Biochimica et Biophysica Acta, 636 (1981) 113–118
Elsevier/North-Holland Biomedical Press

BBA 48070

THIOREDOXIN/FRUCTOSE-1,6-BISPHOSPHATASE AFFINITY IN THE ENZYME ACTIVATION BY THE FERREDOXIN-THIOREDOXIN SYSTEM

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(Received November 25th, 1980)

Key words: Fructose-1,6-bisphosphatase; Thioredoxin; Ferredoxin; Enzyme activation; Light activation; (Spinach)

In this work we analyze the affinity relationship between photosynthetic fructose-1,6-bisphosphatase and ferredoxin and thioredoxin from spinach leaves, two components of the proposed light-activation system of this enzyme, using affinity techniques on ferredoxin- and thioredoxin-Sepharose columns. Oxidized and reduced ferredoxin did not show enzyme affinity, whereas thioredoxin, both the oxidized and the dithiothreitol-reduced form, exhibited a strong bisphosphatase affinity at pH 7.5; this thioredoxin/enzyme affinity appears diminished at pH 8.2. When the affinity experiments were performed in the presence of 5 mM Mg^{2+}, only 30% and 12% of the bisphosphatase remained bound to the thioredoxin-Sepharose at pH 7.5 and 8.0, respectively; these percentages were reduced to 6% when the Mg^{2+} concentration increased to 10 mM. These results suggest that a rise of stromal pH and Mg^{2+} concentration can account for a loosening of the thioredoxin/bisphosphatase linkage, which could be of physiological significance in the dark-light transition. Studies on the nature of the chemical groups responsible for the affinity have shown that the thioredoxin/bisphosphatase linkage is concerned with the existence of hydrophobic clusters. We have found no difference in the behaviour of the chloroplastic thioredoxins f and m, and the cytoplasmic ones c_f and c_m. These results support the existence of an in vivo thioredoxin/fructose-1,6-bisphosphatase interaction, in accordance with the light-activation mechanism by the ferredoxin-thioredoxin system.

Introduction

The light-activation mechanism of photosynthetic fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) is emerging as a controversial subject. At first Buchanan et al. [1] proposed a direct ferredoxin-enzyme interaction, but the need for a chloroplastic 'protein factor' for the reductive ferredoxin activation of spinach leaf fructose-1,6-bisphosphatase was soon found [2]. Later investigations [3] showed that the protein factor was a mixture of two components, assimilation regulatory protein b and assimilation regulatory protein a, the former being identified as thioredoxin [4], a low molecular weight protein previously found in bacteria [5] and animal cells [6], and the latter described as a chromophore-free protein with ferredoxin-thioredoxin-reductase activity [7]. Photochemically reduced ferredoxin transfers its reducing power to thioredoxin by a reductase mediated process, which in turn activates the fructose-1,6-bisphosphatase by reduction of some of the 32 half-cystine residues of the enzyme molecule [8], promoting conformational changes related to the enzyme activity [9,10].

At the same time the appearance of vicinal dithiol groups upon light-induced conformational changes of chloroplast membranes had been reported [11], and Anderson and Avron [12] found that thiol compounds bound to thylakoid lamellae of pea chloroplasts (light effector mediators I and II) activated some chloroplastic enzyme under illumination. Light effector mediator II was reduced in the photosynthetic electron transport at ferredoxin level, and was the

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only one competent in the fructose-1,6-bisphosphatase activation [10]. The requirement of a stromal factor for light effector mediator I activation of NADP-dependent malate dehydrogenase, but not for that of thioredoxin [13], suggests some functional similarities between thioredoxin and the light effector mediator factors. Moreover, 64% of the photoactivation system of spinach chloroplast NADP-malate dehydrogenase seems to be linked to thylakoid lamellae, from which it can be easily released by washing with buffer [14], and Ashton and Brennan [15] found that thioredoxin-like species occur in both the stroma and membranes of pea chloroplasts [15]. However, a protein isolated from spinach chloroplasts, named new protein factor or ferralterin, seems to promote the photochemical activation of fructose-1,6-bisphosphatase in a reaction depending on chloroplast membranes, but not that of ferredoxin, thioredoxin and reductase [16].

