Spinach (*Spinacia oleracea*) chloroplast sedoheptulose-1,7-bisphosphatase

Activation and deactivation, and immunological relationship to fructose-1,6-bisphosphatase

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In this paper we study activation by dithiothreitol and reduced thioredoxins and deactivation by oxidized thioredoxins of sedoheptulose-1,7-bisphosphatase. The behaviour of the enzyme when chromatographed on a thioredoxin–Sephadex column is also described. The enzyme is autoxidizable upon removal of reducing agents, and is activated when reduced by any of the thioredoxins. This mechanism may allow the regulation of the Calvin cycle upon light–dark and dark–light transitions. The formation of a stable complex between enzyme and thioredoxin could explain the inhibitory effect of high thioredoxin concentrations. The use of immunological techniques shows that sedoheptulose-1,7-bisphosphatase and fructose-1,6-bisphosphatase are poorly related immunologically.

INTRODUCTION

Recently we have described the isolation and the purification to homogeneity of spinach chloroplast SBPase [1]. It is a protein of M_r 66 000, made up of two apparently identical subunits. This enzyme is involved in the reductive pentose phosphate cycle, the so-called ‘Calvin cycle’, in C_3 plants. This cycle involves another phosphatase, FBPase, whose properties are relatively well known, whereas very little is known about spinach SBPase. Although the SBPase has been described and characterized in wheat [2–4], to the best of our knowledge only a few data are available concerning the enzyme from spinach [5]. The aim of the present paper therefore is to contribute to knowledge of the spinach enzyme. We report the activation of chloroplast SBPase by thioredoxins in an attempt to understand how thioredoxins could control the activity of this phosphatase. Secondly, we have studied the immunological relationship between this enzyme and FBPase.

MATERIALS AND METHODS

Materials

All chemicals were purchased from commercial sources and were of the highest quality available.

All the proteins were isolated from commercially grown spinach. SBPase was purified by the procedure of Cadet *et al.* [1]. The enzyme, free of FBPase, was homogeneous on SDS/5–25% polyacrylamide-gel electrophoresis. FBPase was purified by a slight modification of the method of Zimmermann *et al.* [6], including a second molecular-sieving over a Sephadex G-200 column. It was not contaminated by SBPase [7].

Thioredoxins were obtained by the method of Buc *et al.* [8].

The antibodies raised against FBPase were obtained as follows. A first injection into a rabbit of the non-activated FBPase (3 mg in 1.5 ml) was done intracutaneously (0.5 ml of protein emulsified in 1 vol. of Freund’s complete adjuvant); after 3 weeks a booster injection (0.5 ml of protein emulsified in 1 vol. of Freund’s incomplete adjuvant) was given, half intracutaneously, half intramuscularly; 10 days later, 250 μ l of enzyme solution was injected intramuscularly, and the day after the remaining 250 μ l was given intravenously. Then 10 days later the rabbit was bled at the ear vein. The serum was collected and adjusted to 40% saturation with $(NH_4)_2SO_4$, and the precipitate, after dissolution in 50 mM-Tris/sodium acetate containing 20 mM-acetic acid, final pH 5.0, was submitted to DEAE-Sephadex A-50 chromatography [9]. IgG fractions were stored at $-20^\circ C$, and before use were dialysed against suitable buffer.

Double immunodiffusion and immunotitrations

Ouchterlony double-immunodiffusion experiments [10] were carried out at room temperature with 9.5 cm $\times$ 4.5 cm glass plates, which were covered with 4 ml of 2% (w/v) agarose in 5 mM-sodium phosphate buffer, pH 7.5, containing 0.78% NaCl. Wells of 6 mm diameter with 6.5 mm distance between well perimeters were used. The precipitin bands were detected after 24 h incubation at room temperature after Coomassie Brilliant Blue staining.

Immunotitrations of both phosphatases by antibodies raised against oxidized FBPase were conducted as a function of antibody concentration and as a function of the incubation time with a known amount of antibody.

