Effect of polyethylene glycol on the activity, intrinsic fluorescence, and oligomeric structure of castor seed cytosolic fructose-1,6-bisphosphatase

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Abstract **The effect of polyethylene glycol (PEG) on the activity, intrinsic fluorescence, and oligomeric structure of homogeneous cytosolic fructose-1,6-bisphosphatase (FBPase$_c$) from endosperm of germinating castor oil seeds has been examined. Increasing the PEG concentration in the FBPase$_c$ reaction mixture elicited a progressive 3-fold decrease in the enzyme's K_m for fructose-1,6-P_2. The presence of PEG also: (i) increased the extent of FBPase$_c$ inhibition by high levels of fructose-1,6-P_2, (ii) enhanced the intensity of the enzyme's fluorescence emission spectra, and (iii) prevents dissociation of the active tetrameric native enzyme into inactive lower M_r forms during gel filtration HPLC. It is concluded that the activity and structure of plant FBPase$_c$ is modified by extreme dilution, probably as a result of partial deaggregation of the native tetrameric enzyme.**

Key words: Fructose-1,6-bisphosphatase; Polyethylene glycol; Enzyme concentration; Enzyme structure; Intrinsic fluorescence; *Ricinus communis*

1. Introduction

FBPase$_c$ (EC 3.1.3.11) is considered to be a key regulatory enzyme of the gluconeogenic pathway. In contrast to the homotetrameric FBPase from most non-plant sources, the purified COS FBPase$_c$ was recently shown to exist as a heterotetramer composed of equal amounts of 41- and 39-kDa polypeptides [1]. However, similar to its counterpart from animals and yeast [2], the COS FBPase$_c$ displays potent synergistic inhibition by F-2,6-P_2 and AMP [3]. Cytosolic enzymes such as FBPase, however, are present in vivo at far higher concentrations than are normally used during routine in vitro assays [4]. This difference is believed to be particularly important for regulatory oligomeric enzymes because their structure, and hence their kinetic properties, may be affected by both homologous and heterologous protein-protein interactions [4–11]. The interactions between proteins that probably exist at high protein concentrations prevailing in vivo can be specifically promoted in vitro by the addition of compatible solutes (i.e. PEG) to the reaction mixture [6]. The mechanism involves exclusion of the protein from the binary solvent, thus increasing the local enzyme concentration and favouring protein aggregation [4–7]. The in vitro activity of homogeneous COS cytosolic pyruvate kinase, for instance [9], as well as that of a variety of other non-plant and plant regulatory enzymes, is enhanced by the presence of PEG [5–7,10,11]. However, a PEG effect on any FBPase has not been documented [5,6]. In this report we demonstrate that the substrate saturation kinetics and intrinsic fluorescence of a plant FBPase$_c$ are significantly altered by the addition of PEG to the assay medium and argue that these effects are elicited via stabilization of the enzyme's native tetrameric structure.

2. Experimental

2.1. Enzyme assay and purification

Enzymic activity was routinely assayed at 30°C in a reaction mixture of 1 ml containing 50 mM Bis-Tris-propane-HCl (pH 7.5), 50 μM F-1,6-P_2, 5 mM $MgCl_2$, 0.2 mM EGTA, 0.15 mM NAD^+, 2 units of hexose-P isomerase, 1 unit of *Leuconostoc mesentroides* glucose-6-P dehydrogenase, and various amounts of PEG. F-1,6-P_2 was acid pretreated as in [1] to remove any contaminating traces of F-2,6-P_2, and its final concentration verified spectrophotometrically as in [12]. Assays were initiated by the addition of 0.1 μg of purified FBPase$_c$. FBPase$_c$ activity is defined as the amount of enzyme resulting in the production of 1 μmol fructose-6-P·min^{-1}. Activity in all assays was proportional to the amount of enzyme added and remained linear with respect to time for at least 4 min when the concentration of F-1,6-P_2 was >10 μM. Because of substrate depletion, however, reaction rates became nonlinear with respect to time when the concentration of F-1,6-P_2 was <10 μM. For K_m(F-1,6-P_2) determinations, reaction rates were therefore estimated by the slopes of tangents drawn on the progress curve, as described by Dixon and Webb [13]. These tangents represent the reaction rates at the corresponding concentrations of substrate remaining [13]. Substrate saturation data were fitted to the Michaelis-Menten equation using a nonlinear least-squares regression computer kinetics program kindly provided by Dr. Steve Brooks [14]. I_{50} values (concentration of inhibitor necessary to reduce enzyme activity by 50%) were obtained by fitting inhibition data to the Job equation using the aforementioned computer kinetics program [14].