The fructose-1,6-bisphosphatase photoactivation by the ferredoxin-thioredoxin system is also supported by the disappearance, after dithiothreitol reduction, of the inhibition that P_i stromal concentration produces in the spinach enzyme, and by the dithiothreitol promoted change from a sigmoidal kinetic to a hyperbolic one, with lower K_m values for Fru-1,6-P_2 and Mg^{2+}, which are brought within the physiological range [17]. However, the slow light activation of the enzyme [9] and the delayed reversion of reduced fructose-1,6-bisphosphatase to the low affinity form in the light-dark transition [18], suggest that changes in its reduction state are too slow to account for the control of the enzyme activity.

Leegood and Walker [19] have recently found up to 6-times greater fructose-1,6-bisphosphatase activity after chloroplast illumination, it being the only stromal enzyme exhibiting a sufficiently increased activity in the dark-light transition to account for the high CO_2 fixation rate that takes place after the induction period. From these results it is suggested that fructose-1,6-bisphosphatase activity is regulated by the electron availability through a modulating mechanism such as the ferredoxin-thioredoxin system [20].

In the present work we analyze the affinity relationships ferredoxin/fructose-1,6-bisphosphatase and thioredoxin/fructose-1,6-bisphosphatase as additional proof of the light-activation mechanism of photosynthetic fructose-1,6-bisphosphatase by the ferredoxin-thioredoxin system. The results we have obtained show the existence of a strong interaction between thioredoxin and fructose-1,6-bisphosphatase isolated from spinach leaves.

Material and Methods

Isolation of fructose-1,6-bisphosphatase, ferredoxin and thioredoxin from spinach. Fresh spinach leaves from the local market were washed with water and kept overnight in plastic bags at 4°C; the petioles and prominent veins were taken out before use. Photosynthetic fructose-1,6-bisphosphatase and ferredoxin were purified to homogeneity as described previously [21,22]. A mixture of spinach leaf thioredoxins was prepared according to Wolosiuk et al. [23], and specific thioredoxins f, m, c_f and c_m were purified as described by Crawford et al. [24]; in both cases the thioredoxin preparations had the same degree of purity as stated in the references.

Assay of fructose-1,6-bisphosphatase activity. Usually the reaction mixture was as follows: 0.1 M Tris-HCl buffer pH 8.8/5 mM Fru-1,6-P_2/5 mM $MgCl_2$/1.6 mM EDTA/5 mM cysteine and the enzyme preparation, in a final volume of 2 ml. After 30 min incubation at 28°C the reaction was stopped with trichloroacetic acid, and the P_i released determined according to Fiske and Subbarow [25].

Affinity techniques. For preparation of ferredoxin-Sepharose, crude thioredoxin-Sepharose and specific thioredoxin-Sepharoses, 15 mg of purified spinach ferredoxin or of the mixture of leaf thioredoxins, or 5 mg of specific thioredoxins, were added to a suspension of 2 g of CNBr-activated Sepharose 4B (Pharmacia, Uppsala) in 15 ml of 0.1 M $NaHCO_3$ buffer, pH 8.0, made up 0.1 M in NaCl. The mixture was gently shaken at 4°C for 12 h, and the unreacted protein removed by washing the gel twice with the above buffer. The remaining free coupling ligands were saturated with an excess of 1 M glycine in the bicarbonate buffer; after shaking overnight at 4°C, the gel was again washed twice with the same buffer. Under these conditions about 6 mg of ferredoxin, 4 mg of crude thioredoxin and 1.2 mg of specific thioredoxins were fixed per g of the Sepharose, as determined by spectrophotometric analysis of the

washings at 422 and 280 nm. Amino acid-Sepharoses and mercaptoethylamine-, glutathione- and propylamine-Sepharoses were prepared by direct saturation of CNBr-activated Sepharose 4B with the corresponding amino-compound, following the above methodology.

Affinity tests were performed by column chromatography on 0.9 X 8 cm gel beds. After packing the columns, the Sepharose gels were equilibrated with 4–5 volumes of 5 mM Tris-HCl buffer, pH 7.5 or 8.2. 0.5 mg of purified fructose-1,6-bisphosphatase in 5 ml of the corresponding Tris-HCl buffer were applied to the column at a rate of 0.2 ml/min, and the nonfixed enzyme removed by washing with the same solution; the bound fructose-1,6-bisphosphatase was then eluted by application of 0.5 M NaCl in the equilibration buffer. In another group of experiments the columns were equilibrated and washed with 5 mM Tris-HCl buffer, pH 7.5 or 8.0, made up 5 or 10 mM in $MgCl_2$, and then eluted with the same solutions of NaCl added to a final 0.5 M concentration. All column operations were performed at 4°C, and monitored by absorption at 280 nm using a continuous-flow cell.