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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Kinetic experiments: SBPase activation and activity measurements

Oxidized SBPase used in all the experiments was the native form of the enzyme, without reducing agent [1]. Activation experiments were performed with a two-step assay: first, the oxidized SBPase was incubated with reductant; secondly, a sample of the incubated fractions was taken out and assayed.

Activity of the enzyme (either oxidized or reduced) was monitored with a Beckman DU8 spectrophotometer and a Perkin-Elmer $\lambda 5$ spectrophotometer in accordance with Woodrow & Walker [2] and Zimmermann *et al.* [6] for SBPase and FBPase respectively.

A unit of activity is defined as 1 μmol of P_i formed/s in the conditions of the assay methods of Zimmermann *et al.* [6] and Woodrow & Walker [2] for the activities of SBPase with FBP and SBP respectively.

Affinity chromatography

The preparation of thioredoxin f_B -Sephacrose was effected by adding the pure protein to a suspension of CNBr-activated Sepharose 4B in the presence of 0.1 M-sodium bicarbonate buffer, pH 8, as described in ref. [11].

Amino acid analysis

Proteins were hydrolysed in 6 M-HCl at 110 °C (for 18 h, 24 h and 48 h) in evacuated sealed tubes. All analyses were performed in a Waters model 510 h.p.l.c. system. The monomer M_r was calculated in accordance with ref. [12].

Fig. 1. Ouchterlony double immunodiffusion of SBPase and FBPase with anti-FBPase antibodies

The middle well was filled with the antiserum (As) raised against pure oxidized chloroplast FBPase. Perimeter wells were filled with 60 μg of pure oxidized SBPase (S) or 2 μg of pure oxidized FBPase (F). The precipitin bands were detected after 24 h incubation at room temperature after Coomassie Brilliant Blue staining.

RESULTS

Immunochemistry

Spinach FBPase and SBPase were compared by testing their immunological properties. Antibodies were raised against the native FBPase. Both enzymes were tested on an Ouchterlony double-diffusion agarose plate (Fig. 1). A single band of precipitin occurred with 2 μg of FBPase and 60 μg of SBPase (at 2 μg no band appeared) and the reaction observed was characteristic of a poor identity between both enzymes. We have checked that proteins occurring in the different reaction mixtures used (phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, pyruvate kinase, phosphofructokinase, lactate dehydrogenase, 60 μg of each) did not react with antibodies raised against FBPase.

Fig. 2(a) shows the results of an immunotitration experiment. Increasing concentrations of antibodies were added to both enzymes and their activities were tested. Although the activity of FBPase decreased with increasing antibody concentration, in the same conditions the activity of SBPase remained constant. When the activities of both enzymes were assayed as a function of time at a fixed IgG concentration, inactivation of FBPase was very fast, whereas the inactivation of SBPase was very slow (Fig. 2b). Antibodies raised against FBPase hardly recognized the other phosphatase. In both experiments we checked that the antibodies were specific to FBPase and did not react with other proteins such as pyruvate kinase, phosphofructokinase etc.

Activation of SBPase

On incubation at pH 7.5 with 20 mM- or 25 mM-dithiothreitol, the enzyme was slowly activated (Fig. 3a). This activation was fully complete after 3 h. It should be noted that higher dithiothreitol concentrations inhibited enzyme. When incubation was carried out in presence of thioredoxins and dithiothreitol (5 mM), the rate of enzyme activation and the maximum activity of the enzyme were significantly increased (Fig. 3b) compared with the control (5 mM-dithiothreitol). As shown in the inset to Fig. 3(b), thioredoxin f_A (58 μM) was able to activate the enzyme fully within 5 min. When the enzyme was incubated with thioredoxin f_B , the activity first increased up to the maximum level, then decreased (Fig. 3b). Results shown in Fig. 3(c) indicate that the greater the thioredoxin f_B /enzyme molar ratio in the incubation medium the greater the rate of activation became, but the rate of subsequent inactivation was also greater. Similar results were obtained with FBPase (results not shown). The inhibition observed cannot be due to the chelation of Mg^{2+} with the thiol groups of dithiothreitol or thioredoxin since the activation experiments were performed in the absence of Mg^{2+} , which is only present in the reaction mixture during the assay.

