Properties of oxidized and reduced spinach (*Spinacia oleracea*) chloroplast fructose-1,6-bisphosphatase activated by various agents

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Fructose-1,6-bisphosphatase (FBPase) can be reduced and activated by either dithiothreitol or reduced thioredoxin. This activation is pH-dependent. An amino acid group with a pK value of 5.55 is involved in this process. Both enzyme forms can also be stimulated by agents such as fructose 1,6-bisphosphate, Mg^{2+} , Ca^{2+} and Ca^{2+} /fructose 1,6-bisphosphate. FBPase reduced by dithiothreitol is more strongly activated than the enzyme reduced by thioredoxin. The specificity constant (k_{cat}/K_m) is enhanced over 2.5–25-fold and 1.5–2-fold (depending on the agent used) for FBPase reduced by dithiothreitol and thioredoxin respectively. In both cases, no new kinetic properties appeared. The pH-activity profile of the stimulated enzyme is slightly shifted towards the acidic side with respect to the reduced enzyme. A lag phase is observed in the progress curve of both enzymic forms, treated or untreated. Each agent used to stimulate must induce a new conformation of the enzyme, more active than the initial one, characterized by a specificity constant and a relaxation time. This lag phase tends to disappear when the assay temperature is increased. Temperature has the same effect on the activity of oxidized, reduced and stimulated FBPase, but different effects on the stability of the different forms.

INTRODUCTION

Fructose-1,6-bisphosphatase (FBPase), one of the two phosphatases of the Calvin cycle, exists in two states, one oxidized and one reduced [1,2]. At pH 8 and low Mg^{2+} concentration (5 mM), the oxidized form is without detectable activity. However, we have recently reported that preincubation of inactive oxidized FBPase with fructose 1,6-bisphosphate (Fru-1,6- P_2) and Ca^{2+} gave rise to the activation of the enzyme [3]. The reduced form is active over a wide range of pH, even at low Mg^{2+} concentration [2,4]. Reduction of the oxidized FBPase can be achieved by dithiothreitol (DTT) or thioredoxin [5–8]. Previously Gontero *et al.* [9], have shown that there is no synergy between Fru-1,6- P_2 and Mg^{2+} in the thioredoxin activation of FBPase, whereas, according to Hertig & Wolosiuk [10], the time-dependent activation of FBPase by DTT and thioredoxin is enhanced synergistically by the presence of Fru-1,6- P_2 and Ca^{2+} during the preincubation. It is not known whether the activity of the reduced FBPase can be enhanced. We have studied the activation by various agents of the reduced and already active FBPase. Some properties of this stimulated enzyme are described and compared with those of the oxidized and reduced enzymes. Since a lag phase occurred in the progress curve of the reduced enzyme, in this paper we also present the results of the study of this lag phase under different conditions. We have extended this study to the stimulated enzyme.

MATERIALS AND METHODS

Spinach leaves were purchased from a local market. All biochemicals were purchased from Boehringer-Mannheim. $CaCl_2$ was obtained from Prolabo (Paris, France) (Gen-Apex grade).

Chloroplast FBPase was purified according to the method of Zimmerman *et al.* [1], but slightly modified to include a second molecular sieving over a Sephacryl S-300 column. The native form of the enzyme obtained without reducing agent present was

oxidized. The purity of the enzyme was checked by SDS/PAGE (7.5%, w/v). Thioredoxins were purified as described by Buc *et al.* [11]. The activity of the enzyme was monitored spectrophotometrically as described by Zimmermann *et al.* [1].

Activation of the chemically reduced FBPase

The enzyme (580 nM) was reduced at constant pH (from 7.1 to 8.4) in a 100 mM-triethanolamine (TEA) buffer containing 50 μ M-EDTA and 23 mM-DTT, at 30 °C, until completely reduced. Reduced enzyme was then separated from the reductant over a Sephadex G-25 column, incubated in the presence of various agents, and assayed as described in the legends.

