cerevisiae* containing either the α- or the *β*-subunit, the glycolytic pathway is still functioning. On the other hand, Pfk-1 composed of only one type of subunits was found to be unstable in vitro and inactivates completely immediately after disruption of the cells (*21*, *22*). Reactivation of the enzyme by mixing cell-free extracts of both single deletion mutants failed, because the individual subunits form aggregates in the course of preparing the extract. We have postulated considering these results that (i) the only stable enzyme form in vitro exists as a heterooligomer, (ii) isolated subunits of Pfk-1 are not able to perform their catalytic function in vitro due to the formation of insoluble aggregates, (iii) Pfk-1 subunits expressed in single-deletion mutants are potentially able to recombine in vitro to an active enzyme under conditions preventing the subunits from aggregation in the assembly reaction.

In this work, we investigated the kinetics of unfolding of purified Pfk-1 and the conditions of refolding and reactivation using GdmCl as denaturant. We have studied these processes by following changes of the catalytic activity and hydrodynamic properties, as well as fluorescence spectroscopy, turbidimetry, and circular dichroism spectroscopy. Denaturation of the enzyme proceeds via intermediate species to monomers, which are rather unstable and form aggregates. The aggregates can be redissolved at 3 M GdmCl to form the unfolded structure. Renaturation of the enzyme yielding a native heterooctameric protein could be observed only after complete unfolding of both types of subunits. The highest yield of reactivation was obtained by addition of ATP and α-cyclodextrin; the latter might act as a molecular chaperone.

MATERIALS AND METHODS

Chemicals. GdmCl (99% grade), α-cyclodextrin, and *γ*-cyclodextrin were purchased from Fluka (Deisenhofen, Germany). 2,2′ Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and all biochemicals were obtained from Boehringer (Mannheim, Germany). Phenylmethanesulfonyl fluoride (PMSF) was from Serva (Heidelberg, Germany). Other substances used were of analytical grade.

Enzyme Preparation. Phosphofructokinase-1 was purified from PS1 (*MATa ura3-52 leu2-3, 112 his4-519 pep4-3 gal2 cir*$^+$), a recombinant strain of *Saccharomyces cerevisiae* carrying additionally *PFK1* and *PFK2* genes in a multicopy vector. PS1 is the *cir*$^+$ form of the strain PS2 as described by Seeboth et al. (*23*) and was kindly provided by Dr. J. Heinisch (Düsseldorf, Germany). The enzyme was purified to homogeneity according to ref (*24*) with the following modifications: Cell disruption was carried out by vigorous shaking of the yeast suspension in a Vibrogen cell mill (E. Bühler, Tübingen, Germany). Fractionated precipitation of the enzyme was performed with poly(ethylene glycol) 6000 [4% (w/w) and 15% (w/w]. The enzyme was desorbed from Cibacron Blue F3G-A−Sephadex G100 gel with 5 mM MgATP instead of ammonium sulfate. The ion- exchange chromatography was performed in HPLC mode on the column Resource Q (Pharmacia, Uppsala, Sweden). The enzyme was stored in 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol, and 0.5 mM PMSF, saturated with ammonium sulfate at 4 °C.

Protein Concentration and Enzyme Activity. The concentration of the native Pfk-1 was determined spectrophotometrically at 279 nm with the molar absorption coefficient of $\epsilon = 736\,000\ M^{-1}\,cm^{-1}$, related to a molecular mass of 835 kDa (*16*). The enzyme activity was measured spectrophotometrically at 25 °C by a coupled assay with aldolase, triosephosphate isomerase (TIM), and glycerolphosphate dehydrogenase (GDH) as auxiliary enzymes. The assay solution contained 100 mM imidazole hydrochloride, pH 7.15, 3 mM Fru 6-P, 0.6 mM ATP, 1 mM AMP, 5 mM $MgSO_4$, 5 mM $(NH_4)_2SO_4$, 0.2 mM NADH, 1 unit of aldolase/mL, 1.5 units of GDH/mL, and 5 units of TIM/mL. The concentration of GdmCl in the assay (maximum concentration 10 mM), which resulted by addition of the denatured Pfk-1 sample into the cuvette, did not disturb the conditions of the enzyme assay.

Inactivation and Reactivation of Phosphofructokinase-1. After exhaustive dialysis of the enzyme against 0.1 M potassium phosphate buffer, pH 7.0, containing 5 mM DTT (buffer A), the enzyme was denatured by GdmCl dissolved in buffer A. Equilibrium denaturation was attained after a 30 min incubation of the enzyme at 25 °C. Protein inactivation was followed by measuring the enzymatic activity.

Reactivation of Pfk-1 was initiated by rapid 100-fold dilution of the denatured samples into buffer A without or with diverse additives as indicated. At different times, aliquots were taken and assayed for Pfk-1 activity.

Fluorescence Measurements. The intrinsic fluorescence was determined with the luminescence Spectrometer LS50B (Perkin-Elmer) at an excitation wavelength of 280 nm. Excitation and emission slits were set to 5 nm. Quartz cuvettes of 10 mm/10 mm were used. All spectra were corrected for the buffer baseline.

The kinetic experiments were performed with an Applied Photophysics BioSequential DX.18 MV stopped-flow spectrometer (Leatherhead, U.K.). The change in total fluorescence above 320 nm (cutoff filter) on excitation at 280 nm was recorded at 20 °C. The photomultiplier was set to about 550 V in all experiments. The path length of the observation chamber was 2 mm/10 mm. All kinetic folding studies were carried out in buffer A. The final protein concentration after 11-fold dilution of a stock solution was 20 or 40 *μ*g/mL.