When the reducing agent was removed from enzyme fully reduced by molecular-sieving on a Sephadex G-25 column, activity quickly declined (Fig. 4a) and was restored when dithiothreitol was added again. If after the removal of dithiothreitol the enzyme was mixed with an excess of oxidized thioredoxin f_A or f_B , the phosphatase activity decreased slightly more quickly than in the absence of thioredoxin (Fig. 4a). Again the activity was restored by adding reducing agent. With low thioredoxin concentration (a thioredoxin f_B /enzyme molar ratio of 9) the enzyme was slightly activated. If dithiothreitol

Fig. 2. Immunotitration curves of FBPase and SBPase with anti-FBPase IgG

(a) Activity of both phosphatases as a function of IgG concentration. SBPase (curve 1, 202 nM) and FBPase (curve 2, 0.6 nM) in 0.5 ml of the medium of Woodrow & Walker [2] for SBPase (except SBP) and 0.5 ml of the medium of Zimmermann *et al.* [6] for FBPase (except FBP) were activated for 15 min at 30 °C in the presence of 10 mM-dithiothreitol. Samples were prepared with increasing concentration of anti-FBPase IgG and immediately assayed for SBPase (○) and FBPase (×) activities by adding either SBP or FBP. Curves 3 (□) and 4 (■): the same experimental conditions were used but anti-FBPase IgG was replaced by IgG from a non-immunized rabbit. Controls were run in the absence of antiserum. (b) Activity of both phosphatases as a function of the incubation time with IgG. FBPase (curves 1 and 3; 18 μg/ml) and SBPase (curves 2 and 4; 14 μg/ml) in 0.1 M-phosphate buffer, pH 7.5, were incubated at 4 °C with 144 μg (curve 1) and 202 μg (curve 2) of anti-FBPase IgG/ml and with IgG from a non-immunized rabbit (curves 3 and 4). After various times samples were assayed for FBPase activity (○ and ▼) in accordance with Zimmermann *et al.* [6] and SBPase activity (△ and ●) in accordance with Walker & Woodrow [2]. In curve 5 (□), pyruvate kinase (14 mg/ml) and phosphofructokinase (0.44 mg/ml) were incubated with 2.9 mg and 3.2 mg of anti-FBPase IgG/ml respectively and at various times samples were assayed for activity in accordance with Woodrow & Walker [2]. Controls were run in the absence of antiserum.

(2.5 mM) was also present during preincubation enzyme was activated to a greater extent (Fig. 4b).

In order to check the affinity between SBPase and thioredoxin f_B , an thioredoxin f_B -Sepharose column was used. SBPase bound to the gel, as shown in Fig. 5, and was eluted with buffer containing 50 mM-NaCl.

Amino acid composition of SBPase

A comparison of the amino acid composition of spinach SBPase, spinach FBPase and pig kidney FBPase was made (Table 1). The differences in the cysteine content are important in the context of thioredoxin activation: four cysteine residues per monomer for SBPase compared with six per monomer for both FBPases. But much more marked differences seem to exist, for example, in the serine, glycine, tyrosine and lysine contents. The two FBPases are certainly more similar one to the other, despite their very different sources, than is either to spinach SBPase.

DISCUSSION

Oxidized SBPase is inactive, and becomes active when reduced by dithiothreitol (Fig. 3a). Reduced thioredoxins are also able to activate it (Fig. 3b), and with more efficiency than the artificial reductant. As shown in the inset to Fig. 3(b), the rate of activation by thioredoxin

f_A is of the same order of magnitude as that of the activation by light [15]. SBPase was also activated by thioredoxin m, unlike FBPase [8] but similarly to NADP⁺-linked malate dehydrogenase [16].