FBPase$_c$ was purified to homogeneity from 5-day-old germinating COS as described by Moorhead et al. [1], except that affinity chromatography on Whatman P-11 P-cellulose preceded anion-exchange chromatography on a Waters Protein Pak Q 8-HR column. This modification allowed for the early removal of a contaminating acid phosphatase that interferes with the FBPase$_c$ assay [1], shortened the time required for purification, and resulted in a preparation having a specific activity of 72 units·mg protein^{-1}. The final preparation was concentrated to 4 mg·ml^{-1}, frozen in liquid N_2, and stored at −80°C in 20 μl aliquots. Prior to kinetic studies, aliquots of the final preparation were diluted into 5 mM Bis-Tris propane-HCl (pH 7.5) containing 1 mM DTT.

2.2. Fluorescence spectroscopy

Intrinsic fluorescence measurements were performed with a Perkin-Elmer LS-50 spectrofluorometer using a 0.15 ml quartz micro cuvette thermostatted at 30°C and an excitation wavelength of 280 nm. Emission spectra were corrected using buffer as a blank. Excitation and emission slit widths were 5 nm. Purified FBPase$_c$ was diluted to 0.2 mg·ml^{-1} in 50 mM Bis-Tris-propane-HCl (pH 7.5) containing 1 mM DTT in the presence and absence of 10% (w/v) PEG or 8 M urea. The intensity of fluorescence emission was linear with respect to

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Abbreviations: COS, castor oil seed endosperm; FBPase$_c$, cytosolic fructose-1,6-bisphosphatase; F-1,6-P_2, fructose-1,6-P_2; F-2,6-P_2, fructose-2,6-P_2; PEG, polyethylene glycol (average M_r 8000).

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$FBPase_c$ concentrations over 0 to 0.3 $mg \cdot ml^{-1}$. All solutions were filtered through 0.22 μm membranes prior to use. The FL Data Manager (version 3.0) from Perkin-Elmer was used to acquire and analyse the data.

2.3. Gel filtration HPLC

Gel filtration was performed at room temperature using a Pharmacia Superose 12 HR 10/30 column connected to a Waters 600E HPLC system and equilibrated with 50 mM HEPES-NaOH (pH 7.5) containing 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.04% (w/v) NaN_3 in the presence or absence of 5% (w/v) PEG. Aliquots of purified $FBPase_c$ were diluted to 0.75 $mg \cdot ml^{-1}$ with column buffer prior to HPLC. Flow rate was 0.25 $ml \cdot min^{-1}$, sample volume was 20 μl, and elution was monitored at 280 nm using a Waters 486 detector. The following standards were used in the calibration of the column: apoferritin (443 kDa), catalase (232 kDa), rabbit muscle pyruvate kinase (228 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa). Blue dextran was used to determine the column's void volume.

3. Results

3.1. Effect of PEG on $FBPase_c$ kinetic properties

Increasing the concentration of PEG in the $FBPase_c$ reaction mixture progressively elicite: (i) a substantial increase in susceptibility of the enzyme to inhibition by high concentrations of F-1,6-P_2 (Fig. 1A), and (ii) an approximate 3-fold decrease in the enzyme's K_m for F-1,6-P_2 (Fig. 1B). V_{max} was unaltered, except at 20% (w/v) PEG where it was reduced by about 35% (Fig. 1A), possibly as a result of the pronounced F-1,6-P_2 inhibition that occurred under these conditions. By contrast, inhibition of the $FBPase_c$ by F-2,6-P_2 was unaffected by the presence of PEG. The I_{50} for F-2,6-P_2 at 50 and 5 μM F-1,6-P_2 was 50 and 60 nM, respectively, in the presence of 0%, 10%, or 20% (w/v) PEG.