Protein folding occurred as a multistep transition and can be described by

$$y(t) = \Sigma y_i e^{-k_i t} + y(\infty) \quad (1)$$

where $y(t)$ and $y(\infty)$ are amplitudes of the optical sample at time t and at equilibrium, respectively, while y_i is the amplitude and k_i is the first-order rate constant of phase *i*. Data were fitted to consecutive first-order reactions. Unfolding experiments were performed by an 11-fold dilution of Pfk-1 into buffer A containing different concentrations of GdmCl. Refolding experiments were performed by an 11-fold dilution of GdmCl-unfolded enzyme into buffer A. At least 4−7 traces were used for averaging.

Circular Dichroism Measurements. The circular dichroism (CD) experiments were performed with an Aviv CD spectropolarimeter at 20 °C using cuvettes of 1 mm (far UV) and 10 mm (near UV) optical path length. Spectra were acquired at a scan speed of 20 or 50 nm/min, respectively, a slit wide of 1 nm, and a response time of 1 s. All spectra were corrected for buffer baseline.

Turbidity Measurements. Protein aggregation was measured by following the increase of absorbance at 400 nm at 25 °C on a Uvikon 930 spectrophotometer (Kontron Instruments). The denatured Pfk-1, dissolved in 3 M GdmCl, was diluted 60-fold into buffer A without or with additives to a final concentration of 0.07 mg /mL.

Size-Exclusion HPLC. Isocratic size-exclusion chromatography (SEC) was performed at 20 °C on a Bio-Silect SEC 400-5 column combined with a SEC 400 guard column (Bio-Rad, München, Germany) and coupled to the Beckman HPLC System Gold. The column was equilibrated and run in 0.1 mM potassium phosphate buffer, pH 7.0, 2 mM DTT, and additives as indicated in the legends of the figures at a constant flow rate of 1 mL/min. The absorbance of the eluate was monitored at 280 nm and fractions of 0.25 or 0.5 mL were collected. The standard protein mixture (Bio-Rad) and samples of native octameric Pfk-1 (21 S Pfk-1) and of the truncated tetrameric half-molecule (12 S Pfk-1) (*25*) were used for a molecular weight estimation of the peak fractions.

ELISA of Pfk-1. The assay was performed in a sandwich mode. Nunc-Immuno-Plates I (96 wells with high binding capacity) were coated overnight at 4 °C with sheep polyclonal Pfk-1 antibodies (1 μg/mL in 0.1 M sodium carbonate buffer, pH 9.6), purified by immunosorption on Pfk-1−Sepharose. After being washed three times with 50 mM sodium phosphate buffer, pH 7.5, containing 155 mM sodium chloride and 0.05% Tween 20 (PBST), the wells were filled with 0.2 mL samples in duplicate. The samples from the size-exclusion HPLC were diluted 1:2 with PBS containing 0.1% Tween 20. After incubation for 1.5 h at 25 °C in a moist chamber, the plates were washed three times as described above. Subsequently, 0.2 mL of rabbit anti-Pfk-1−peroxidase conjugate (IgG fraction purified by immunosorption on Pfk-1−Sepharose and labeled with horseradish peroxidase, diluted 1:6000 with PBST) were poured into the wells. After a second incubation for 1.5 h at 25 °C in a moist chamber, the wells were rinsed 3−4 times with PBST and then filled with 0.2 mL of substrate solution [2 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 3.25 mM sodium perborate, 39.8 mM citric acid, and 60 mM sodium phosphate buffer, pH 4.4]. The reaction was allowed to proceed for 10 min at 25 °C to obtain sufficient absorbance at 405 nm. Samples without antigen were used as a blank.

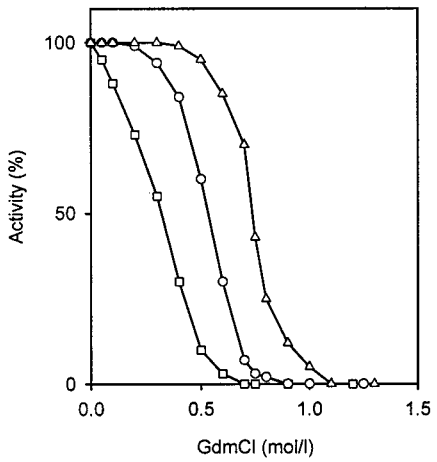


FIGURE 1: GdmCl-dependent inactivation of phosphofructokinase. The dialyzed enzyme was diluted into buffer A at different concentrations of GdmCl to a final protein concentration of 2 μg/mL. (○) Without effectors; (□) with 3 mM ATP; (△) with 3 mM Fru 6-P. Pfk-1 activity was determined after incubation for 30 min at 25 °C. The values are means of three experiments.

Analytical Ultracentrifugation. Analytical ultracentrifugation was carried out with a Beckman XL-A ultracentrifuge equipped with a UV absorption scanner optics at 280 nm. The temperature was set at 20 °C. Sedimentation velocity experiments were performed at 42 000 rpm by using a double sector cell of 12 mm light path. The Optima XL-A analysis software version 2.0 from Beckman Instruments was used for the calculation of the apparent sedimentation coefficients. Prior to centrifugation, the enzyme was exhaustively dialyzed against buffer A, diluted to 2 mg/mL, and incubated for 3 h at room temperature at the respective GdmCl concentration.

RESULTS

Inactivation of Pfk-1. The enzyme inactivation started at about 0.3 M GdmCl. This process was complete at 0.8 M denaturant. Pfk-1 activity disappeared with increasing concentrations of GdmCl via a cooperative transition as demonstrated in Figure 1. The addition of 2 mM Fru 6-P to the denaturation sample caused a shift of the inflection point of the inactivation curve to a higher concentration of GdmCl (0.75 M), while an addition of ATP decreased the stability of Pfk-1 significantly. A 1000-fold increase of the enzyme concentration as demonstrated in Figure 1 revealed only a small shift of the midpoint of transition from 0.55 to 0.65 M GdmCl. This result is in accordance with the high structure stability of the enzyme in dependence on its concentration (*26*).

