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Influence of Phosphoenolpyruvate and Magnesium Ions on the Quaternary Structure of Enzyme I of the Phosphotransferase System from Gram-Positive Bacteria†

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ABSTRACT: Solution X-ray scattering patterns of enzyme I of the phosphotransferase system from *Staphylococcus carnosus* indicate an increase in radius of gyration and molecular mass in the presence of Mg^{2+} or both Mg^{2+} and phosphoenolpyruvate, indicating a partial dimerization of enzyme I. Mg^{2+} ions are essential for both the dimerization and the activation, whereas the substrate phosphoenolpyruvate shifts the monomer−dimer equilibrium to the enzymatically active dimer by decreasing the dissociation rate of the phosphorylated dimer.

The phosphoenolpyruvate (PEP)[1]-dependent protein kinase enzyme I is part of the PEP-dependent phosphotransferase system (PTS) which catalyzes carbohydrate uptake in microorganisms. In the reaction sequence of PTS, enzyme I, which requires Mg^{2+} for its activity, catalyzes the phosphorylation of a phospho carrier protein (HPr) from where the phospho group is transferred to the sugar. It was shown by several authors that enzyme I is exclusively active as a dimer (Kukuruzinska et al., 1980, 1982, 1984; Hoving et al., 1982; Weigel et al., 1982; Waygood, 1986; Han et al., 1990). Because this dimerization is slow compared to the catalytic reaction, a lag in product formation can be expected (Kukuruzinska et al., 1980) if the reaction is started with a fraction of monomeric enzyme I. Due to these properties, enzyme I appears to be a target for regulation of the activity of the whole PTS. A central point of this regulation is the influence of substrates and effectors on the dimerization of enzyme I. Using gel filtration, Hoving (Hoving et al., 1982) found for the *Escherichia coli* enzyme that Mg^{2+} and especially the phosphorylation of enzyme I stabilizes the dimer. In contrast to these results, Kukuruzinska et al. (1984) observed a significant decrease in the dimer concentration for the enzyme from *Salmonella typhimurium* when both ligands, Mg^{2+} and PEP, were present. To elucidate the influence of Mg^{2+} and PEP on the quaternary structure of the enzyme from *Staphylococcus carnosus*, the structural changes of enzyme I were investigated by X-ray solution scattering at protein concentrations of 3.1 and 5.2 mg/mL, respectively. To extend the investigations to lower protein concentrations, the kinetics of the dimerization and dissociation of enzyme I were investigated by activity measurements.

MATERIALS AND METHODS

Purification of Enzyme I from S. carnosus. *E. coli* strain TG_1 cells harboring the plasmid pUC19-ptsI2.6X (Kohlbrecher et al., 1992) were grown in the presence of isopropyl thiogalactoside in a rich medium. Cells (10 g wet wt) were suspended in 20 mL of standard buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride), disrupted by sonication, and centrifuged at 25000*g*. The supernatant was applied to a Q-Sepharose column (2.5 × 7.5 cm) using a gradient of 500 mL of 0.2−0.6 M NaCl in standard buffer. Enzyme I activity was detected with the mutant complementation assay. The enzyme I pool was adjusted to 20% ammonium sulfate, applied to a butyl-TSK column (5 × 22 cm; Merck, Darmstadt), and eluted with a linear gradient of 1200 mL from 20% to 0% ammonium sulfate in standard buffer. Fractions containing activity were concentrated by ammonium sulfate precipitation and applied to a Sephacryl S200 column (5 × 90 cm) equilibrated with standard buffer. The fractions containing activity were loaded on a Whatman DE52 cellulose column (12 × 2.5 cm) and eluted with a gradient of 500 mL of standard buffer (gradient 0−0.5 M NaCl). The fractions containing enzyme I were pooled and desalted by gel filtration with a Sephadex G25 column using 50 mM ammonium hydrogen carbonate as elution buffer. The pure desalted protein was lyophilized. The final yield amounted to 200 mg of pure protein. HPr from *S. carnosus* was purified according to Kruse et al. (1993).

