pH and kinetic studies of chloroplast sedoheptulose-1,7-bisphosphatase from spinach (*Spinacia oleracea*)

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The aim of this paper is to study some steady-state kinetic properties of sedoheptulose-1,7-bisphosphatase, its pH-dependence and the effect of a substrate analogue, fructose 2,6-bisphosphate. Studies were carried out with sedoheptulose 1,7-bisphosphate and with fructose 1,6-bisphosphate, an alternative substrate. The pK values are identical for both substrates, and fructose 2,6-bisphosphate behaves like a competitive inhibitor. These results suggest that there exists a unique active site for either sedoheptulose 1,7-bisphosphate or fructose 1,6-bisphosphate on the enzyme molecule. Increasing Mg^{2+} concentrations shifted the optimum pH. As for fructose-1,6-bisphosphatase, we believe that this shift is due to the neutralization of negative charges near the active centre [Cadet, Meunier & Ferte (1987) Eur. J. Biochem. **162**, 393–398]. The free species of sedoheptulose 1,7-bisphosphate and fructose 1,6-bisphosphate are not the usual substrates of enzyme, nor is Mg^{2+} . But the kinetics relative to the $(Mg^{2+}$ –substrate $^{4-})^{2-}$ complex is not consistent with this complex being the substrate. An explanation of this discrepancy is proposed, involving both the negative charges near the active centre and the positive charges of Mg^{2+} . The observed V_{max} of the reduced enzyme is 65 % of the theoretical V_{max} for both substrates, but the observed V_{max} relative to sedoheptulose 1,7-bisphosphate is 3 times the one relative to fructose 1,6-bisphosphate. The specificity constant (k_{cat}/K_m), $1.62 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ with respect to sedoheptulose 1,7-bisphosphate compared with $5.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ with respect to fructose 1,6-bisphosphate, indicates that the enzyme specificity towards sedoheptulose 1,7-bisphosphate is high but not absolute.

INTRODUCTION

We have shown in the preceding paper [1] that some peculiar molecular properties of SBPase are different from their counterparts in FBPase: SBPase was activated by all the thioredoxins whereas FBPase was not activated by thioredoxin m; FBPase was not autoxidizable whereas SBPase was. But there are no striking differences at the molecular level, although the two are poorly related immunologically. In the present paper we study the steady-state kinetics of SBPase with respect to Mg^{2+} , substrates and $(Mg^{2+}$ –substrate $^{4-})^{2-}$ complexes in order to discriminate which one is the usual substrate. To obtain some information about the active site of the enzyme, the pH-dependence of the enzyme and also the effects of a FBP analogue (fructose 2,6-bisphosphate) were studied. The results obtained are compared with those reported for the spinach FBPase [2,3].

MATERIALS AND METHODS

Reduced SBPase (0.15 μM), used in the pH and kinetic experiments, was obtained on incubation of the oxidized enzyme with 12.5 mM-dithiothreitol for 4 h at 30 °C in 0.1 M-sodium phosphate buffer, pH 7.5, containing 2 mM-EDTA. Oxidized SBPase was the native form of the enzyme, obtained as described in the preceding paper [1].

For the steady-state kinetic study at pH 8.2, a sample of the reduced enzyme was pipetted into the cuvettes under different Mg^{2+} and substrate concentrations.

The calculations of the concentrations of the $(Mg^{2+}$ –SBP $^{4-})^{2-}$ and $(Mg^{2+}$ –FBP $^{4-})^{2-}$ complexes and of the free species were in accordance with McGilvery [4], Woodrow *et al.* [5] and Gardemann *et al.* [6].

To study the effect of Mg^{2+} on the reaction rate as a function of pH (from pH 7 to 10), at each pH Mg^{2+} concentrations were varied from 0.5 to 20 mM with SBP (0.6 mM) or from 2 to 100 mM with FBP (4 mM) as substrate. To calculate the pK values of the reduced enzyme–substrate complexes, the maximum velocities (from pH 7 to 10) were measured at saturating concentrations of both substrates (FBP and SBP) and Mg^{2+} .