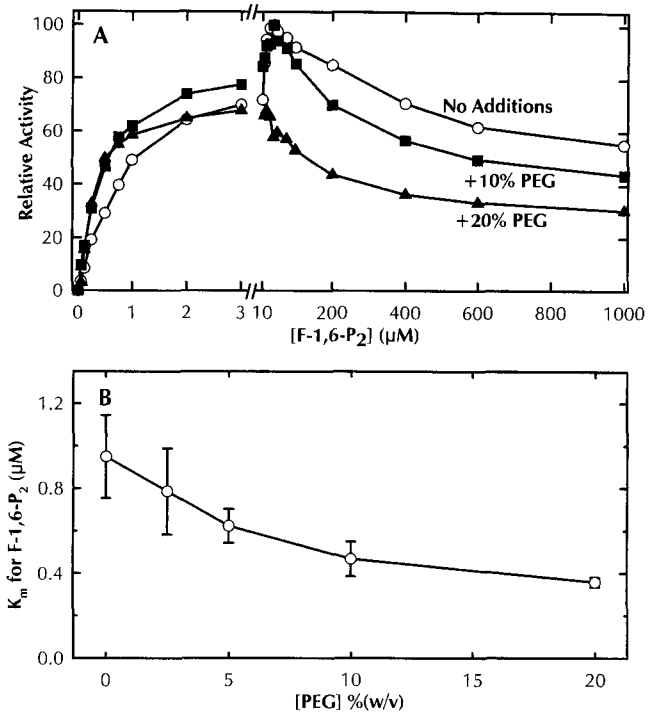

Fig. 1. (A) The effect of PEG on the F-1,6-P_2 saturation kinetics of germinating castor seed $FBPase_c$. (B) Effect of PEG on the enzyme's K_m for F-1,6-P_2. All values represent the means ± S.E. of 3 separate determinations. K_m values at 5, 10 and 20% (w/v) PEG are significantly lower than the corresponding value at 0% PEG ($P < 0.05$, 0.01 and 0.005, respectively), using Student's t-test.

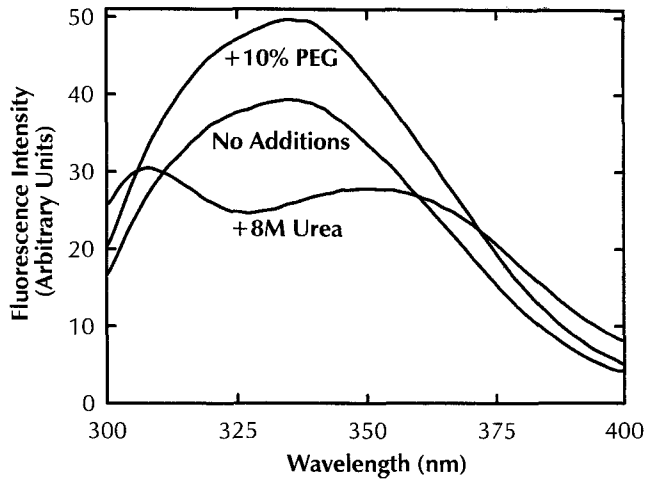

Fig. 2. The effect of 10% (w/v) PEG and 8 M urea on the fluorescence emission spectrum of castor seed $FBPase_c$. Emission spectra were recorded using an excitation wavelength of 280 nm and detecting emission between 300 and 400 nm as described in section 2.