Hydrodynamic Studies. Sedimentation velocity runs and size-exclusion HPLC of Pfk-1 at increasing concentrations of the denaturant indicated a stepwise denaturation of the enzyme (Table 1). Until 0.5 M GdmCl, the sedimentation coefficients and the retention time of the enzyme corresponded to that of the native octamer. Between 0.5 and 1.5 M GdmCl protein aggregates were formed, which quickly sedimented in the cell during acceleration of the ultracentrifuge. According to Figure 1, the concentration range of GdmCl between 0.5 and 0.8 M seems to be the critical one for destabilizing the oligomeric structure of Pfk-1. Therefore,

Table 1: Apparent Sedimentation Coefficient and Retention Time in SEC−HPLC[a] at Different Concentrations of GdmCl

GdmCl (M)	s_{app} (S)	retention time of peak fractions (min)
without	20.8 ± 0.2	9.6 ± 0.1
0.3	−	9.8 ± 0.1
0.5	20.2 ± 0.4	9.8 ± 0.1
0.7	nd[b]	9.8 ± 0.2 and 11.4 ± 0.2
1.0	nd[b]	6.2 ± 0.3
1.5	21 and 40	6.2 ± 0.2[c]
2.0	18	6.7 ± 0.2
2.5	2.0 ± 0.2	
3.0	1.8 ± 0.2	9.4 ± 0.4
5.0	1.3 ± 0.2	

[a] BioSilect SEC 400 column. [b] nd, not detectable; protein sediments already during acceleration. [c] Broad peak.

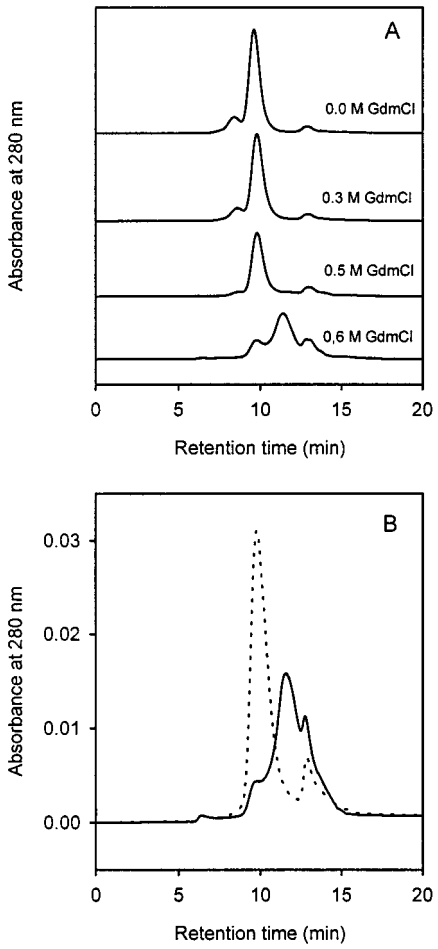


FIGURE 2: Analysis of unfolding of phosphofructokinase-1 by gel-permeation chromatography. (A) Dialyzed Pfk-1 was diluted into buffer A containing GdmCl as indicated to a final concentration of 2 mg/mL. After incubation at 25 °C for 30 min, 0.05 mL samples were loaded onto a Bio-Silect SEC 400-5 column equilibrated with the respective denaturation buffer. Protein absorbance was recorded at 280 nm. (B) Dialyzed enzyme was diluted into buffer A containing 0.6 M GdmCl to a final protein concentration of 2 mg/mL. After 30 min of incubation at 25 °C, 3 mM Fru 6-P was added and the sample allowed to stand at the same temperature for 1.5 h. (—) Without Fru 6-P; (···) with 3 mM Fru 6-P. The column was equilibrated with denaturation buffer without and with 3 mM Fru 6-P, respectively.

denaturation was followed in SEC−HPLC by a stepwise increase of GdmCl in a narrow range. As depicted in Table 1 and Figure 2A, only one main peak was eluted below 0.5 M GdmCl; its retention time t_R = 9.6−9.8 min corresponded

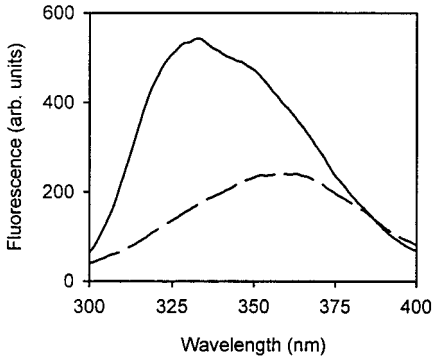


FIGURE 3: Fluorescence spectra of phosphofructokinase-1 in the absence and presence of 3 M GdmCl. The dialyzed enzyme was diluted into buffer A giving a final concentration of 0.01 mg/mL, and denatured with 3 M GdmCl in the same buffer at 25 °C. After 30 min of incubation, samples were excited at 280 nm with a slit width of 5 nm. (− − −) 3 M GdmCl; (—) control without GdmCl.

to the octameric enzyme. The small peak in front of the main fraction represents self-associating Pfk-1 as was observed earlier (*26*). This species disappeared with an increasing concentration of GdmCl. The minor peak detected at t_R = 13 min resulted from small impurities. It should be mentioned that there was a loss of some protein due to aggregation and precipitation even at 0.5 M GdmCl. Between 0.5 and 0.7 M GdmCl protein precipitation increased. The peak corresponding to the octamer decreased in the remaining enzyme solution, which paralleled to the appearance of a protein fraction with a retention time t_R = 11.4 min (Figure 2B). This peak corresponds to a molecular weight species of about 200 kDa. Precipitated Pfk-1 redissolved completely at 2 M GdmCl and the protein appeared in the breakthrough fraction (t_R = 6.2 min). However, the retention time of the enzyme changed from 6.2 to 9.4 min at 3 M GdmCl and indicated the final hydrodynamic state of the unfolded Pfk-1.

