REVERSIBLE DENATURATION WITH UREA OF RABBIT LIVER FRUCTOSE-1,6-BISPHOSPHATASE*

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Denaturation of fructose-1,6-bisphosphatase (Fru-P$_2$-ase, EC 3.1.3.11) by urea and renaturation of denatured enzyme has been investigated. Denaturation lowers the specific activity of the enzyme but even at 8 M urea concentration in the presence of sucrose the activity of the enzyme is detectable. Centrifugation of the enzyme in a sucrose density gradient at 4 M urea reveals one peak of protein corresponding to a dimer. Denaturation increases intensity of intrinsic fluorescence of Fru-P$_2$-ase and causes a red shift of fluorescence peak of the thioisindole derivative of the enzyme. Renaturation of the denatured enzyme followed as the reappearance of enzymatic activity in the presence and absence of bovine serum albumin (BSA) is characterised by first order kinetics, k = 1.78 $\times$ 10$^{-3}$ s$^{-1}$. The presence of BSA does not affect the rate of renaturation but perceptibly increases the recovery of enzymatic activity. A 100% recovery of Fru-P$_2$-ase activity is observed at 0.5 $\mu$g/mL concentration of the enzyme and 2 mg/mL of BSA.

Reversible denaturation of a number of biologically active proteins was investigated [1]. Rabbit liver Fru-P$_2$-ase is a regulatory enzyme of gluconeogenesis (for review see [2, 3, 4]). The specific activity, pH optimum and allosteric properties of the enzyme are dependent on bivalent and monovalent ions and the presence of chelators. The effect of denaturing agents on Fru-P$_2$-ase was reported: Grazi et al. [5] found that the dimer of immobilised Fru-P$_2$-ase is active. Kolb [6] determined molecular weight

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of Fru-P$_2$-ase monomers at 8 M urea. The kinetics of renaturation of rabbit liver Fru-P$_2$-ase have not been investigated. Our recent study on the effect of urea on structural and allosteric properties of Fru-P$_2$-ase [7], prompted us to investigate the dissociation and reassociation of the enzyme. The conditions for reversible denaturation enabling kinetic studies were established. The results on the effect of urea on the activity, tetrameric structure and fluorescence properties of Fru-P$_2$-ase are also presented. The experiments were performed on the enzyme of specific activity equal to 8 i.u./mg of protein and pH optimum around 6.8 when determined in the presence of 2 mM EDTA and absence of potassium ions.

MATERIALS AND METHODS

Rabbit liver Fru-P$_2$-ase was obtained according to Pontremoli et al. [8], with a slight modification described earlier [7]. BSA was purchased from International Enzymes (Windsor, England). o-Phtalaldehyde and coupling enzymes were from Sigma (U.S.A.). Urea from Merck (Darmstadt, F.R.G.). All other reagents were of the highest purity commercially available. Fru-P$_2$-ase concentration was determined spectrophotometrically by using absorption coefficient: $A_{280}^\text{nm} = 6.3$ [8].

Enzyme assay. The rate of fructose-1,6-bisphosphate hydrolysis was measured spectrophotometrically or colorimetrically. Spectrophotometric determination was applied to measure the activity of the native and renatured enzyme, to follow the renaturation process and to determine $S_{0,5}$ for the inhibition of native and renatured enzyme with AMP. Activity of native and renatured enzyme was also measured colorimetrically. Besides that colorimetric determination was used to determine activity of Fru-P$_2$-ase in the presence of urea. Details of spectrophotometric determinations are given in the description of the denaturation and renaturation process. The procedure for colorimetric determination is described in the Legend to Fig. 1.

Sucrose density gradient centrifugation. Centrifugation of Fru-P$_2$-ase in 5 to 30% sucrose density gradient was performed in SW-50 rotor with the Beckman L-5-50 ultracentrifuge at 45,000 r.p.m. for 12 h at 25°C. Details in the Legend to Fig. 2.

Fluorescence measurements. Perkin-Elmer MPF-44 spectrofluorometer was used to determine the intrinsic fluorescence intensities of native and modified with o-phtalaldehyde Fru-P$_2$-ase. The procedure previously described [7] was applied for modification of the enzyme.