Reduced ferredoxin-Sepharose and thioredoxin-Sepharose were prepared, respectively, by passing through the columns 10 ml of 5 mM sodium dithionite, or 10 mM dithiothreitol, in 5 mM Tris-HCl buffer, pH 7.5. In both cases the fructose-1,6-bisphosphatase was dissolved in this buffer made up 2.5 mM with dithionite or 10 mM with dithiothreitol, respectively, also using both solutions for washing and, after addition of 0.5 NaCl, for elution. Binding experiments with carboxymethylated and with *p*-hydroxymercuribenzoate-derivatives of cysteine-Sepharose, mercaptoethylamine-Sepharose and glutathione-Sepharose were performed after treatment with iodoacetate [26] or *p*-hydroxymercuribenzoate [27].

Amino acid composition. The amino acid composition of photosynthetic spinach leaf fructose-1,6-bisphosphatase was determined in triplicate in a two column JEOL model JLC-6AH autoanalyzer after acid hydrolysis in HCl for 24 h at 100°C. Half-cystine residues were determined separately after performic acid oxidation [28], and tryptophan after acid hydrolysis in the presence of mercaptoacetic acid [29].

Results and Discussion

According to the Buchanan's laboratory proposal concerning the light-activation mechanism of photosynthetic fructose-1,6-bisphosphatase by the ferredoxin-thioredoxin system [4], the existence of some type of affinity between fructose-1,6-bisphosphatase and its close reductant thioredoxin seems feasible. Affinity experiments on thioredoxin-Sepharose columns have shown that the enzyme is indeed strongly bound at pH 7.5 by the oxidized thioredoxin as much as by the dithiothreitol-reduced one (Fig. 1); on the other hand, fructose-1,6-bisphosphatase was not fixed at all on ferredoxin-Sepharose, both in the oxidized form and in the dithionite-reduced form, which is at odds with the old hypothesis of a direct ferredoxin-enzyme interaction [1]. When the thioredoxin-Sepharose gel and the fructose-1,6-bisphosphatase solution were equilibrated in 5 mM Tris-HCl buffer, pH 8.2, the thioredoxin/fructose-1,6-bisphosphatase affinity was diminished. Even though there is an enzyme transition from a polymeric to a protomeric form at pH above 7.5 [21], such pH-de-

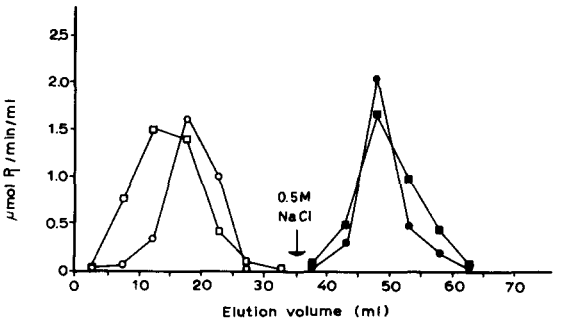

Fig. 1. Elution profiles of fructose-1,6-bisphosphatase by affinity chromatography on ferredoxin- and thioredoxin-Sepharose. 0.5 mg of enzyme in 5 mM Tris-HCl buffer, pH 7.5, was applied to each of several 0.9 X 8 cm columns of ferredoxin-Sepharose (12 mg of ferredoxin) or thioredoxin-Sepharose (9 mg of thioredoxin) equilibrated with the above buffer. This was also used for washing the non-fixed enzyme, and the bound fructose-1,6-bisphosphatase then eluted with the same solution made up 0.5 M with NaCl. Experiments with reduced ferredoxin-Sepharose and thioredoxin-Sepharose were performed with the above solutions made up 5 mM with sodium dithionite or 10 mM with dithiothreitol, respectively. □——□, ferredoxin$_{ox}$; ○——○, ferredoxin$_{red}$; ●——●, thioredoxin$_{ox}$; ■——■, thioredoxin$_{red}$.