To check the reversibility of denaturation of Pfk-1 under that conditions, where the enzyme dissociated preferably into dimers, we added Fru 6-P preceding the denaturation at 0.6 M GdmCl. As seen in Figure 2B, Fru 6-P shifted the protein fraction of 200 kDa completely to the octamer, which paralleled a recovery of the enzymatic activity of this fraction. The activity, which decreased to 20% of the control during a 30 min denaturation by GdmCl, recovered to 95%.

Spectroscopic Studies. The fluorescence emission spectrum of the native enzyme revealed a maximum at 332 nm and a shoulder at 345 nm (Figure 3), which characterized at least two classes of tryptophan residues. The first class at 332 nm comprises residues in a nonpolar environment. The second class of tryptophans having a maximum at 345 nm comprises residues located at the surface of the protein, which are surrounded by bound water dipoles with low mobility (*27*). On denaturation of the enzyme by 3 M GdmCl, a significant decrease of the fluorescence intensity was observed, which is accompanied by a red shift to a maximum at 357 nm.

Native Pfk-1 showed a typical far-ultraviolet circular dichroism spectrum as observed for proteins with a high α-helix content (Figure 4). After incubation with at least 3 M GdmCl, the CD spectrum drastically changed and points to an extensive unfolding.

As depicted in Figure 5A, no significant changes of the intrinsic fluorescence were observed until 0.3 M GdmCl.

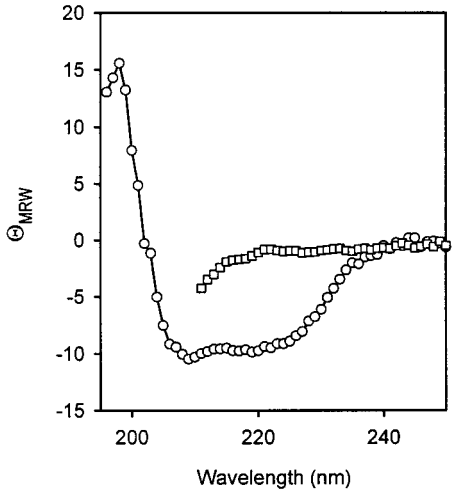


FIGURE 4: Circular dichroism of native and GdmCl-denatured phosphofructokinase. The dialyzed enzyme was diluted into 0.1 M buffer A without or with 3 M GdmCl and allowed to stand at 25 °C for 30 min. The optical path length was 1 mm (far UV) and 10 mm (near UV), respectively. The spectra were recorded at 20 °C and averaged 5 times. The protein concentration was 0.2 mg/mL. (○) Control without denaturant; (□) with 3 M GdmCl.

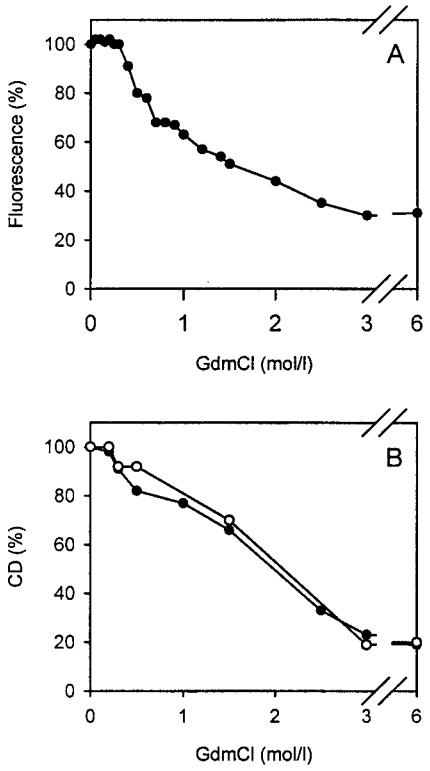


FIGURE 5: Equilibrium unfolding of phosphofructokinase-1 monitored by intrinsic fluorescence at 330 nm and far- and near-UV circular dichroism. After dialysis, the enzyme was diluted into buffer A containing GdmCl as indicated. (A) Relative fluorescence of Pfk-1 (10 μg/mL) at 330 nm as a function of the GdmCl concentration. (B) Relative circular dichroism of Pfk-1 (0.2 mg/mL) at 220 nm (○) and 280 nm (●).

Above 0.3 M GdmCl, a denaturant concentration where the enzyme started to inactivate, the relative fluorescence intensity decreased, reaching a small plateau at 0.7 M GdmCl. This indicated obviously an intermediate structure of the protein, which paralleled the dissociation of the enzyme and the formation of aggregates.

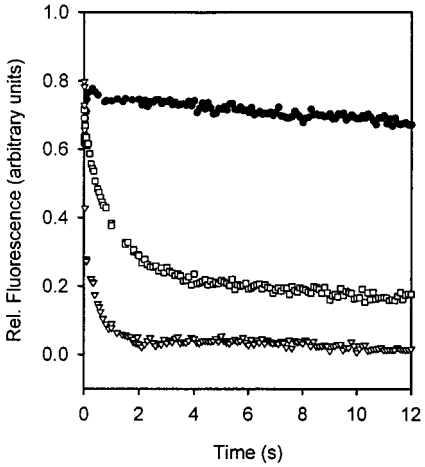


FIGURE 6: Kinetics of unfolding of phosphofructokinase-1 monitored by stopped-flow fluorescence. The reaction was started by mixing dialyzed Pfk-1 (0.42 mg/mL) with 10 volumes of buffer A containing different concentrations of GdmCl. The excitation wavelength was 280 nm; the emission cutoff filter was set at 320 nm. Concentration of GdmCl in the buffer: (●) 0.8 M GdmCl; (□) 1.5 M GdmCl; (▽) 3.0 M GdmCl.