All the initial-rate measurements were made at 30 °C in the reaction mixtures described by Zimmermann *et al.* [7] and Woodrow & Walker [8] for FBP and SBP respectively. A Perkin–Elmer spectrophotometer ($\lambda 5$) was used.

In the pH experiments Tris/HCl buffer was used from pH 7 to 8.6 and glycine/NaOH buffer at above pH 8.6. Results were identical around this pH with both buffers. Treatment of the pH data were effected in accordance with Segel [9] as in ref. [3].

Abbreviations used: SBP, sedoheptulose 1,7-bisphosphate; SBPase, sedoheptulose-1,7-bisphosphatase; FBP, fructose 1,6-bisphosphate; FBPase, fructose-1,6-bisphosphatase.

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RESULTS AND DISCUSSION

When the kinetic results were expressed with respect to the concentration of the substrate SBP (Fig. 1) we observed a Michaelian behaviour. But when they were expressed with respect to the concentration of total Mg^{2+} (Fig. 2) or of free Mg^{2+} (results not shown) there was a deviation from Michaelian behaviour (positive kinetic co-operativity). It has been postulated but not demonstrated by Woodrow *et al.* [5] that $(\text{Mg}^{2+}\text{-SBP}^{4-})^{2-}$ is the usual substrate of SBPase. More recently Gardemann *et al.* [6], using a crude stroma extract, proposed that the $(\text{Mg}^{2+}\text{-FBP}^{4-})^{2-}$ complex is the usual substrate of FBPase. They obtained a single saturation curve plotted versus the concentration of $(\text{Mg}^{2+}\text{-FBP}^{4-})^{2-}$ whatever the Mg^{2+} and FBP concentrations used, with the use of a 'short-time assay'. These authors showed a reaction presenting a 'burst' in its progress curve and did not find a single saturation curve for steady-state rates plotted versus the concentration of $(\text{Mg}^{2+}\text{-FBP}^{4-})^{2-}$. They found a single curve only with initial rates. They postulated, but did not demonstrate, that the free species FBP^{3-} has an allosteric inhibitory effect upon the reaction by transforming an active enzyme species into a less active one [6]. With pure FBPase (oxidized or reduced by thioredoxin in the presence of dithiothreitol) and with pure reduced SBPase a burst has never been found in the progress curve [8,10,11]. Therefore the results given in Figs. 3, 4 and 5 cannot be explained by an allosteric inhibitory effect of SBP^{3-} and FBP^{3-} . Apparently these results were in contradiction with the complex $(\text{Mg}^{2+}\text{-SBP}^{4-})^{2-}$ or $(\text{Mg}^{2+}\text{-FBP}^{4-})^{2-}$ being the usual substrate,

since we did not obtain a single saturation curve. For FBPase, from results given in ref. [2], we have calculated $(\text{Mg}^{2+}\text{-FBP}^{4-})^{2-}$ complex concentrations and the same type of kinetics as for SBPase was found (results not shown). Since we did not get a single curve (Figs 3, 4 and 5) with $(\text{Mg}^{2+}\text{-SBP}^{4-})^{2-}$ and $(\text{Mg}^{2+}\text{-FBP}^{4-})^{2-}$ complexes, we thought that the free species should be the substrate and not the complex forms [it was also checked that $(\text{Mg}^{2+}\text{-SBP}^{3-})^{-}$ or $(\text{Mg}^{2+}\text{-FBP}^{3-})^{-}$ was not the substrate;

Fig. 2. Kinetic behaviour of reduced SBPase as a function of Mg^{2+} concentration: effect of SBP on Mg^{2+} saturation curves

The reaction rate was determined at pH 8.2 as described in the Materials and methods section, but at different total Mg^{2+} concentrations. Total substrate concentrations were: curve 1, 0.02 mM; curve 2, 0.025 mM; curve 3, 0.03 mM; curve 4, 0.05 mM; curve 5, 0.1 mM; curve 6, 0.2 mM. Hill coefficients were equal to 2.3 for all the curves. The enzyme concentration in the cuvette was 3 nM.