SBPase was able to bind strongly to thioredoxin, as shown in Fig. 5. A stable complex was apparently formed between these two proteins. Results shown in Fig. 4 suggest that there exists the equilibrium:

where oPh and rPh represent the oxidized (inactive) and reduced (active) phosphatase respectively, and rTf_B and oTf_B the reduced and oxidized thioredoxin f_B respectively. A similar mechanism has been proposed for the activation-deactivation of FBPase [17] and the non-specific chloroplast phosphatase [18]. The equilibrium is assumed to be shifted towards the left, because activation of the enzyme by reduced thioredoxin f_B required either a large excess of thioredoxin or another strong reductant (dithiothreitol). This equilibrium leads to the prediction that if reduced SBPase and oxidized thioredoxin are mixed the enzyme must become inactive. This is what has been observed. Unlike FBPase a spontaneous deactivation of the phosphatase was observed. However, in presence of thioredoxins f_B and f_A , the rate of deactivation was slightly greater. This inactivation is reversible and activity can be restored by

Fig. 3. Activation kinetics of SBPase

(a) Activation kinetics of SBPase by dithiothreitol. SBPase (0.3 μM) was incubated at 30 °C in 0.1 M-phosphate buffer, pH 7.5, with the following concentrations of dithiothreitol: ▼, 20 mM; ■, 25 mM; ●, 50 mM; ○, 250 mM. The enzyme concentration in the cuvette was 3 nM. At the times indicated samples were taken and assayed for activity. (b) Activation kinetics of SBPase by thioredoxins. SBPase (0.3 μM) was incubated with 5 mM-dithiothreitol and 2.6 μM-thioredoxin: ■, thioredoxin f_B; ●, thioredoxin m; ▲, thioredoxin f_A. □, Control (5 mM-dithiothreitol); ▼, activation kinetics with 25 mM artificial reductant. The enzyme concentration in the cuvette was 3 nM. At the times indicated samples were taken and assayed for activity. Inset: activation by thioredoxin f_A at high concentration (58 μM). Dithiothreitol was also 5 mM. (c) Activation kinetics by thioredoxin f_B. Enzyme at several concentrations was mixed with 2.5 mM-dithiothreitol and 2.6 mM-thioredoxin f_B and assayed. Curve 1 (▲), 0.606 μM-enzyme; curve 2 (●), 0.303 μM-enzyme; curve 3 (■), 0.152 μM-enzyme. Enzyme concentration in the cuvette was 3 nM.

either dithiothreitol or reduced thioredoxin. The rapid decrease in the activity of SBPase after the removal of reductants may be responsible for the breakdown of the Calvin cycle when light is turned off.

The reduction of some non-specific disulphide bridges, perhaps followed by aggregation of the protein molecules as for spinach NADP⁺-linked malate dehydrogenase [19], might contribute to the inhibition of enzyme activity by high dithiothreitol concentrations (Fig. 3a). Since it

appears that reduced thioredoxin cannot reduce the non-specific disulphide bridges of the enzyme (as dithiothreitol does), we believe that the inhibition of enzyme activity by thioredoxin (shown in Fig. 3c) may involve the equilibrium:

where (oPh-rTf_B)_i represents the inactive complex and (rPh-oTf_B)_a the active complex between the reduced (r)

Fig. 4. Activation-deactivation of SBPase by thioredoxins

(a) Deactivation of reduced SBPase by an excess of oxidized thioredoxin. Curve 1. SBPase ($0.67 \mu\text{M}$) was reduced by dithiothreitol (25 mM) for 2 h 30 min in 0.1 M-phosphate buffer, pH 7.5, containing 2 mM-EDTA at 30°C . Dithiothreitol was removed from the enzyme by chromatography on a Sephadex G-25 column and enzyme activity was assayed. Curve 2. After the removal of reductant, oxidized thioredoxin f_A ($5.1 \mu\text{M}$) was added to the enzyme solution ($0.042 \mu\text{M}$) and activity was assayed. Curve 3. After the removal of reductant, oxidized thioredoxin f_B ($1.5 \mu\text{M}$) was added to the enzyme solution ($0.15 \mu\text{M}$) and activity was assayed. (b) Activation of SBPase by reduced thioredoxin f_B in the presence and in the absence of dithiothreitol. SBPase ($0.3 \mu\text{M}$) was incubated at 30°C (pH 7.5) with either $2.6 \mu\text{M}$ reduced thioredoxin f_B (curve 1) or 2.6 mM reduced thioredoxin f_B plus 2.5 mM -dithiothreitol (curve 2). Curve 3 was a control (2.5 mM -dithiothreitol, $0.3 \mu\text{M}$ -enzyme). SBPase activity was assayed at the times indicated.