Thioredoxin-mediated reduction of FBPase

Oxidized FBPase (290 nM) was incubated in a 100 mM-sodium phosphate or 100 mM-TEA buffer at pH 7.5 containing 50 μ M-EDTA, at 30 °C, in the presence of 3 μ M-thioredoxin f_a and 12 mM-DTT. Under these conditions, the enzyme was fully activated within 45 min.

Heat-inactivation study

Small assay tubes containing the enzyme were incubated at various temperatures (40–55 °C). At intervals, one of the assay tubes was taken out and quickly cooled (15 min) on ice. The remaining activity was monitored at a constant temperature (test temperature) of 30 °C. The remaining enzyme activity reached a constant value immediately after cooling on ice and maintained this activity for at least several hours.

Data analysis

Progress curves were recorded using a DU 70 Beckman u.v.-visible spectrophotometer, and were analysed by fitting the data to eqn. (1) as described by Neet & Ainslie [12], using the DUD (Doesn't Use Derivative) method of the SAS-STAT software.

$$P = C_1 - [C_2 \exp(-\lambda t)] + vt \quad (1)$$

Abbreviations used: FBPase, fructose-1,6-bisphosphatase; Fru-1,6- P_2 , fructose 1,6-bisphosphate; DTT, dithiothreitol; Fru-2,6- P_2 , fructose 2,6-bisphosphate; TEA, triethanolamine.

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where P is the quantity of product at time t , C_1 is the amplitude factor, and λ and v are the time constant and the steady-state rate of the enzyme reaction respectively.

pH kinetic data of activity were fitted to eqn. (2) as described by Segel [13].

$$V_{app.} = \frac{V_{app.} (H^+) K_{es_1}}{K_{es_1} (H^+) + (H^+)^2 + K_{es_1} K_{es_2}} \quad (2)$$

K_{es_1} and K_{es_2} are the true dissociation constants of the enzyme-substrate complexes.

pH kinetic data of activation were fitted to eqn. (3):

$$v = \frac{V}{1 + (H^+)/K_{es_1}} \quad (3)$$

The kinetic (V and K_m) and activation parameters were calculated from the least-square-fitted linear Lineweaver-Burk and Arrhenius plots respectively.

The data of the heat-inactivation study were fitted to eqn. (4):

$$R(t) = R_1 e^{-kt} \quad (4)$$

where R_1 is the specific activity of the native form and k is the deactivation constant.

RESULTS

Lag phase in progress curves of the reduced FBPase

When inactive oxidized FBPase was reduced at pH 8 by DTT or by reduced thioredoxin, activity appeared slowly (Fig. 1a). In both cases the progress curves showed a lag phase. This lag phase exhibited one time constant. The time constants of the lag phase [see eqn. (1)] varied with respect to the incubation time with both DTT and thioredoxin concentrations (Fig. 1b), i.e. they were inversely related to the reduced enzyme concentration. Controls were also performed by varying the concentrations of the coupling enzymes of the assay over a wide range to check that the activity was always proportional to the FBPase concentration, and that the lag phase (i.e. the induction time and the time constant λ) were not influenced by the enzyme concentrations of the coupled assay. Since the enzyme, before assay, was incubated at 30 °C for several minutes, the lag phase was not due to a temperature jump.

When the enzyme was fully reduced by DTT and assayed at pH 8, the time constant of the lag phase was about $4.45 \times 10^{-2} \text{ s}^{-1}$. In the pH range 7.1–8.4, the time constants did not vary at fixed Mg^{2+} and Fru-1,6- P_2 concentrations.

Fig. 2. pH-dependence of FBPase activation by DTT

At each indicated pH value, a kinetic study of activation of oxidized FBPase by DTT was carried out, as described in the legend to Fig. 1(a). The reaction rate was measured by measuring the hydrolysis of Fru-1,6- P_2 at pH 7.5, 30 °C. Under these conditions, oxidized FBPase was inactive. The maximum rate determined at each pH value was plotted against pH.

pH-dependence of activation of FBPase

As shown in Fig. 2, the activation of FBPase by DTT depended on the pH of the incubation medium. The rate of reaction of the reduced enzyme increased with an increase in pH and reached a plateau at pH 7. By fitting the data to eqn. (3), a pK value of 5.55 ± 0.11 was found for the transition.