3.2. Effect of PEG on $FBPase_c$ intrinsic fluorescence

Fluorescence emission spectra of COS $FBPase_c$ were recorded using an excitation wavelength of 280 nm (Fig. 2). Under these conditions most of a protein's intrinsic fluorescence can be attributed to tryptophan residues [15]. Consistent with our previous results [1], the COS $FBPase_c$ demonstrated an emission maximum at approximately 335 nm when excited at 280 nm (Fig. 2). The presence of 10% (w/v) PEG caused the intensity of the fluorescence emission spectra of COS FBPase to increase by approximately 30% without affecting the emission maximum (Fig. 2). By contrast, denaturation of the $FBPase_c$ with 8 M urea caused the intensity of the enzyme's fluorescence emission to be reduced by about 30%, and generates an emission spectrum exhibiting two maxima at about 310 and 355 nm (Fig. 2).

3.3. Effect of PEG on $FBPase_c$ oligomeric structure

Investigation of the quaternary structure of COS $FBPase_c$ by gel filtration HPLC confirmed a previous report [1] that a variety of oligomeric forms of this enzyme can occur. The injection of 15 μg of homogeneous $FBPase_c$, followed by elution with the standard aqueous buffer, resulted in most of the protein being equally distributed between an active tetramer of approximately 140 kDa and an inactive low M_r form (Fig. 3A, trace a, peaks I and II, respectively). A number of inactive smaller peaks were also observed. The elution of an inactive form at about 18.1 ml (i.e. peak II) which corresponds to a very low apparent M_r (i.e. <1 kDa) implies that an interaction between the chromatography media and this form of $FBPase_c$ occurred. Immunoblotting using rabbit anti-(COS $FBPase_c$) IgG [1] confirmed the presence of immunoreactive polypeptides corresponding to the COS $FBPase_c$ subunits in the various A_{280} absorbing peaks shown in Fig. 3A (trace a)(results not shown). Reinjecting 20 μl of the active peak I observed in Fig. 3A (trace a) generated the smaller, inactive, low M_r forms (Fig. 3A, trace b). The addition of 5% (w/v) PEG to the eluent caused the bulk of the $FBPase_c$ to elute as a single active form, relative to inactive smaller M_r forms (Fig. 3B, trace c, peak I). Moreover, when a 20 μl aliquot of the active peak observed in Fig. 3B

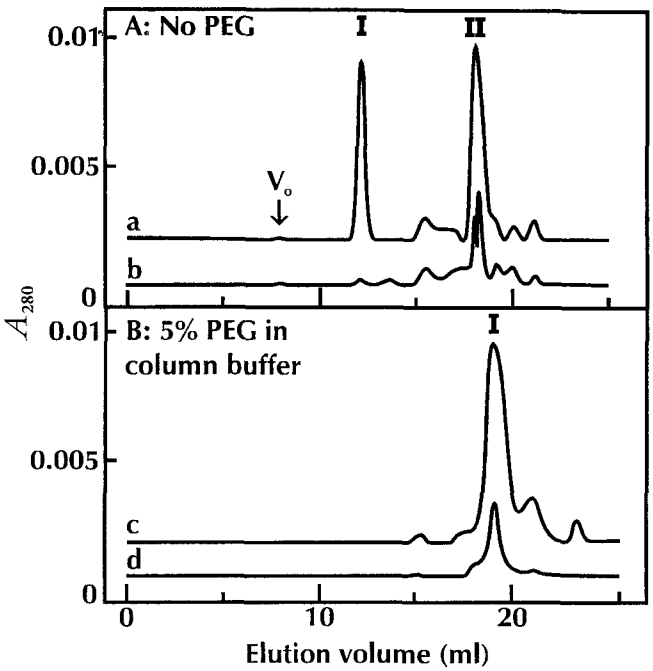

Fig. 3. The effect of PEG on the oligomeric structure of castor seed $FBPase_c$ as determined by Superose 12 HR 10/30 gel filtration chromatograpy. The enzyme (15 μg at 0.75 mg/ml) was chromatographed without (panel A, trace a) or with 5% (w/v) PEG (panel B, trace c) in the column buffer. An aliquot (20 μl) of the active peak I from traces (a) and (c) was reinjected onto the column to produce traces (b) and (d), respectively. V_0 designates the void volume.