Fig. 1. Kinetic behaviour of reduced SBPase as a function of SBP concentration: effect of Mg^{2+} on substrate saturation curves

The reaction rate was determined at pH 8.2 as described in the Materials and methods section, but at different substrate concentrations. Total Mg^{2+} concentrations were: curve 1, 1.5 mM; curve 2, 2 mM; curve 3, 2.5 mM; curve 4, 3 mM; curve 5, 3.5 mM; curve 6, 5.5 and 10 mM. Hill coefficients are 1 for all the curves. They were determined at half-saturation of substrate saturation curves. The enzyme concentration ($[E]_0$) in the cuvette was 3 nM.

Fig. 3. Kinetic behaviour of reduced SBPase as a function of $(\text{Mg}^{2+}\text{-SBP}^{4-})^{2-}$ concentration: effect of Mg^{2+} on saturation curves of complex

Data were recalculated from Fig. 1. Total Mg^{2+} concentrations were: curve 1, 1.5 mM; curve 2, 2 mM; curve 3, 2.5 mM; curve 4, 3 mM; curve 5, 3.5 mM; curve 6, 5.5 and 10 mM. The Hill coefficients were equal to 1 for all the curves.

Fig. 4. Kinetic behaviour of reduced SBPase as a function of $(\text{Mg}^{2+}\text{-SBP}^{4-})^{2-}$ concentration: effect of SBP concentration on saturation curves of complex

Data were recalculated from Fig. 1. Total SBP concentrations were: curve 1, 20 μM ; curve 2, 100 μM ; curve 3, 200 μM ; curve 4, 800 μM .

results not shown]. Nonetheless, when the activity was plotted against the concentration of SBP^{3-} or FBP^{3-} and of SBP^{4-} or FBP^{4-} , an inverse relationship was obtained, excluding these species as substrate (e.g., plot against the concentration of SBP^{4-} is given in Fig. 6). To explain the kinetic behaviour, we propose the involvement of the negative charges near the active centre [3,12]. With this hypothesis, $(\text{Mg}^{2+}\text{-SBP}^{4-})^{2-}$ or $(\text{Mg}^{2+}\text{-FBP}^{4-})^{2-}$ could be the substrate. The neutralization of the negative charges by free Mg^{2+} would allow a better access of the substrate $(\text{Mg}^{2+}\text{-SBP}^{4-})^{2-}$ or $(\text{Mg}^{2+}\text{-FBP}^{4-})^{2-}$ to the active centre: the higher the Mg^{2+} concentration, the higher the rate. This is what we observed (Figs. 3, 4 and 5). This explanation therefore holds for FBPase [3] and also for SBPase, since the results shown in Figs. 7 and 8 indicate that increasing Mg^{2+} concentration shifts the optimum pH. It is likely that negative charges are involved near the active centre of SBPase. The enzyme behaviour with respect to the concentration of total Mg^{2+} (Fig. 2) or free Mg^{2+} (results not shown) could be explained by a co-operative effect in the neutralization of the negative charges by Mg^{2+} .

The inhibition observed in Fig. 4 appears to be the consequence of two effects: for a same complex concentration, from curves 1 to 4 the concentration of free SBP increases and that of free Mg^{2+} decreases. Increasing the concentration of free SBP results in a saturation of the active centres of the enzyme by sugar, without hydrolysis. Free Mg^{2+} can neutralize negative charges near the active centre of SBPase; therefore, if its

Fig. 5. Kinetic behaviour of reduced SBPase as a function of $(\text{Mg}^{2+}\text{-FBP}^{4-})^{2-}$ concentration: effect of Mg^{2+} on saturation curves of complex

Data were recalculated from the plots of rate against [FBP] (or $[\text{Mg}^{2+}]$) (not shown). Total Mg^{2+} concentrations were: curve 1, 1.5 mM; curve 2, 2.5 mM; curve 3, 3.5 mM; curve 4, 4.5 mM; curve 5, 5.5 mM; curve 6, 7.5 mM; curve 7, 9.5 mM. The Hill coefficients were equal to 1 for all the curves.