Stimulation of FBPase reduced by DTT or thioredoxin

Chemically reduced FBPase was incubated at pH 8 in the presence of various agents such as Mg^{2+} , Ca^{2+} and Fru-1,6- P_2 for 15 min, and then assayed. Initial rates were measured. The activity of the enzyme was increased over 1.25-, 1.40- and 1.90-fold for Fru-1,6- P_2 , Mg^{2+} and Ca^{2+} respectively. Saturation of the response was reached in the concentration range 5–10 mM, except for Ca^{2+} . Ca^{2+} is known to be a strong inhibitor of the reaction (K_i approx. 50 μM) [14]. When the enzyme was incubated with 8 mM- Ca^{2+} , the concentration of the free cation in the assay medium was 40 μM . Therefore it is impossible to study the effect of Ca^{2+} at higher concentrations, although at 8 mM the stimu-

Fig. 1. Relationship between the activation of oxidized FBPase and the lag in the progress curve

Oxidized FBPase (580 nm) was incubated at 30 °C for various time-periods with 23 mM-DTT in a 100 mM-TEA/HCl buffer, pH 8, containing 2 mM-EDTA. (a) The reaction rate was measured by measuring the hydrolysis of Fru-1,6- P_2 at pH 8 and 30 °C (5 mM- Mg^{2+} , 0.5 mM-Fru-1,6- P_2). The enzyme concentration $[E_0]$ in the assay was equal to 1.74 nM. (b) The time constant, λ , was determined by fitting the data of the progress curves to eqn. (1).

Table 1. Kinetic constants of the reaction catalysed by DTT-reduced FBPase activated by various agents

The kinetic parameters (V , K_m) were obtained by fitting the data to the Michaelis-Menten equation. The values of K_m are relative to the $(\text{Mg}^{2+}/\text{Fru-1,6-}P_2^{4-})^{2-}$ complex concentration.

Parameter	Reduced FBPase activated with:			
	None	10 mM-Mg ²⁺	5 mM-Ca ²⁺	1 mM-Ca ²⁺ plus 10 mM- Fru-1,6- P_2
K_m (μM)	58.7	32	9.54	3.75
k_{cat} (s^{-1})	285	366	397	451
k_{cat}/K_m ($\text{M}^{-1}\cdot\text{s}^{-1}$)	4.9×10^6	11×10^6	41×10^6	120×10^6

lation was not at a maximum. All the progress curves were also characterized by a lag phase. The data could be fitted to eqn. (1), i.e. the lag phase was characterized by one time constant. The relaxation times (the reciprocal of λ) were lower than that of the control (reduced FBPase: 19.02 ± 3.13 s), 10 ± 1.42 s for Mg^{2+} , 7.59 ± 0.25 s for Ca^{2+} and 7.07 ± 0.43 s for $\text{Ca}^{2+}/\text{Fru-1,6-}P_2$. As shown in Fig. 3(a), the effect of fructose 2,6-bisphosphate (Fru-2,6- P_2) plus Mg^{2+} on the activity of the reduced FBPase was weak. However, the lag phase was more pronounced (with respect to the control) than for the other agents (Fig. 3b). The relaxation time varied from 19 (control) to 125 s.

On the other hand, the rate of the activation of the reduced enzyme was slow. For example, the half-time of the activation by Mg^{2+} is about 2 min (results not shown). This activation is followed by a decrease in activity after 20 min of incubation in the presence of Mg^{2+} . This latter finding is consistent with the previous work of Gontero *et al.* [9].

The kinetics of the activated reduced enzyme were always Michaelian (results not shown), and the results in Table 1 indicate that the k_{cat}/K_m ratios of the activated forms are significantly greater than that of the non-activated enzyme (K_m was decreased and V increased).