(trace c, peak I) was rechromatographed in the PEG-containing buffer, essentially all of the protein eluted at the same position in an active form (Fig. 3B, trace d). Calibration of the column in the presence of 5% (w/v) PEG was negated by the anomalous elution volume of several of the protein standards relative to their respective M_rs. However, in the presence of 5% (w/v) PEG the elution volumes of the active FBPase form (i.e. Fig. 3B, peak I) and rabbit aldolase (158 kDa) were 18.9 and 18.6 ml, respectively. It is therefore assumed that the active form of $FBPase_c$ obtained in the presence of 5% (w/v) PEG (Fig. 3B, peak I) corresponds to the native tetramer.

4. Discussion

Inert polymers like PEG are solvent-excluding agents that increase the fractional volume occupied by proteins. The change in excluded volume is believed to promote protein self-association by increasing the activity coefficient of protein monomers in equilibrium with the corresponding oligomers [6,7]. Indeed, PEG has been found to promote the self-association and/or activation of a number of regulatory enzymes in dilute solutions [5–11]. However, the present study demonstrates for the first time that PEG can also markedly influence the kinetic and structural properties of FBPase. Our results also indicate that the native COS $FBPase_c$ exists as a 140 kDa tetramer that can be readily converted into inactive lower M_r forms by dilution.

The addition of PEG to the COS $FBPase_c$ assay mixture elicited a significant increase in the enzyme's affinity for F-1,6-P_2, as well as in its susceptibility to substrate inhibition (Fig. 1). Varying degrees of substrate inhibition have been reported for several non-plant FBPases [2,16]. However, in contrast to our results (Fig. 1A), Kruger and Beevers [3] failed to observe significant F-1,6-P_2 inhibition of an impure preparation of COS $FBPase_c$. One general mechanism of substrate inhibition is substrate 'crowding' wherein two substrate molecules simultaneously bind to the active site in such a way that neither is able to react [13]. Conversely, chemical modification studies have revealed that high levels of F-1,6-P_2 inhibit mammalian FBPase by binding to the F-2,6-P_2 allosteric inhibitor site [16]. However, F-2,6-P_2 binding sites of mammalian FBPases are controversial and include the active site in addition to a distinct allosteric site (as cited in [17]). In fact, the X-ray crystal structure of pig kidney FBPase complexed with F-2,6-P_2 suggests that this negative modulator binds at the active site, and a distinct allosteric site [17]. Interestingly, although PEG significantly enhanced inhibition of the COS $FBPase_c$ by high levels of F-1,6- P_2 (Fig. 1A), it had no effect on inhibition of the enzyme by F-2,6-P_2. These data suggest differing mechanisms for the inhibition of the COS $FBPase_c$ by F-1,6-P_2 vs. F-2,6-P_2.

The effects of PEG on the F-1,6-P_2 saturation kinetics of the COS $FBPase_c$ were correlated with a PEG-mediated: (i) increase in the intensity of the enzyme's intrinsic fluorescence emission spectrum (Fig. 2), and (ii) inhibition of the dilution-dependent dissociation of the active tetrameric native enzyme into inactive lower M_r forms (Fig. 3). The former result provides evidence of gross conformational changes in the enzyme such that fluorescent amino-acid side chains shift to a more hydrophobic environment in the presence of PEG [15]. This implies that PEG promotes tighter folding of the COS $FBPase_c$. The consistent emission maxima obtained in the presence and absence of PEG indicates that no drastic structural changes to $FBPase_c$ occurred upon the addition of PEG. By contrast, denaturation of the enzyme with 8 M urea caused the intensity of its fluorescence emission to substantially decrease, and elicited pronounced shifts in its emission maximum (Fig. 2).