TABLE I

EFFECT OF Mg^{2+} CONCENTRATION ON THE THIOREDOXIN/FRUCTOSE-1,6-BISPHOSPHATASE AFFINITY

0.5 mg of enzyme in 5 ml Tris-HCl buffer, pH 7.5 or 8.0, made up 5 or 10 mM in $MgCl_2$, or 10 mM in NaCl, was applied to each of several 0.9 × 8 cm columns of thioredoxin-Sepharose (with approx. 8 mg of thioredoxin) equilibrated with the above solutions, which were also used for washing the non-fixed enzyme. The bound enzyme was then eluted with the same solutions made up 0.5 M with NaCl, and both the linked and non-linked enzyme assayed for fructose-1,6-bisphosphatase activity.

Affinity conditions			Bound enzyme (%)
pH	$MgCl_2$ (mM)	NaCl (mM)	
7.5	–	–	100 *
8.0	–	–	100
7.5	5	–	30
8.0	5	–	12
7.5	10	–	6
8.0	10	–	6
7.5	–	10	100
8.0	–	10	100

* Actual value: 2.8 μmol of P_i released per min.

pendent change of affinity cannot be considered of physiological significance because of the high pH value at which the thioredoxin/fructose-1,6-bisphosphatase binding decreases. However, affinity experiments at pH 7.5 or 8.0 in the presence of 5 mM Mg^{2+} only showed 30% and 12%, respectively, of enzyme bound to the thioredoxin-Sepharose, which was decreased to 6% when at both these pH values the Mg^{2+} concentration rose to 10 mM (Table I). This loss of binding capability in the presence of 5–10 mM Mg^{2+} cannot be related to an increase of ionic strength, since the fructose-1,6-bisphosphatase was still strongly bound with the buffer made up 10 mM in NaCl. Although the basal concentration of stromal Mg^{2+} has not yet been well established, light induced rises between 13 mM [20] and 2 mM [31] have been reported, as well as a stromal pH increase from approximately 7.0 to nearly 8.0 [32]. So a rise of stromal pH and Mg^{2+} concentration in the dark-light transition could account for a loosening of the thioredoxin/fructose-1,6-bisphosphatase linkage through induced conformational changes of the enzyme molecule, releasing the photoactivated fructose-1,6-bisphosphatase in the stromal space.

Anderson and Ben-Bassat [33] have found that some light-modulated enzymes of the reductive pentose-phosphate cycle are partially linked to chloroplast lamellae in the dark, from which they can be released to the stromal solution after illumination. If, as has been earlier shown by Ashton and Brennan [15], there is some type of thioredoxin-like species bound to the thylakoid membranes, this thioredoxin-enzyme affinity could account for such a linkage.

Thioredoxin from *Escherichia coli* can fully replace native thioredoxin in the light in vitro activation of spinach fructose-1,6-bisphosphatase [4]. Because *E. coli* thioredoxin shows two half-cystine residues per molecule [5], it is possible that the fructose-1.6-bisphosphatase/thioredoxin linkage takes place through the -SH groups. To check this assumption we have tested the fructose-1,6-bisphosphatase interaction with cysteine-Sepharose, and additionally with mercaptoethylamine-Sepharose and glutathione-Sepharose. Surprisingly, the enzyme was not fixed at all on cysteine-Sepharose, but was strongly bound by the other two amino compounds (Table II); however, these linkages cannot be related to the free -SH groups, because the enzyme was also completely bound by the *S*-carboxymethylated and the *p*-hydroxymercuribenzoate-treated compounds.

In order to discover the nature of the thioredoxin binding group in the thioredoxin/fructose-1,6-bisphosphatase complex, we have studied the affinity properties of the enzyme with some amino acids. Table II shows that fructose-1,6-bisphosphatase was strongly bound by tyrosine, isoleucine, lysine, arginine and proline, and loosely linked by tryptophan, phenylalanine, valine, leucine and histidine; moreover, the enzyme was also completely bound by the non-physiological compound propylamine. From these data we can conclude that the thioredoxin/fructose-1,6-bisphosphatase affinity depends on a nonspecific interaction concerned with nonpolar groups of both components. The amino acid composition of spinach chloroplast fructose-1,6-bisphosphatase (Table III) does not show a very high number of nonpolar amino acids; on the contrary, glutamic and aspartic acids and glycine appear as the most abundant. The same can be stated concerning thioredoxin, assumed to have an amino acid composition (for the spinach leaf

TABLE II

AFFINITY PROPERTIES OF FRUCTOSE-1,6-BISPHOSPHATASE WITH AMINO ACIDS AND SOME OTHER AMINO COMPOUNDS

0.5 mg of enzyme in 5 mM Tris-HCl buffer, pH 7.6, was applied to each of several 0.9 × 8 cm columns of CNBr-Sepharose 4B saturated with the corresponding amino compound, and then equilibrated with the above buffer. The non-fixed enzyme was washed with the same solution, and the bound enzyme eluted with the buffer made up 0.5 M with NaCl. Both the fixed and non-fixed enzyme were assayed for fructose-1,6-bisphosphatase activity. PHMB, *p*-hydroxymercuribenzoate.