The activity of FBPase reduced by thioredoxin was increased over 1.23-, 1.30-, 1.30- and 1.36-fold when incubated in the presence of Fru-1,6- P_2 , Mg^{2+} , Ca^{2+} and $\text{Ca}^{2+}/\text{Fru-1,6-}P_2$ respectively. The activity plateau was reached in the concentration range 10–15 mM. The kinetics of the activated enzyme were in all cases Michaelian (results not shown). The specific constant (k_{cat}/K_m) was enhanced over 1.51-, 2.00- and 1.91-fold for Mg^{2+} , Ca^{2+} and $\text{Ca}^{2+}/\text{Fru-1,6-}P_2$ respectively (Table 2).

From the results in Fig. 4, which gives the pH profile of the activity of FBPase reduced by thioredoxin and activated by

Fig. 3. Effects of $\text{Mg}^{2+}/\text{Fru-2,6-}P_2$ on the activity and the progress curve of the reduced FBPase

(a) FBPase fully reduced by DTT was incubated for 15 min at 30 °C in a 100 mM-TEA/HCl buffer, pH 8, containing 50 μM -EDTA, in the presence of a constant Mg^{2+} concentration (10 mM) and various Fru-2,6- P_2 concentrations. Curve 1 (○), rate of the reaction, measured by hydrolysis of Fru-1,6- P_2 at pH 8 (1 mM-Fru-1,6- P_2 , 10 mM- Mg^{2+}). Curve 2 (●), time constant of the progress curves, calculated by fitting the data to eqn. (1). The enzyme concentration $[E_0]$ in the cuvette was equal to 0.625 nM. (b) Curve 1, progress curve of the reduced enzyme. Curve 2, progress curve of the reduced enzyme incubated in the presence of 60 μM -Fru-2,6- P_2 and 10 mM- Mg^{2+} .

Mg^{2+} , we have calculated the optimum pH value and the apparent and real pK values of the enzyme. These are listed in Table 3.

Effect of temperature on the activity and lag phase of FBPase reduced by DTT and on the stimulated reduced FBPase

The optimum temperature of the reaction catalysed by reduced FBPase was about 45 °C (as for the oxidized enzyme). The Arrhenius relationship is linear over the temperature range

Table 2. Kinetic constants of the reaction catalysed by FBPase that had been reduced by thioredoxin and activated by various agents

The kinetic parameters have been calculated by fitting the results (not shown) to the Michaelis-Menten equation.

Parameter	Reduced FBPase activated with:				
	None	10 mM- Fru-1,6- P_2	10 mM- Mg^{2+}	1 mM- Ca^{2+}	1 mM- Ca^{2+} + 10 mM- Fru-1,6- P_2
K_m (μM)	6	11	4.7	53.9	4.8
k_{cat} (s^{-1})	433	535	518	564	664
k_{cat}/K_m ($\text{M}^{-1}\cdot\text{s}^{-1}$)	72×10^6	48×10^6	109×10^6	144×10^6	138×10^6

Fig. 4. Comparison of the pH-dependence of the activity of the reduced and stimulated FBPase

Oxidized FBPase ($0.29 \mu\text{M}$) was fully reduced by thioredoxin f_a ($3 \mu\text{M}$) in the presence of DTT (12 mM) in a 100 mM -sodium phosphate buffer, pH 7.5, containing $50 \mu\text{M}$ -EDTA. ■, pH maximum rate profile of the thioredoxin-reduced enzyme. □, pH maximum rate profile of the reduced enzyme incubated in the presence of 10 mM - Mg^{2+} for 10 min. Buffer in the assay medium was 100 mM -TEA/HCl below pH 8.9, and glycine/NaOH from 8.9 to 9.8. The concentration of Fru-1,6- P_2 was saturating and equal to 1 mM . Mg^{2+} concentration was saturating at each pH (50 mM from 6.0 to 7.2, 20 mM from 7.2 to 7.6, 10 mM from 8.0 to 8.2, 5 mM from 8.3 to 9.8). The concentration of the enzyme in the assay medium $[E_0]$ was equal to 0.58 nM . The data were fitted to eqn. (3). The curves are theoretical.