Kinetic Measurements. The activity of enzyme I was measured by following the initial rate of PEP-dependent phosphorylation of HPr. The product pyruvate was monitored spectrophotometrically using the lactate dehydrogenase (Serva)/NADH (Sigma) system (Waygood et al., 1979).

To determine the kinetics of dissociation of Mg^{2+}-activated enzyme I, dissociation was started by a 10-fold dilution of a solution of Mg^{2+}-activated enzyme I. Samples were transferred at successive times immediately from this solution to the standard activity assay.

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[1] Abbreviations: PTS, phosphotransferase system; PEP, phosphoenolpyruvate; HPr, phospho carrier protein.

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The kinetics of enzyme I association were initiated by addition of Mg^{2+} and Mg^{2+}/PEP, respectively. After different time intervals, samples were withdrawn from the respective incubation mixture and immediately completed to the standard activity assay. Since dissociation and association proceed slowly in the activity assay and the observed progress curves are thus not absolutely linear, the initial 10 s of the assay was used for calculating the rates of dissociation and association, respectively. Within this time interval the change in the degree of oligomerization is negligible.

For determination of the concentration dependence of the activity, enzyme I was preincubated at different concentrations in the presence of 10 mM Mg^{2+} for 30 min at 20 °C. In the assay solution identical enzyme I concentrations of 0.2 μg/mL were obtained by appropriate dilution of the incubation mixture.

X-ray Solution Scattering. Enzyme I was dissolved in a minimum volume of the elution buffer (0.1 M MOPS, pH 7.1, containing 1 mM dithioerythrol and additives as indicated in Table 1) and equilibrated using a PD 10 column (Pharmacia Biotech). The protein-containing fractions were adjusted to a final concentration of 3.1 and 5.2 mg of enzyme I/mL, respectively. After preincubation for 10 h at 20 °C, the solution-scattering measurements were performed at 20 °C on the X 33 camera (Koch & Bordas, 1983) of the EMBL in HASYLAB on the storage ring DORIS of the Deutsches Elektronen-Synchrotron (DESY) at Hamburg using the standard data acquisition and evaluation system (Boulin et al., 1986, 1988). The X-ray scattering patterns of enzyme I solutions and the corresponding buffers were collected in the range 0.01 $nm^{-1} < x < 0.3$ nm^{-1} ($s = 2 \sin \theta/\lambda$, where 2θ is the scattering angle and λ is the wavelength of 0.15 nm) in 10 frames of 1 min to monitor possible radiation damage. Data reduction, background subtraction, and correction for the detector response and sample transmission were done using the program SAPOKO (Svergun and Koch, unpublished information) following standard procedures (Koch, 1991). Radius of gyration and forward scattering were calculated using the program GNOM (Svergun et al., 1988). The molecular mass was obtained by comparison of the forward scattering of the solutions with that of a solution of known concentration of lysozyme (10 mg/mL).

RESULTS

To characterize the Mg^{2+}- and PEP-induced changes in the quaternary structure of enzyme I of the PTS, enzyme I was investigated by X-ray solution scattering as well as by kinetic measurements of the rate of association and dissociation.

The scattering patterns of enzyme I in the presence and absence of additives are significantly different (Figure 1). As shown in Table 1, the radius of gyration and the molecular mass of enzyme I increase in the presence of 5 mM Mg^{2+}. The highest molecular mass is obtained in the presence of both Mg^{2+} and PEP. The samples were measured at 3.1 and 5.2 mg/mL, and no significant differences were found in the scattering curves. The concentrations used are low by the standard of most solution-scattering experiments. To monitor possible radiation damage, the catalytic activities of the solutions were determined after the X-ray measurements. No loss of enzyme activity could be detected.