Compound	Bound enzyme (%)	Compound	Bound enzyme (%)	Compound	Bound enzyme (%)
Alanine	0	Leucine	5	Glutathione (*S*-carboxymethyl)	100
Arginine	100	Lysine	100	Glutathione (PHMB-derivative)	100
Aspartic acid	0	Methionine	0	Mercaptoethylamine	100
Cysteine	0	Phenylalanine	29	Mercaptoethylamine (*S*-carboxymethyl)	100
Glutamic acid	0	Proline	83	Mercaptoethylamine (PHMB-derivative)	100
Glycine	0	Serine	0	Propylamine	100
Histidine	3	Threonine	0		
Isoleucine	100 *	Tryptophan	52		
		Tyrosine	100		
		Valine	9		
		Glutathione red.	100		

* Actual value: 1.15 μmoles of P_i released per min.

TABLE III

AMINO ACID COMPOSITION OF SPINACH LEAF PHOTOSYNTHETIC FRUCTOSE-1,6-BISPHOSPHATASE

Samples were hydrolyzed with 6 N HCl in sealed tubes under vacuum. Half-cystine was determined as cysteic acid after performic acid oxidation, and tryptophan after acid hydrolysis in the presence of mercaptoacetic acid. The number of residues were obtained on the basis of minimum molecular weight (one histidine residue per mol). The multiplying factor used for calculation (n = 16) was determined from the molecular weight of the enzyme found by other techniques [34].

Amino acid	Number of residues	Amino acid	Number of residues
Lysine	64	Alanine	80
Histidine	16	Valine	80
Arginine	32	Methionine	0
Aspartic acid	112	Isoleucine	80
Threonine	48	Leucine	80
Serine	96	Tyrosine	32
Glutamic acid	128	Phenylalanine	32
Proline	48	Half-cystine	32
Glycine	112	Tryptophan	0

thioredoxin) similar to that of the *E. coli* protein [5]; however, the existence of hydrophobic properties in the thioredoxin molecule can be deduced because of the purification procedure of *E. coli* thioredoxin by affinity chromatography on octil-Sepharose [35]. We can thus assert that the thioredoxin/fructose-1,6-bisphosphatase affinity is linked to the presence of local hydrophobic clusters, rather than to a high content of nonpolar residues.

Wolosiuk et al. [36] found that chloroplast thioredoxin is a mixture of the named thioredoxins f and m, of which the former preferentially activated fructose-1,6-bisphosphatase and other enzymes of the photosynthetic reduction cycle. Moreover, a thioredoxin c found in the cytoplasm of photosynthetic tissues has also been resolved in thioredoxins c_f and c_m ; as in the case of their chloroplast counterparts, thioredoxin c_f showed a preferential activation of photosynthetic fructose-1,6-bisphosphatase [24]. Affinity experiments with specific thioredoxins have shown that the chloroplastic thioredoxins f and m bind quantitatively the photosynthetic spinach fructose-1,6-bisphosphatase, with 100% effectiveness.

Concerning the cytoplasmic thioredoxins c_f and c_m, they also strongly bind the enzyme, with yields of 100% and 98%, respectively. These results are in accordance with the more specific fructose-1,6-bisphosphatase activation by thioredoxins f and c_f, but also explain the residual activation by thioredoxins m and c_m. From a structural point of view this behaviour suggests similarities between the four thioredoxins concerning amino acid composition and the existence of hydrophobic clusters.

In conclusion, the present results provide additional evidence of the light-activation mechanism of photosynthetic fructose-1,6-bisphosphatase by the ferredoxin-thioredoxin system. The physicochemical characteristics of the thioredoxin/fructose-1,6-bisphosphatase complex, and the use of this binding property to outline new purification procedures for fructose-1,6-bisphosphatase and thioredoxins, are now under further investigation.

Acknowledgements

The authors are indebted to Miss Matilde Garrido for her skillful technical assistance. This research has been supported by grants from Fundación Juan March and Comisión Asesora de Investigación Científica y Técnica (Spain).

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