The near- and far-UV CD signal decreased continuously (Figure 5B). Above 3 M GdmCl, the relative fluorescence and circular dichroism did not further change, indicating a stable state of the protein.

The stopped-flow fluorescence technique was applied to study the kinetics of denaturation at different denaturant concentrations. As seen from the change in the integral fluorescence intensity in Figure 6, several phases can be observed in the denaturation of Pfk-1 in dependence on the denaturant concentration. Up to 0.4 M GdmCl only a little increase of the optical signal could be observed. Between 0.4 an 0.8 M GdmCl this increase is followed by a slower decrease, which can be fitted to double-exponential first-order reactions. However, from 1 to 3 M GdmCl, only a decrease of the integral fluorescence is observed, which can be fitted to triple exponential first-order reactions. The unfolding proceeds as single-exponential first-order reaction at even higher denaturant concentrations. All rate constants of the detected unfolding phases are denaturant-dependent. They are summarized in Table 2 together with the relative amplitudes of the corresponding phase.

Reactivation of Pfk-1. We have studied the renaturation of Pfk-1 after 100-fold dilution of the denatured sample into renaturation buffer. Starting from various concentrations of GdmCl, which resulted in a complete inactivation of the enzyme, a significant reactivation was found only after preceding denaturation at GdmCl concentrations above 2.5 M. The reactivation of Pfk-1 after denaturation by 3 M GdmCl is shown in Figure 7. However, the kinetics of reactivation is characterized by a small lag phase (Figure 7, inset), and the yield of the active enzyme depends on the temperature, the protein concentration and on several additives. Maximum recovery of activity (about 65%) was achieved in the presence of ATP and α-cyclodextrin (Figure 7A), at protein concentrations between 0.01 and 0.02 mg/mL and at a temperature of 10 °C. An increase of the temperature or the protein concentration reduced the yield of active enzyme (Table 3). On the other hand, the time required for obtaining the maximum activity decreased with

Table 2: Relative Amplitudes[a] and Rate Constants for the Unfolding of Phosphofructokinase

GdmCl (M)	relative amplitude A_1 (%)	rate constant k_1 (s^{-1})	relative amplitude A_2 (%)	rate constant k_2 (s^{-1})	relative amplitude A_3 (%)	rate constant k_3 (s^{-1})
0.80	100	0.03				
1.00	15.4	0.32	84.6	0.05		
1.25	13.3	1.54	58.6	0.51	28.1	0.034
1.50	10.6	4.78	63.2	0.89	26.2	0.045
1.75	45.8	3.55	32.9	0.98	21.2	0.057
2.00	53.2	4.80	27.3	0.98	19.5	0.061
2.50	48.8	10.80	30.2	2.26	21.0	0.152
3.00	61.6	38.06	23.3	5.50	15.0	0.553
4.00	77.0	50.93	22.9	5.45		
5.00	100	32.56				

The relative amplitude of a phase is defined as the relation of its absolute amplitude divided by the total absolute amplitude (sum of the absolute amplitudes of all phases detected in the progress curve).

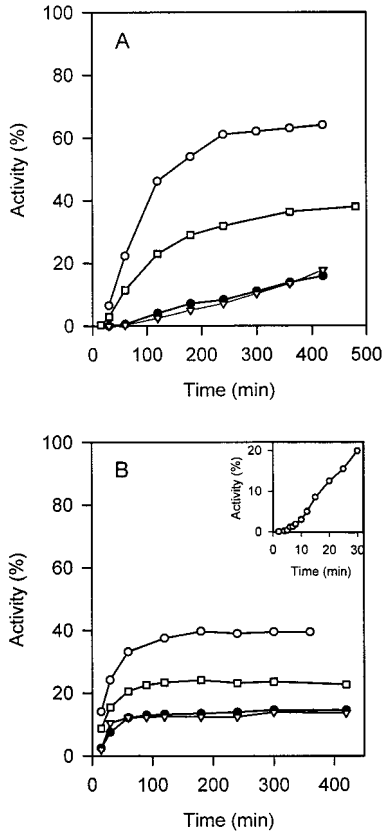


FIGURE 7: Reactivation kinetics of GdmCl-denatured phosphofructokinase-1. Denaturation of the enzyme (2 mg/mL) was performed at 3 M GdmCl. The reactivation was started by a 100-fold dilution of the sample into buffer A without or with additives as indicated. (A) Reactivation at 10 °C; (B) reactivation at 25 °C. (●) Without additions; (▽) with 40 mM α-cyclodextrin; (□) with 3 mM ATP; (○) with 3 mM ATP and 40 mM α-cyclodextrin. The inset contains the expanded time scale of reactivation in the presence of ATP and α-cyclodextrin.

increasing temperature ($t_{1/2}^{25°C} \sim 25$ min; $t_{1/2}^{10°C} \sim 90$ min). A further decrease of the temperature below 10 °C had no influence on the yield of the recovery, but the time to obtain the maximum activity was prolonged.

The recovery of active enzyme appears to be dependent on the proportion of the concentrations of α-cyclodextrin and Pfk-1. While at 0.02 mg/mL Pfk-1 a concentration of 40 mM α-cyclodextrin was found to be sufficient for a maximum reactivation, 100 mM α-cyclodextrin was necessary at 0.1 mg/mL Pfk-1. An addition of ATP is essential for an increase of Pfk-1 reactivation, but Fru 6-P was found to be inefficient (data not shown). α-Cyclodextrin alone did not cause a higher yield of the reactivated enzyme; in combination with ATP, however, its effect was maximal (Figure 7).