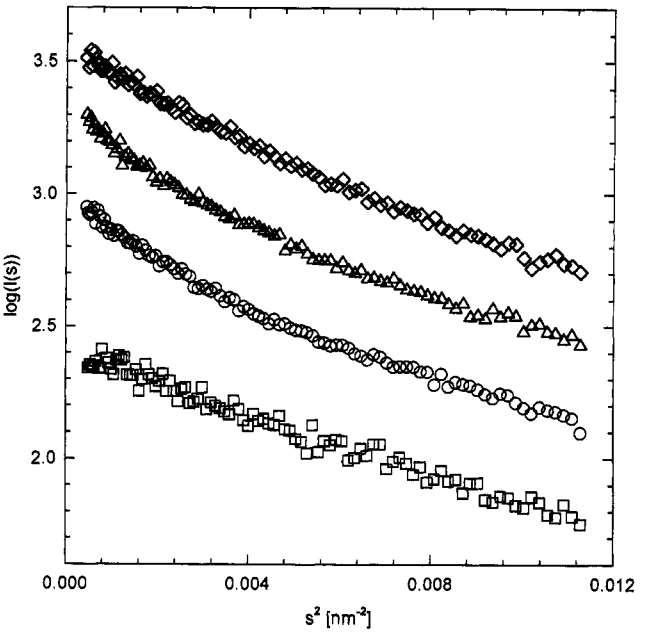

FIGURE 1: Guinier plot of the scattering pattern scaled for concentration of enzyme I in 0.1 M MOPS, pH 7.1, containing 1 mM dithioerythrol and 3.1 mg of enzyme I/mL (□); 1 mM dithioerythrol, 5 mM Mg^{2+}, and 3.1 mg of enzyme I/mL (◇); 1 mM dithioerythrol, 2 mM PEP, 5 mM Mg^{2+}, and 3.1 mg of enzyme I/mL (△); and 1 mM dithioerythrol, 2 mM PEP, 5 mM Mg^{2+}, and 5.2 mg of enzyme I/mL (○). Successive curves have been multiplied by 2 for better visualization.

Table 1: Apparent Relative Molecular Masses and Radii of Gyration of Enzyme I[a]

	radius of gyration (nm)	M_r (kDa)
enzyme I	3.6 ± 0.1	58.0 ± 3.3
enzyme I + 5 mM Mg^{2+}	4.1 ± 0.1	93.0 ± 2.0
enzyme I + 5 mM Mg^{2+} + 2 mM PEP	4.3 ± 0.1	100.0 ± 2.0

[a] Data were calculated from X-ray scattering patterns in the range of 0.025 $nm^{-1} \leq s \leq 0.205$ nm^{-1} assuming a maximum dimension of 13 nm. Enzyme I (3.1 mg/mL) was incubated at 20 °C in 0.1 M MOPS buffer, pH 7.1, containing 1 mM dithioerythrol and additives as indicated in the table.

Enzyme I is a rather elongated molecule with a maximum dimension in solution of about 12 nm, as calculated using the program GNOM (Svergun et al., 1988).

X-ray scattering indicates a Mg^{2+}- and PEP-induced change in quaternary structure of enzyme I. Since enzyme I is only active as a dimer, the influence of Mg^{2+} and PEP on the kinetics of dimerization can be followed by the change in activity (Kukuruzinska et al., 1980, 1982, 1984; Hoving et al., 1982; Weigel et al., 1982; Waygood, 1986; Han et al., 1990). A series of studies was performed where the loss of activity after dilution of Mg^{2+}-activated enzyme I (Figure 2) and the recovery of activity after addition of Mg^{2+} and Mg^{2+}/PEP (Figure 3) were investigated.

Figure 2 illustrates that the decrease in enzyme activity after a 10-fold dilution of a solution containing enzyme I and 16 mM Mg^{2+} is dependent on the additives in the dilution buffer. In the presence of PEP, the enzyme activity decreases more slowly than in its absence. Under conditions where the concentration of Mg^{2+} after the dilution step is very low, a fast decrease in enzyme activity is observed followed by a slow decrease.