Fig. 6. Kinetic behaviour of reduced SBPase as a function of the concentration of free SBP^{4-}

$(\text{Mg}^{2+}\text{-SBP}^{4-})^{2-}$ concentration was 100 μM . Data are recalculated from Fig. 1.

concentration is increased, the neutralization effect of this metal ion on the enzyme (negatively charged) is less important. Therefore the sigmoidal pattern obtained could be the result of the two observations described above and not the result of subunit interactions.

In order to perform a study of the effect of pH on enzyme activity, a pH-stability study is necessary. Usually the stability curve is obtained by preincubating the enzyme at the indicated pH for a period as long as the usual assay time. Enzyme activity is then monitored at the optimum pH. The pH-stability study proves that the decline of enzyme activity observed in the pH-activity

Fig. 7. pH-dependence of reduced SBPase with SBP

Curve 1, 0.5 mM- Mg^{2+} ; curve 2, 5 mM; curve 3, 9.5 mM; curve 4, 20 mM; curve 5, Mg^{2+} was saturating at each pH (20 mM from pH 7.05 to 7.85, 10 mM from pH 8 to 8.20, 5 mM from pH 8.25 to 9.90). The buffer was 0.1 M-Tris/HCl below pH 8.6 and 0.1 M-glycine/NaOH above pH 8.6. In all cases the substrate was saturating (0.6 mM). The enzyme concentration was 3 nM.

Fig. 8. pH-dependence of reduced SBPase with FBP

Curve 1, 2 mM- Mg^{2+} ; curve 2, 10 mM; curve 3, 40 mM; curve 4, 100 mM; curve 5, Mg^{2+} was saturating at each pH (100 mM from pH 6.9 to 7, 80 mM from pH 7 to 7.4, 60 mM from pH 7.4 to 7.6, 40 mM from pH 7.6 to 9.9). The buffer was 0.1 M-Tris/HCl below 8.6 and 0.1 M-glycine/NaOH above pH 8.6. In all cases the substrate was saturating (4 mM). The enzyme concentration was 3 nM.

Table 1. pK and pH optimum values of reduced SBPase with both substrates

From curves of apparent $V_{\max}$ (Figs. 7 and 8), the observed pK, the observed pH optimum and the optimum apparent rate values (apparent $V_{\max}$) were determined. pK (ES_1) and pK (ES_2) refer to the true pK of the enzyme-substrate complex and theoretical $V_{\max}$ to the rate of the reaction if all the enzyme was in the active form. The values of pK and the theoretical $V_{\max}$ were calculated in accordance with Segel [9].

Parameter	Reduced enzyme with	
	FBP	SBP
pK ₁ (observed)	7.40	7.40
pK ₂ (observed)	9.15	9.20
pH optimum	8.28	8.30
pK (ES_1)	7.70	7.67
pK (ES_2)	8.85	8.93
$V_{\max}$ (apparent) (s^{-1})	25	81
$V_{\max}$ (theoretical) (s^{-1})	38	119

Fig. 9. Hydrolysis of FBP in the presence of fructose 2,6-bisphosphate

Curve 1, no fructose 2,6-bisphosphate; curve 2, 2 mM-fructose 2,6-bisphosphate; curve 3, 6 mM-fructose 2,6-bisphosphate. The buffer was 0.1 M-Tris/HCl, pH 8.2. The enzyme concentration was 3 nM.

curves did not result from irreversible enzyme inactivation.