Table 3: Effect of Protein Concentration on Phosphofructokinase-1 Reactivation[a]

protein concentration (μg/mL)	recovery of the enzymatic activity (%)
5	65 ± 5
20	65 ± 4
30	45 ± 3
50	31 ± 3
75	19 ± 3
100	16 ± 2

[a] After denaturation of the enzyme at 3 M GdmCl, the reactivation was initiated by dilution into buffer A containing 3 mM ATP and 40 mM α-cyclodextrin. The activity was measured 300 min after dilution and compared with that of Pfk-1 treated under the same conditions but without GdmCl.

Spectroscopic Studies. Refolding of Pfk-1 was also followed by monitoring the change in far-UV circular dichroism at 220 nm immediately after 10-fold dilution of the denatured sample. The CD signal, representative for the secondary structure, increased to the value of the native state within 30 s after dilution (result not shown). The time scale of this process was found to be much shorter than that for regaining the total fluorescence signal. Stopped-flow fluorescence measurements revealed a lag phase of about 80 s after dilution of the denatured protein before the intrinsic fluorescence increased and reached a plateau after about 10 min (Figure 8). The curve fits well to a first-order reaction. The reaction rate was found to be dependent on the denaturant concentration but independent of the protein and α-cyclodextrin concentrations (results not shown) The signal amplitude of the refolding reaction starting from fully unfolded Pfk-1 corresponded to that of the unfolding reaction at high denaturant concentration.

Hydrodynamic Studies. The mass distribution of the refolded Pfk-1 was analyzed by size-exclusion HPLC. The activity profile of the enzyme, obtained 5 h after 100-fold dilution of the denatured sample (Figure 9), was identical with the elution profile of the native 800 kDa Pfk-1, which was run as a control under the same conditions. The protein maximum of the renatured sample coincided with the maximum of the enzymatic activity (Figure 9, fractions 21 and 22). However, in analyzing a sample obtained 45 min after starting renaturation, the protein profile obtained

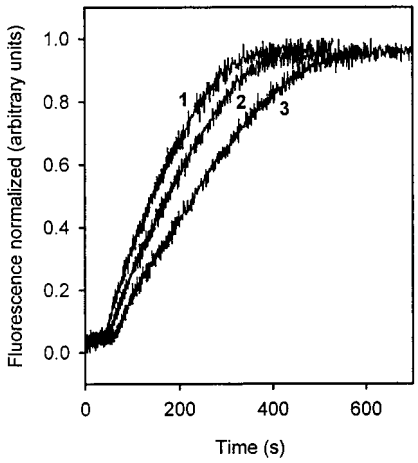


FIGURE 8: Kinetics of refolding of phosphofructokinase-1 monitored by stopped-flow fluorescence. The dialyzed enzyme was denatured in buffer A containing GdmCl. Refolding reaction was started by mixing the denatured protein (0.4 mg/mL) with 10 volumes of buffer A at 20 °C. Concentration of GdmCl in the denaturation buffer: (1) 3 M GdmCl; (2) 4 M GdmCl; (3) 5 M GdmCl. The excitation wavelength was set at 280 nm, and the cutoff filter was set at at 320 nm.

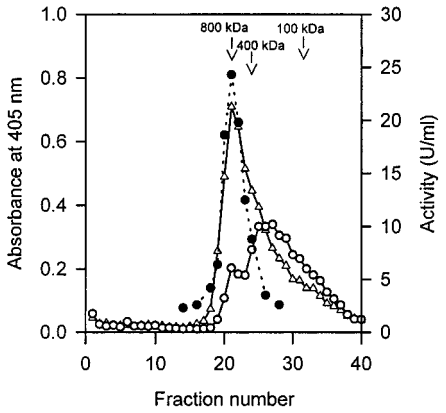


FIGURE 9: Reassociation of phosphofructokinase as monitored by size-exclusion HPLC. Dialyzed Pfk-1 was denatured at 3 M GdmCl dissolved in buffer A. The final protein concentration was 2 mg/mL. The enzyme was diluted 100-fold into buffer A containing 3 mM ATP and 40 mM α-cyclodextrin. After 45 and 300 min, respectively, samples of 0.05 mL were taken and loaded onto a Bio-Silect SEC 400 column, equilibrated with buffer A containing 0.1 M $(NH_4)_2SO_4$. The flow rate was 1 mL/min and fractions of 0.25 mL were collected. (○) Pfk-1 protein (ELISA) 45 min after dilution; (△) Pfk-1 protein (ELISA) 300 min after dilution; (●) enzyme activity measured 300 min after dilution.

revealed only a small amount of protein with a molecular mass of 800 kDa. A larger portion of Pfk-1 was eluted as a broad peak corresponding to masses between 200 and 400 kDa without enzymatic activity. When the time of refolding was prolonged, the lower molecular weight fraction disappeared and the fraction corresponding to a molecular mass of 800 kDa increased. It should be emphasized that in this experiment the elution of Pfk-1 protein was followed by applying an immunological assay, because of the low concentration of the renatured Pfk-1 after 100-fold dilution of the sample.