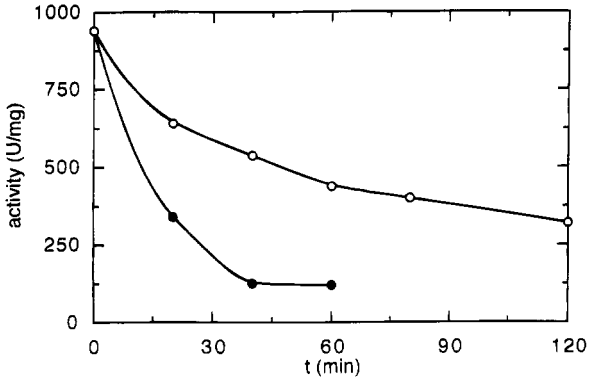

FIGURE 2: Dissociation of Mg^{2+}-activated enzyme I in the presence and absence of PEP. Enzyme I (8 μg/mL) was preincubated with 16 mM Mg^{2+} in 0.1 M MOPS buffer, pH 7.1, for 30 min. Dissociation was initiated by a 10-fold dilution with 0.1 M MOPS buffer, pH 7.1 (●), or 0.1 M MOPS buffer, pH 7.1, containing 2 mM PEP (○). After the respective times, samples were withdrawn and the initial rate was determined in the standard assay.

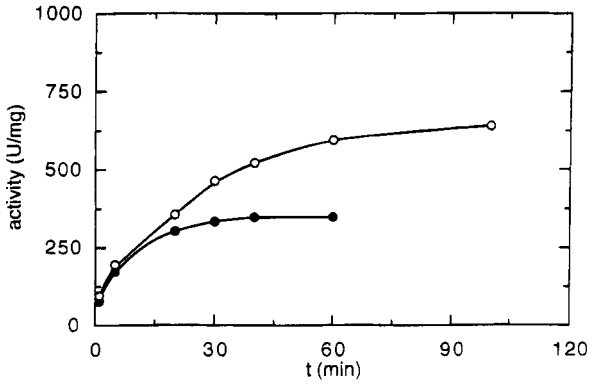

FIGURE 3: Time course of enzyme activity after incubation of enzyme I (2.5 μg/mL) with 5 mM Mg^{2+} (●) or 5 mM Mg^{2+} and 2 mM PEP (○) in 0.1 M MOPS buffer, pH 7.1.

In the case where a solution containing 10 μg of enzyme I/mL and 10 mM Mg^{2+} in 0.1 M MOPS buffer, pH 7.1, was diluted 50-fold in 0.1 M MOPS buffer containing no Mg^{2+} (0.2 mM residual Mg^{2+} is present from the preincubation mixture), the activity decreases within the mixing time to 30% relative to the experiment where 5 mM Mg^{2+} was present in the dilution buffer. On the other hand, addition of Mg^{2+} immediately after the dilution step leads to a recovery of the full activity. However if Mg^{2+} is added after completion of the slow phase, only a slow reactivation occurs (data not shown).

The time course of Mg^{2+}-induced activation of enzyme I in the absence and presence of PEP is depicted in Figure 3. At prolonged incubation with Mg^{2+}, the enzyme activity increases in the absence and presence of PEP. In the presence of PEP, the final enzyme activity is 2.5-fold increased compared to the experiment where PEP was absent. In both cases semilogarithmic plots provide straight lines with slopes of 0.1 min^{-1} in the absence and 0.04 min^{-1} in the presence of PEP.