Denaturation and renaturation of Fru-P$_2$-ase. Fru-P$_2$-ase, BSA and coupling enzymes were dialysed against 20 mM 1,3-bis[tris(hydroxymethyl)methyl-
amino]propane (BTP), 2 mM EDTA, pH 6.8, and 20 μL of dialysed native Fru-P₂-ase was added to 500 μL solution containing 8.33 M urea, 20 mM BTP, 2 mM EDTA, 50 mM 2-mercaptoethanol, pH 6.8 and was incubated for 0.5 h at 25°C. Then 10 μL of the above solution of denatured Fru-P₂-ase was added to 1 mL of the assay mixture containing: 100 mM BTP, 5 mM MgCl₂, 2 mM EDTA, 2 mM fructose-1,6-bisphosphate, 2 mM NADP, 5 μg glucose 6-phosphate dehydrogenase, 5 μg of glucose 6-phosphate isomerase and 2 mg/mL BSA. Renaturation was followed by the reappearance of enzymatic activity as monitored by the changes in absorbance at 340 nm and 25°C. Measurements were performed with ACTA M VI Beckman instrument or Specord M 40 Carl Zeiss (Jena G.D.R.). Parallel experiments were performed without BSA. Denatured Fru-P₂-ase 10 μL was added to 1 mL of the same assay mixture but without BSA. The control experiments were done as follows: 20 μL of the native dialysed Fru-P₂-ase was added to 500 μL of dialysis buffer and after 0.5 h of incubation at 25°C the activity was measured in the identical assay mixture as described above with or without BSA. The final concentrations of Fru-P₂-ase in the cuvette were: 0.05, 0.1, 0.5 and 1 μg/mL. In each case the increase of absorbance was linear and the recovery of enzymatic activity was calculated from its slope. The rate constant of reactivation was calculated from the equation ln (100 − R) = kt, where R = % of reactivation at time t calculated vs the final recovery reached at each concentration of the enzyme.

To compare the properties of renatured enzyme with those of native one, renaturation of Fru-P₂-ase was performed in the following way: Fru-P₂-ase (50 μg/mL) was incubated in 8 M urea, 20 mM BTP, 2 mM EDTA, 50 mM 2-mercaptoethanol, pH 6.8, at 25°C for 0.5 h. Then the enzyme was dialysed exhaustively against 20 mM BTP, 2 mM EDTA, 50 mM 2-mercaptoethanol, pH 6.8. The specific activity, S₀.₅ for inhibition by AMP, and spectroscopic properties of the renatured enzyme were determined.

RESULTS

The dependence of Fru-P₂-ase activity on urea concentration in the presence and absence of sucrose were presented in Fig. 1. As it was shown earlier [7], up to 5 M of urea the enzyme was active, irrespective of the presence or absence of protecting agents. In the presence of sucrose and at 8 M urea about 1% of the initial activity was detectable. It may be concluded that even at high concentration of the denaturant the enzyme was not fully unfolded when sucrose was present. Results of sucrose density gradient centrifugation presented in Fig. 2 indicate that at 4 M urea the enzyme existed as a dimer, as one can judge on the basis of comparison of sucrose density gradient centrifugation of Fru-P₂-ase with BSA centrifuged
under the same conditions. Molecular weight of tetramer of Fru-P₂-ase was 144,000. Thus molecular weight of a dimer was similar to that of BSA. At 5 M urea two protein peaks of dissociated Fru-P₂-ase were seen, supposedly corresponding to an equilibrium mixture of dimer and monomer. Only one peak of protein was observed at 6 M urea. Intrinsic fluorescence intensity of native Fru-P₂-ase slightly increased with the increasing concentration of urea as can be seen in Fig. 3. A higher increase was observed at urea concentration higher than 5 M. Changes in intrinsic fluorescence intensity were parallel to red shift of the fluorescence peak of thioisouindole derivative of Fru-P₂-ase presented in Fig. 4. We have shown previously [7] that Fru-P₂-ase at pH 6.3 is highly resistant to prolonged exposure to urea. Therefore the effect of urea on Fru-P₂-ase activity, tetrameric structure, and fluorescence properties was investigated at pH 6.3. The results obtained at pH 6.8 were nearly the same, the same was the shape of the curve for Fru-P₂-ase activity in the presence of urea, the red shift of thioisouindole derivative of the enzyme and the dissociation of Fru-P₂-ase by urea in sucrose density gradient. Therefore we decided to perform kinetic study of renaturation of the denatured enzyme at pH 6.8, the value equal to optimum pH of Fru-P₂-ase measured in the presence of 2 mM EDTA and absence of potassium ions. The presence of 0.08 M urea in the assay mixture did
Fig. 2. Sucrose density gradient centrifugation of Fru-P$_2$-ase and BSA at different concentration of urea. The protein 75 µg was placed on the top of 5 - 30% sucrose density gradient (3.8) mL in 100 mM BTP, 2 mM EDTA, 50 mM 2-mercaptoethanol, pH 6.3. Fractions of 5 drops were collected through a hole punched in the bottom of the tube. Protein was determined by measurements of intrinsic fluorescence of collected fractions. Excitation wavelength 280 and 290 nm, emission wavelength 305 and 340 for Fru-P$_2$-ase and BSA respectively. A, Fru-P$_2$-ase at 0 M urea; B, Fru-P$_2$-ase at 4 M urea; C, BSA at 4 M urea; D, Fru-P$_2$-ase at 5 M urea; E, Fru-P$_2$-ase at 6 M urea;
not affect the Fru-P$_2$-ase activity. Nevertheless for each concentration of Fru-P$_2$-ase with and without BSA, control determination of Fru-P$_2$-ase activity was performed. In agreement with previous reports [3], BSA activated Fru-P$_2$-ase but only at concentration of at least 0.5 µg/mL of the enzyme. The progress of renaturation measured by the reappearance of activity in