Dissolving Aggregates. Reactivation of GdmCl-denatured proteins by dilution competes with the formation of insoluble aggregates (*28*, *29*). To follow this nonproductive side reaction, we measured the increase in turbidity in the course

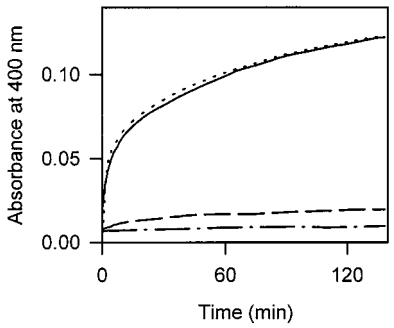


FIGURE 10: Aggregation kinetics of phosphofructokinase-1 in the absence and presence of additives monitored by turbidimetric measurements at 400 nm. The dialyzed enzyme (7 mg/mL) was denatured with 3 M GdmCl, dissolved in buffer A, at 25 °C for at least 2 h and afterward diluted 100-fold into buffer A without or with additives. (—) No additives; (···) 3 mM ATP and 3 mM Fru 6-P; (– – –) 40 mM α-cyclodextrin; (– · –) native Pfk-1.

of renaturation and in dependence on different additives. A significant increase in the turbidity was observed after dilution of the GdmCl-denatured Pfk-1 in the absence of additives, but also in the presence of ATP as demonstrated in Figure 10. On the other hand, α-cyclodextrin depressed the formation of aggregates and the enzyme sample behaved similarly to the control.

Additionally, we tried to reactivate the aggregates obtained by thermal denaturation of Pfk-1. The aggregates, formed during a complete inactivation of Pfk-1 (4 mg/mL) by heating in buffer A at 60 °C for 1 h, were redissolved in 3 M GdmCl at 25 °C for 1 h. Afterward, this solution was 100-fold diluted into buffer A containing 3 mM ATP and 40 mM α-cyclodextrin. After 4 h of agitation we found a recovery of the enzymatic activity of about 40% at 10 °C. Dilution of the thermally denatured enzyme without preceding dissolution in 3 M GdmCl did not regain the enzymatic activity.

DISCUSSION

We could demonstrate at first that the heterooctameric Pfk-1 with a molecular mass of 835 kDa is able to reconstitute to its native form after complete unfolding of the subunits. Unfolding of Pfk-1 can be achieved by increasing the concentration of the denaturant. Up to 0.3 M GdmCl, the octameric enzyme showed neither significant changes of the structure nor inactivation as demonstrated for a broad concentration range. Pfk-1 was found to dissociate predominantly into dimers from 0.5 to 0.6 M GdmCl. This process is reversible, since the addition of Fru 6-P caused a reshifting to the octameric structure accompanied by a reactivation. At 0.7 M GdmCl, the formation of insoluble aggregates became significant, probably via the monomeric forms of the enzyme. A further increase of the denaturant resulted in a resolution of these aggregates as indicated by hydrodynamic measurements. The stepwise structural alterations were recorded by the change of the intrinsic fluorescence. The enzyme has reached its unfolded state at 3 M GdmCl, since a further increase of GdmCl changed neither its circular dichroism spectrum nor the intrinsic fluorescence and hydrodynamic properties.

Dissociation of the oligomeric Pfk-1 in the concentration range from 0.5 to 1.0 M GdmCl did not generate stable α- and β-monomers, respectively. The dissociation of the enzyme into monomers promotes the formation of ag-

gregates, governed mainly by increased exposure of apolar surfaces (*1*). It should be mentioned that a spontaneous aggregation of Pfk-1 subunits has also been observed after cell lysis of Pfk-1 single deletion mutants (*21*). Therefore, we conclude that monomeric subunits of Pfk-1 are unstable under in vitro conditions.

A reactivation of Pfk-1 has been observed either at moderate concentrations of GdmCl, where the enzyme is arrested at the dimeric state, or after complete unfolding of the subunits by at least 3 M GdmCl. Protein aggregates obtained at GmdCl concentrations from 0.7 to 1.5 M or by increasing temperature could not be reactivated. In contrast to the slow rate of reactivation initiated after dilution of the denaturant, the recovery of the CD and the intrinsic fluorescence signals of Pfk-1 were rather fast. The CD signal at 220 nm was regained within 30 s after dilution of the sample, indicating a fast formation of the secondary structure of the subunits. On the other hand, the recovery of the protein fluorescence signal showed a lag phase of approximately 80 s, comprising that time required for the formation of secondary structure mentioned above. The subsequent increase of the signal indicated the formation of tertiary structure (polarity or environment of the aromatics as in the native state) and finished after 10 min without any indication of an intermediate folding state as found in the course of equilibrium denaturation. However, at that time point only about 10% of the maximum attainable Pfk-1 activity could be measured at 25 °C (Figure 7B). These findings point to the assumption that the assembly of the subunits is rate-limiting in the reactivation process. Indeed, besides octamers, transient intermediates having lower molecular masses also could be detected in the course of renaturation (Figure 9).

The overall assembly of Pfk-1 is thought to proceed via several steps: (i) formation of homodimers or heterodimers, (ii) formation of heterotetramers, and (iii) dimerization of heterotetramers to the octamer. Each of the association steps is second-order in kinetics. As found earlier, the formation of homotetramers can be excluded (*30*).

We have carefully analyzed the recorded reactivation kinetics (Figure 7) by determining the reaction order. Taking into account all experimental points (including the lag phase), the curve fits well to a second-order process of reactivation as shown in Figure 11. By assuming a second-order reaction with a lag phase, the sum of residuals decreased less than 2%. A first-order plot did not fit well; in particular, the first data points were outside of the model curve.

It should be mentioned that the recombination of isolated subunits of Pfk-1 in vitro by mixing cells of single-deletion mutants prior to disruption proceeds rather fast (*21*). This result indicates that a recombination of folded Pfk-1 subunits in cell extracts of single-deletion mutants may be governed by specific cellular chaperones of yeast as detected, for example, for the folding and assembly of ornithine transcarbamoylase (*31*). Alternatively, the subunits obtained immediately after cell disruption might be more competent for the formation of oligomers than those generated from the complete unfolded state.