In an additional series of experiments, the concentration dependence of enzyme I activity was determined. In this case different concentrations of enzyme I were preincubated with 10 mM Mg^{2+} in 0.1 M MOPS buffer, pH 7.1, for 30 min before aliquots of the incubation mixture, giving identical final concentrations of enzyme I in the test mixture (0.2 μg/mL), were used for determination of enzyme activity. Since decrease in activity by dissociation of dimers is slow compared to the activity assay (Figure 2), apparently no dissociation occurred during the assay. The activity measured in this way reflects the amount of dimers relative to the initial concentration of monomers in the preincubation mixture. Activity increases proportionally to the concentration of enzyme I in the preincubation mixture and reaches a value of 1430 U/mg of enzyme I/mL.

DISCUSSION

This work examines the influence of the substrates PEP and Mg^{2+} on the quaternary structure of enzyme I of the PTS from *S. carnosus* and their correlation with the enzyme activity. X-ray solution scattering indicates that enzyme I exists as a monomer at concentrations ca. 3 mg/mL in the absence of Mg^{2+} or PEP. The calculated molecular mass of ca. 58 kDa (Table 1) is in accordance with that calculated from the nucleotide sequence of the structural gene (Kohlbrecher et al., 1992). In the presence of Mg^{2+}, an increased molecular mass of 104 kDa is calculated from the scattering patterns of enzyme I. This indicates that the subunits of enzyme I associate largely into dimers. This dimerization seems additionally to be favored by the substrate PEP. This value of the relative molecular mass in Table 1 is obtained from the extrapolated forward scattering which solely depends on the concentration and the contrast and volume of the solute and not on the details of its structure. It thus provides a direct measure of the equilibrium between monomers and oligomers. The increase in radius of gyration from 3.6 to 4.3 nm upon addition of Mg^{2+} is compatible with a side by side association of monomers. The behavior of the Guinier plots near the origin further indicates that the effect is mainly due to specific rather than nonspecific aggregation. The increase in molecular mass is less than the doubling expected for complete dimerization, and one can estimate that only 60−80% of the monomers have associated into dimers. By following the kinetics of enzyme I association at different Mg^{2+} and PEP concentrations (data not shown), 5 mM Mg^{2+} or 2 mM PEP was determined to be saturating concentrations for enzyme I from *S. carnosus*. In the X-ray scattering experiments, the enzyme concentration was below 2 mM, and therefore enzyme I was completely saturated with the cofactors. The partial dissociation of enzyme I thus does not result from partial saturation of the enzyme by the cofactors. An association constant between 5 × 10^3 and 5 × 10^4 L/mol can be estimated from these experiments when Mg^{2+} and PEP are present.

To answer the question of whether the large association constant in the presence of PEP results from a higher association rate or from a lower dissociation rate of the dimers, the time courses of both dimerization and dissociation were investigated by monitoring the time course of enzyme activity. As shown in Figure 2, the dissociation after dilution of enzyme I is retarded by the substrate PEP. The integrated rate equation for the association to dimers where the concentration of the dimer at the start of the reaction is zero is described by

$$\ln \frac{D_{equ}(M_O^2 - DD_{equ})}{M_O^2(D_{equ} - D)} = \frac{M_O^2 - D_{equ}^2}{D_{equ}} k_D t \qquad (1)$$

with M_O as initial concentration of monomeric enzyme I, D as concentration of the dimer, D_{equ} as equilibrium concentration of the dimer, and k_D as intrinsic rate constant of dimerization.

As the association constant is in the range of 10^4 L/mol, the equilibrium concentration of the dimer in the kinetic experiments is much smaller than the concentration of the monomer, and eq 1 simplifies to

$$\ln \frac{D_{equ}}{D_{equ} - D} = \frac{M_O^2}{D_{equ}} k_D t \quad (2)$$

Under our experimental conditions, the activation of enzyme I by Mg^{2+} in the presence and absence of PEP is a pseudo-first-order reaction with an observed rate constant given by

$$k_{obsd} = \frac{M_o^2 k_D}{D_{equ}} \quad (3)$$

It follows from this equation that k_{obsd} is inversely proportional to the equilibrium concentration of the dimer, which is in agreement with the experimental data (Figure 3). This relationship between the observed rate constants and the equilibrium concentration of the dimer indicates that the intrinsic rate constant of dimerization is not influenced by PEP. Possibly, the dimerization reaction is a prerequisite for phosphorylation of enzyme I, and the dimer is stabilized by this modification. Both the results from X-ray solution scattering and the kinetics of activation and dissociation indicate a stabilization of the dimer of enzyme I by Mg^{2+} and PEP. This is in accordance with results published by Hoving et al. (1982) for the enzyme from *E. coli*.