Table 1

<table>
<thead>
<tr>
<th>Fru-P$_2$-ase concentration µg/mL</th>
<th>First order rate constant s$^{-1}$</th>
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<tbody>
<tr>
<td></td>
<td>A*</td>
</tr>
<tr>
<td>0.05</td>
<td>$1.67 \times 10^{-3}$</td>
</tr>
<tr>
<td>0.1</td>
<td>$2.00 \times 10^{-3}$</td>
</tr>
<tr>
<td>0.5</td>
<td>$1.98 \times 10^{-3}$</td>
</tr>
<tr>
<td>1.0</td>
<td>$1.80 \times 10^{-3}$</td>
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A* determined in the presence of BSA
B* determined in the absence of BSA

the presence and absence of BSA is presented in Fig. 5 A and B, respectively. The recovery of enzymatic activity corresponded to first order kinetics. The reactivation constants were calculated for each concentration of Fru-P$_2$-ase and are presented in Table 1. Within the range of experi-
mental error they were the same for each concentration of the enzyme irrespective of the presence or absence of BSA. The renatured Fru-P₇-ase was indistinguishable from the native enzyme in terms of its specific activity and spectroscopic properties. Allosteric properties were also conserved and $S_{0.5}$ for AMP of the renatured enzyme was not increased.

**DISCUSSION**

Renaturation of oligomeric enzymes is a complicated process [1], kinetics of renaturation first of all depends on properties of the protein and the denaturant as well as on the presence of a ligand with affinity to the renatured protein. The recovery of initial activity depends on the stability of intermediates or on the ratio of the velocity of formation of native
structures to the velocity of aggregation, as in the case of LDH renaturation [9]. Fru-P$_2$-ase is highly resistant to denaturation by urea. We have shown previously [7] that Fru-P$_2$-ase displays allostERIC properties up to 4 M urea. We found that at 4 M urea the enzyme exists as a dimer, it can be therefore concluded that dimer is allosterically inhibited by AMP. According to Grazi et al. [5] dimer of Fru-P$_2$-ase is active and is slightly inhibited by AMP whereas, monomers are inactive. We found that Fru-P$_2$-ase activity was detectable even at 8 M urea but only in the presence of sucrose. Supposedly the active center of Fru-P$_2$-ase at 8 M urea is partially conserved when sucrose is present.

Kolb reported [6] that 8 M urea dissociates Fru-P$_2$-ase to monomers. Centrifugation in sucrose density gradient of Fru-P$_2$-ase revealed one peak of protein at 6 M urea (Fig 2) and at 7 and 8 M urea (not shown). Since the Fru-P$_2$-ase activity is detectable over this range of urea concentration
it seems reasonable to postulate that monomers of rabbit liver Fru-P$_2$-ase are active.

Only slight changes of spectrofluorimetric properties of the enzyme are observed during dissociation into dimer. Dissociation of Fru-P$_2$-ase to monomer results in more profound spectrofluorimetric changes and complete loss of enzymatic activity when measured in the absence of protecting agents. Irrespective of the enzyme concentration, the renaturation of Fru-P$_2$-ase is characterized by the first order kinetics. This suggests that the observed process of renaturation involves a conformational change, leading to restoration of the active center; the velocity of this change is much lower than association of dimers to tetramers.

The determined rate constant of reactivation is similar to the analogous constant determined for a similar process of renaturation of glyceraldehyde-3-phosphate dehydrogenase [10]. The presence of BSA does not affect the velocity of reactivation but perceptibly increases the recovery of enzymatic activity. It can be concluded that BSA stabilizes the intermediates of Fru-P$_2$-ase renaturation. Interaction between BSA and Fru-P$_2$-ase resulting in activation of the enzyme was observed earlier. Benkovic & De Maine [3], suggested that this interaction may be related to stabilization of the more active conformational form of the enzyme.

Rabbit liver Fru-P$_2$-ase is resistant not only to urea: heat treatment is successfully applied during purification of the enzyme [7, 8], pointing to higher thermal stability of Fru-P$_2$-ase than of other cytoplasmic proteins. The presence of sucrose at two first steps of the purification procedure not only stabilizes of lysosomes against disruption but also directly stabilizes Fru-P$_2$-ase structure.

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