It is well-known that refolding of denatured proteins strongly competes with a nonproductive aggregation (*4*). As shown in this paper, the yield of reactivation of Pfk-1 depended not only on the initial protein concentration and temperature but also on the addition of ATP and α-cyclodextrin, acting in a cooperative manner. ATP alone was not able to depress aggregation but increased the reactivation moderately. α-Cyclodextrin, when added separately, abolished aggregation but did not enhance the yield of active enzyme. A combination of both additives led to maximum reactivation. To explain both effects, we suggest that α-cyclodextrin may trap the folded monomers or some kinds of intermediates and, in this way, prevent the aggregation, but it does not influence the oligomerization. ATP, on the other hand, assists the formation of a heterodimer ($\alpha\beta$), which is an important intermediate in the assembly pathway of the enzyme. In contrast, Fru 6-P was found to have no effect on the reactivation kinetics. The opposite effects of both substrates of Pfk-1 on denaturation (Figure 1) and renaturation of the enzyme (Figure 7) is surprising. ATP probably governed exclusively the formation of $\alpha\beta$ dimers (or some kinds of intermediates) in the absence of the denaturant, while Fru 6-P shifted the association of the enzyme to the octamer under denaturating conditions only.

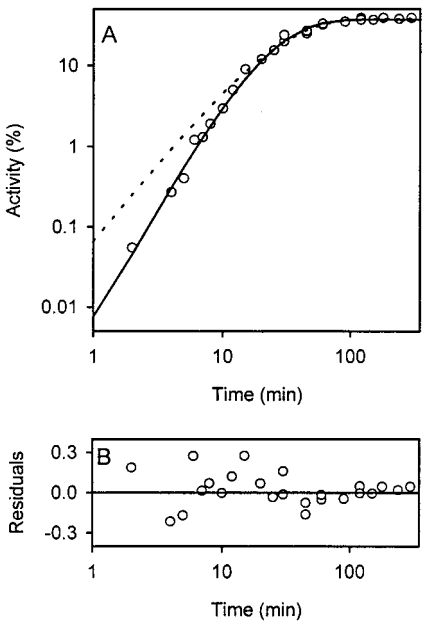
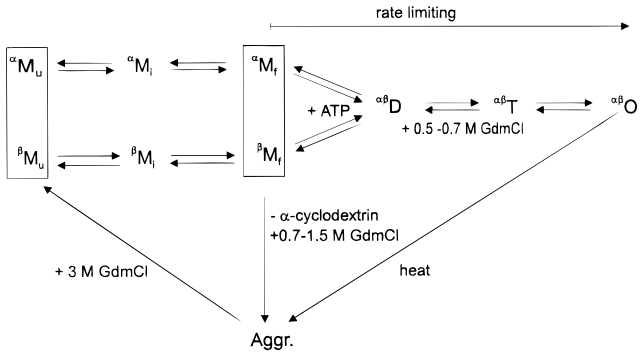


FIGURE 11: Demonstration of the fit of the reactivation kinetics. (A) Kinetics of reactivation of Pfk-1 at 25 °C in the presence of 40 mM α-cyclodextrin and 3 mM ATP were fitted to an unimolecular reaction [$v = V_{max}(1 - e^{-t/\tau})$ (•••)] and to a bimolecular reaction [$v = V_{max}(1 - e^{-t/\tau})^2$ (—)], where v and V_{max} represent the actual velocity and the maximum velocity of the reaction, respectively, t is the time, and τ is the half-time. The calculated values for the bimolecular reaction are $V_{max} = 38.1 \pm 0.6\%$ and $\tau = 23.6 \pm 0.8$ min. (○) Experimental data. (B) Residual plot for the bimolecular fit. (○) Difference between the calculated and the experimental data.

The "chaperonelike" function of different cyclodextrins is well documented. The rate of reactivation of creatine kinase (*32*), carbonic anhydrase (*33*), phosphoglycerate kinase (*34*), and citrate synthetase (*35*) is increased by prevention of the enzymes from aggregation in the course of refolding. Aggregation of proteins during refolding may result from the exposure of hydrophobic surfaces. Cyclodextrin may be able to mask such exposed hydrophobic residues and might act as a small-chaperone mimic in the folding process (*33, 34*). In extension of the mechanism of this artificial chaperone, α-cyclodextrin was thought to strip off the detergent from soluble protein−detergent complexes in such a way that the protein can fold correctly (*32, 35*−

Scheme 1



37). However, the described effect of α-cyclodextrin on Pfk-1 may be based on a simple prevention of aggregation of the subunits. It should be mentioned that γ-cyclodextrin, applied under the same conditions, did not increase the yield of reactivation.

Our results on denaturation and renaturation of Pfk-1 from *Saccharomyces cerevisiae* are summarized in Scheme 1, where M_u symbolizes the unfolded monomers, M_i is an intermediate state (formation of secondary structure), and M_f represents the folded assembly-competent monomers. D, T, and O are dimers, tetramers, and the octamer, respectively.

The inactivation paralleled the dissociation of the enzyme. Monomers of the α- and β-species ($^{\alpha}M_f$ and $^{\beta}M_f$) are not populated, because of their spontaneous aggregation behavior, but they are redissolved at higher denaturant concentration.

In the course of reactivation the transition $M_u \rightarrow M_i$, where most of the secondary structure of the subunits is formed, is rather fast. The next step, the generation of folded monomers (M_f) with tertiarylike structures, is indicated by the increasing fluorescence signal. We suggest the next step, the formation of heterodimers $^{\alpha\beta}D$ is essential for the overall assembly, because the interaction prevents nonproductive aggregation of the monomers (*21*). The dimerization reaction might be one of the rate-limiting steps of the oligomerization ensemble and is governed mainly by ATP. The recovery of activity shows second-order kinetics and coincides with the kinetics of formation of dimers, tetramers, and octamers.

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