Fig. 5. Thioredoxin-Sepharose affinity chromatography of SBPase

The column (3 cm $\times$ 1.5 cm) was equilibrated in 5 mM-phosphate buffer, pH 7.5, containing 0.1 mM-EDTA and rinsed with the same buffer. Elution was effected with a linear gradient (from 0 to 100 mM) of NaCl in the same buffer. The rate of elution was 6 ml/h, and 0.5 ml fractions were collected. —, Protein; ●—●, SBPase activity.

and oxidized (o) species. Soulié *et al.* [20] have proved that there exists on FBPase, besides active sites that bind the substrate, one thioredoxin site per tetramer distinct from the catalytic sites (with a dissociation constant of 800 nM). The existence of these two types of sites suggests that, *in vivo*, reaction is mediated by the

FBPase-thioredoxin complex rather than by the free enzyme [21]. By using the affinity-chromatography method, we found that a stable complex was formed between SBPase and thioredoxin (Fig. 5). Therefore a similar functional complex with this enzyme might be involved. Since reduced thioredoxin was liable to oxidation in the presence of O_2 (results not shown), the equilibrium should be shifted in the reverse direction to the left as a function of time, and the higher the thioredoxin concentration the faster the inactivation should become. Our results support this hypothesis.

From the amino acid composition a minimum M_r was calculated in accordance with Delaage [12], to be 35415. This value agrees with that obtained by molecular sieving, by polyacrylamide-gel electrophoresis and with the dimeric structure of SBPase [1].

Spinach SBPase and FBPase show different amino acid compositions, indicating that there is little or no homology between them. Indeed polyclonal antibodies directed against FBPase confirmed that these two proteins are not structurally related.

An Ouchterlony experiment indicates that SBPase reacted slightly with the antibodies raised against FBPase at a high concentration ($60 \mu\text{g}/\text{well}$) whereas FBPase reacted strongly at $2 \mu\text{g}/\text{well}$. At the same concentration ($60 \mu\text{g}/\text{well}$) control proteins did not react at all. Therefore there is only a poor identity between the two proteins. This conclusion is supported by the immunotitration experiments. Whereas SBPase bisphosphatase activity was not decreased for a short incubation time with different IgG concentrations the

Table 1. Amino acid content of SBPase (spinach) and FBPase (spinach and pig kidney)

The monomeric M_r for spinach enzymes were determined in accordance with ref. [12] and the one of mammalian enzyme from the sequence [14]. Abbreviation: N.D., not determined.

Amino acid	Amino acid composition (mol of residue/mol)		
	FBPase		SBPase
	Spinach [13]	Pig kidney [14]	Spinach
Lys	21	25	13
His	3	4	6
Arg	10	13	13
Asx	31	35	23
Thr	13	15	20
Ser	25	18	38
Glx	40	27	42
Pro	11	15	16
Gly	27	26	57
Ala	21	30	26
Cys	6	6	4
Val	23	29	23
Met	5	9	5
Ile	22	24	16
Leu	27	28	25
Tyr	16	15	6
Phe	10	10	13
Trp	2*	0	N.D.
Total	313	329	346
Monomeric M_r	35700	36534	35415

* Determined by fluorimetry.

phosphatase activity became slightly inhibited with respect to time at a fixed antibody concentration. In these conditions FBPase was immediately inactivated. Therefore the two phosphatases are different proteins exhibiting only a small degree of homology. But knowledge of their sequences will be required to provide further information about this question.

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