The above findings indicate that the physical and kinetic properties of COS $FBPase_c$ are markedly influenced by its own concentration. Our results also show that only from the tetrameric form could $FBPase_c$ activity be recovered after gel filtration HPLC. This suggests that physical and kinetic data obtained with COS $FBPase_c$ in a dilute aqueous medium may not accurately reflect the in vivo situation. Indeed, based upon the assumptions of Podestá and Plaxton [9] and a maximal extractable activity for the COS $FBPase_c$ of 1 unit·g fresh wt^{-1} (Hodgson and Plaxton, unpublished data), the cytosolic soluble protein and $FBPase_c$ concentrations of 5-day-germinated COS are as high as 100 and 0.1 $mg \cdot ml^{-1}$, respectively. Thus, the $FBPase_c$ concentration present during routine in vitro activity assays (i.e. 0.05 to 0.1 $\mu g \cdot ml^{-1}$) may be up to 2,000- and 2,000,000-fold lower, respectively, than the $FBPase_c$ and total soluble protein concentrations prevailing in situ. This suggests that an increase in homologous protein–protein interactions, probably promoting subunit aggregation, is responsible for the PEG-mediated activation and inhibition of COS $FBPase_c$ at low and high concentrations of F-1,6-P_2, respectively. By contrast, most other enzymes that have been examined in the presence of PEG, or by various in situ approaches, do not display any significant alteration in their kinetic behaviour [5,6]. The exception appears to be certain regulatory oligomers such as

mammalian phosphofructokinase and pyruvate kinase [5,6], as well as plant cytosolic pyruvate kinase, ribulose 1,5-P_2 carboxylase activase, and PP_i-dependent phosphofructokinase [9–11]. This correlation places COS $FBPase_c$ amongst the regulatory enzymes and thus further emphasizes its preeminent role in the regulation of the gluconeogenic pathway in the plant cytosol [1,3,18].

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References

[1] Moorhead, G.B.G., Hodgson, R.J. and Plaxton, W.C. (1994) Arch. Biochem. Biophys. 312, 326–335.
[2] Tejwani, G.A. (1983) Adv. Enzymol. 54, 121–194.
[3] Kruger, N.J. and Beevers, H. (1984) Plant Physiol. 76, 49–54.
[4] Fulton, A.B. (1982) Cell 30, 345–347.
[5] Medina, R., Aragon, J.J. and Sols, A. (1985) FEBS Lett. 180, 77–80.
[6] Aragon, J.J. and Sols, A. (1991) FASEB J. 5, 2945–2950.
[7] Minton, A.P. (1981) Biopolymers 20, 2093–2120.
[8] Timasheff, S.N. (1992) Curr. Opin. Struc. Biol. 2, 35–39.
[9] Podestá, F.E. and Plaxton, W.C. (1993) Plant Physiol. 103, 285–288.
[10] Salvucci, M.E. (1992) Arch. Biochem. Biophys. 298, 688–696.
[11] Podestá, F.E., Moorhead, G.B.G. and Plaxton, W.C. (1994) Arch. Biochem. Biophys. 313, 50–57.
[12] Passoneau, J.V. and Lowry, O.H. (1993) Enzymatic Analysis; A Practical Guide. Humana Press, Totowa, NJ.
[13] Dixon, M. and Webb, E.C. (1979) Enzymes, Academic Press, NY.
[14] Brooks, S.P.J. (1992) Biotechniques 13, 906–911.
[15] Lackowiz, J.R. (1983) Principles of Fluorescence Spectroscopy, Plenum Press, NY.
[16] Reyes, A., Burgos, M.E., Hubert, E. and Slebe, J.C. (1987) J. Biol. Chem. 262, 8451–8454.
[17] Liang, J.-Y., Huang, S., Zhang, Y., Ke, H. and Lipscomb, W.N. (1992) Proc. Natl. Acad. Sci. USA 89, 2404–2408.
[18] Stitt, M. (1990) Annu. Rev. Plant Physiol. Mol. Biol. 41, 153–185.