Table 3. pK and pH optimum values of thioredoxin-reduced FBPase and thioredoxin-reduced FBPase activated by 10 mM - Mg^{2+}

From curves of apparent V_{max} (Fig. 4), the observed pK , the observed pH optimum and the apparent optimum rates (apparent V_{max}) were determined. The values of pK_{es} and the theoretical V_{max} were calculated according to Segel [13] by fitting the data to eqn. (2).

Parameter	Enzyme reduced with:		
	Thioredoxin f_a	Thioredoxin f_a + 10 mM - Mg^{2+}	Thioredoxin f_b [15]
pK_1 observed	7.18	7.00	7.30
pK_2 observed	9.45	9.10	9.60
pH optimum	8.30	8.00	8.10
$pK_{\text{es}1}$	7.30 ± 0.17	7.10 ± 0.12	7.60
$pK_{\text{es}2}$	9.30 ± 0.17	9.00 ± 0.14	8.70
$V_{\text{max, app.}} (\text{s}^{-1})$	380	545	635
$V_{\text{max, theor.}} (\text{s}^{-1})$	548 ± 60	689 ± 60	1028

$15\text{--}45^\circ\text{C}$. The activation energy was calculated to be $55.8 \text{ kJ} \cdot \text{mol}^{-1}$ ($51 \text{ kJ} \cdot \text{mol}^{-1}$ for oxidized enzyme and $55 \text{ kJ} \cdot \text{mol}^{-1}$ for oxidized FBPase activated by Ca^{2+} /Fru-1,6- P_2). The thermodynamic activation parameters of the reaction over the temperature range ($15\text{--}45^\circ\text{C}$) were nearly identical and equal to $53 \text{ kJ} \cdot \text{mol}^{-1}$, $61 \text{ kJ} \cdot \text{mol}^{-1}$ and $25.8 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ for $\Delta H^\ddagger$, $\Delta G^\ddagger$ and $\Delta S^\ddagger$ respectively (at 25°C). The values of the time constant of the lag phase were determined at different temperatures. The Arrhenius activation energy (for the lag phase) was calculated to

Fig. 5. Thermostability of the reduced FBPase

Enzyme activity is shown as a function of the deactivation time. The deactivation temperatures were varied over $40\text{--}60^\circ\text{C}$: ○, 40°C ; ●, 45°C ; □, 50°C ; △, 55°C ; ■, 60°C . The curves are theoretical and obtained by fitting the data to eqn. (4).

be 23.4 and $33.0 \text{ kJ} \cdot \text{mol}^{-1}$ for the reduced enzyme and the enzyme stimulated by Mg^{2+} /Fru-2,6- P_2 respectively. In both cases there was a tendency for the lag phase to disappear when the temperature increased.

Heat-inactivation study

In Fig. 5, the effects of increasing temperatures on enzyme activity (reduced FBPase) with respect to incubation time were studied. The plots of $\ln R(t)$ versus incubation time were characterized by one straight line (not shown). The deactivation constant k was determined by fitting the data to eqn. 4. The activation energy, with respect to k , was calculated to be 831 and $873 \text{ kJ} \cdot \text{mol}^{-1}$ when activity was assayed at pH 8 and 8.8 respectively. The Arrhenius activation energy associated with denaturation of the oxidized form was calculated to be $302 \text{ kJ} \cdot \text{mol}^{-1}$. In both cases the heat inactivation was described by first-order kinetics.

Whereas Fru-1,6- P_2 was capable of stabilizing oxidized FBPase treated for 10 min at 50°C , Ca^{2+} and Mg^{2+} had no effect. Enzyme reduced by DTT and incubated in the presence of 10 mM - Mg^{2+} and 2 mM -Fru-2,6- P_2 was stable for at least 20 min when heated at 60°C .