Results in Table 1 show that the differences between the observed pK values (defined as pH corresponding to apparent $V_{\max}/2$; Figs. 7 and 8) were below 2 in every case. Therefore the real pK values were calculated in accordance with Segel [9]. The very slight differences between the pK values for the reduced enzyme relative to FBP and SBP are a proof of the existence of one site on the enzyme molecule for both substrates. Further proof was given by the symmetrical optimum pH shift observed with FBP and SBP as the substrate. If there were two sites, there would be no reason for symmetrical behaviour. The finding that fructose 2,6-bisphosphate behaved as a competitive inhibitor (Fig. 9) with either

Table 2. Kinetic constants of the reactions catalysed by FBPase, SBPase and non-specific chloroplast phosphatase

For kinetic measurements of hydrolysis of SBP and FBP by reduced SBPase see Figs. 3 and 5. The kinetic constants followed by a number in brackets have been calculated from the results given in these references.

Parameter	Oxidized phosphatase			Reduced phosphatase		
	FBPase with		SBPase with	Non-specific phosphatase with		SBPase with
	$(Mg^{2+}-FBP^{4-})^{2-}$	$(Mg^{2+}-SBP^{4-})^{2-}$	$(Mg^{2+}-FBP^{4-})^{2-}$	$(Mg^{2+}-FBP^{4-})^{2-}$	$(Mg^{2+}-SBP^{4-})^{2-}$	$(Mg^{2+}-SBP^{4-})^{2-}$
K_m (mM)	0.13 [2]	3 [3]	0.18	0.006 [15]	0.42 [15]	0.05
k_{cat} (s^{-1})	400 [3]	220 [3]	0.84	30 [15]	30 [15]	81
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	3.07×10^6	7.3×10^4	4.6×10^3	5×10^8	7.1×10^4	1.62×10^6

substrate was also in favour of this hypothesis. We had previously obtained similar results for FBPase [2]. In the case of SBPase we also found the same striking result already reported for FBPase: the apparent V_{max} was far below (65 % and 68 %) the theoretical V_{max} for FBP and SBP respectively (about 60 % for FBPase relative to both substrates). Oxidized SBPase exhibits no significant activity from pH 7 to 10 (results not shown).

Fructose 2,6-bisphosphate has been described as a competitive inhibitor of FBPase [3,13]. Results shown in Fig. 9 indicate that, with FBP as substrate, fructose 2,6-bisphosphate also behaves as a competitive inhibitor of SBPase; similar results are also observed with SBP (results not shown here). One should notice the high concentrations of fructose 2,6-bisphosphate used and the Michaelian kinetics observed (as with spinach and *Euglena gracilis* FBPases [3,14]). SBPase was much less sensitive to fructose 2,6-bisphosphate than was FBPase; K_i was 2.30 mM and 28.50 mM (with FBP and SBP as substrate respectively), against 0.4 μ M and 25 μ M for reduced FBPase [3]. From the results shown in Table 2, SBPase was more specific towards SBP and FBPase more specific towards FBP. One should also notice the very low activity of oxidized SBPase at pH 8.2 compared with the reduced enzyme. Table 1 also shows that k_{cat}/K_m , which is the apparent second-order rate constant for the reaction of free enzyme with free substrate [16], may be as high as $1 \times 10^8 M^{-1} \cdot s^{-1}$ (for FBPase with FBP). In this case the rate-determining step for this parameter is close to the diffusion-controlled encounter of the enzyme and the substrate.

In conclusion, one can say that the two enzymes, although poorly related immunologically, have many common molecular properties: similar effects of pH and fructose 2,6-bisphosphate upon enzyme activities and similar kinetic behaviours with respect to free substrate species and $(Mg^{2+}$ -substrate $^{4-})^{2-}$ complexes. However, although oxidized FBPase is active in the range pH 7–10, oxidized SBPase is not.

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