Only a partial dimerization occurs in the investigated concentration range as indicated by the X-ray solution-scattering experiments. From this point of view, a very low dimer concentration can be expected in the presence of Mg^{2+} if the concentration of enzyme I is small compared to that in the X-ray experiments. As the dimers are the catalytically active species, the specific activity should increase proportionally to the concentration of enzyme I. In fact, in the concentration range from 5 to 160 nM for the monomer of enzyme I, the resulting specific activity is proportional to the concentration of enzyme I in the incubation mixture.

The role of Mg^{2+} in the mechanism of phosphorylation of HPr by enzyme I remains a central question. Considering that the dissociation of the dimer is slow (Figure 2), the decrease in activity after a drastic decrease of the Mg^{2+} concentration is too fast to be explained by dissociation of the dimer in the absence of Mg^{2+}. Because the activity reaches the initial 100% value immediately after Mg^{2+} addition to the Mg^{2+}-free test solution, this fast process reflects the binding of Mg^{2+} to the enzyme I and not a dimerization process of enzyme I. From this result we conclude that the presence of Mg^{2+} is not only a prerequisite for the formation of the catalytically active dimer but is also essential in the catalytic mechanism of enzyme I from *S. carnosus*.

REFERENCES

Boulin, C., Kempf, R., Koch, M. H. J., & McLaughlin, S. M. (1986) *Nucl. Instrum. Methods Phys. Res. A249*, 399−407.
Boulin, C., Kempf, R., Gabriel, A., & Koch, M. H. J. (1988) *Nucl. Instrum. Methods Phys. Res. A269*, 312−320.
Han, M. K., Knutson, J. R., Roseman, S., & Brand, L. (1990) *J. Biol. Chem. 265*, 1996−2003.
Hoving, H., Koning, J. H., & Robillard, G. T. (1982) *Biochemistry 21*, 3128−3136.
Koch, M. H. J. (1991) in *Handbook on Synchrotron Radiation* (Ebashi, S., Koch, M., & Rubenstein, E., Eds.) Vol. 4, pp 241−264, Elsevier Science Publishers, New York.
Koch, M. H. J., & Bordas, J. (1983) *Nucl. Instrum. Methods 208*, 461−469.
Kohlbrecher, D., Eisermann, R., & Hengstenberg, W. (1992) *J. Bacteriol. 174*, 2208−2214.
Kruse, R., Hengstenberg, W., Beneicke, W., & Kalbitzer, H. R. (1993) *Protein Eng. 6*, 417−423.
Kukuruzinska, M. A., Harrington, W. F., & Roseman, S. (1982) *J. Biol. Chem. 257*, 14470−14476.
Kukuruzinska, M. A., Turner, B. W., Ackers, G. K., & Roseman, S. (1984) *J. Biol. Chem. 259*, 11679−11681.
Misset, O., Brouwer, M., & Robillard, G. T. (1980) *Biochemistry 19*, 883−890.
Svergun, D. I., Semenyuk, A. V., & Feigin, L. A. (1988) *Acta Crystallogr. A44*, 244−250.
Waygood, E. B. (1986) *Biochemistry 25*, 4085−4090.
Waygood, E. B., Meadow, N. D., & Roseman, S. (1979) *Anal. Biochem. 95*, 293−304.
Weigel, N., Kukuruzinska, M. A., Nakazawa, A., Waygood, E. B., & Roseman, S. (1982) *J. Biol. Chem. 257*, 14477−14491.

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