DISCUSSION

The finding that the time constant of the lag phase in the progress curve increased with respect to the concentration of reduced enzyme (Fig. 1b) means that the reductive activation of FBPase does not obey a simple mechanism, i.e.

where E_{ox} stands for oxidized and E_r for reduced enzyme, but a more complex one involving the successive appearance of different enzyme forms. No conclusions can be made as to the nature of the conformational differences of these forms at this time but it has previously been shown that the overall process of reduction of this enzyme is linked to a conformation change [6].

It is possible that some effectors (Fru-1,6- P_2 and/or Mg^{2+}) are able to shift the reduced enzyme towards a more active form. The constant value of the time constant with respect to pH indicates that the transition between the two different forms is not due to a change in the protonation state of the enzyme. These forms might be two different conformational forms of the enzyme. The nature of the transition $E \rightleftharpoons E'$ is not clear, but its positive activation energy excludes the possibility that exposure of hydrophobic residues in the protein, and thus the ordering of water molecules, occurs during the transition.

The ionization state of an amino acid group (with a pK of 5.55) should define the capacity of FBPase to be activated by DTT. The situation appears different for oxidized FBPase activated by Ca^{2+} /Fru-1,6- P_2 . An abrupt transition appears in the pH range 6.8–7 and the enzyme could not be activated below pH 6.8.

Among agents capable of stimulating FBPase reduced by DTT, Ca^{2+} was the most efficient (about 100% increase). To a lesser extent, FBPase reduced by thioredoxin was prone to activation at pH 8 by the same agents. Other examples of activation of a phosphatase by different agents are the activation by Mg^{2+} of the 'alternate' phosphatase [9] and that of oxidized FBPase by Ca^{2+} and Fru-1,6- P_2 [3].

The kinetic properties of the stimulated enzyme were not very different from those of the non-stimulated enzyme {Michaelian kinetics with respect to the (Fru-1,6- P_2^{4-} / Mg^{2+}) $^{2-}$ complex, true substrate of the enzyme [15]}. No differences were found in the effects of temperature on the activities and the transition, $E \rightleftharpoons E'$, of the reduced and stimulated enzymes.

The pH-activity profile of the thioredoxin-reduced enzyme activated by Mg^{2+} was shifted slightly towards the acidic side (Fig. 4 and pK values of Table 3). For the DTT-reduced enzyme treated with various agents, the k_{cat}/K_m ratio was enhanced in a significant way (from 2.2 to 25 times). The increase in the k_{cat}/K_m ratio was less with the enzyme reduced by thioredoxin.

The reduced enzyme is known to be more sensitive to alkaline pH than the oxidized one [16]. It was also less thermostable ($t_{1/2}$ equal to 1 and 7 min at 50 °C for the reduced and the oxidized FBPase respectively). The enzyme stimulated by Mg^{2+} /Fru-2,6- P_2 was much more thermostable than the two other forms. In contrast with guinea-pig liver transglutaminase [17] and *Bacillus licheniformis* α -amylase [18], for which the kinetics of deactivation are biphasic, those of FBPase are monophasic and follow the simple model, $N \rightleftharpoons D$.

In order to explain the stimulation by various agents, the simplest model requires that the effectors shift the equilibrium

between two conformational forms to a form that appears to be more active than the initial one. The finding that the time constant and the specificity constants depend on the agent used to stimulate FBPase is in favour of a new ligand-induced conformation of the enzyme.

Upon a Mg^{2+} /Fru-1,6- P_2 concentration jump, which occurs in the assay, reduced and stimulated FBPase changed their activities relatively slowly (19 s for reduced FBPase, 125 s for reduced enzyme treated by Mg^{2+} /Fru-2,6- P_2). When preincubated in a suitable medium, e.g. Mg^{2+} , reduced FBPase still increased its activity relatively slowly ($t_{1/2} = 2$ min). In both cases, the enzyme most probably changed its conformation. It therefore behaves as an hysteretic enzyme [19].

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