PHOTOSYNTHETIC REGULATORY PROTEIN FROM RABBIT LIVER IS IDENTICAL WITH THIOREDOXIN

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1. Introduction

Experiments initiated over ten years ago [1] have led to the finding of a light-dependent mechanism for the regulation of enzymes of photosynthetic CO_2 assimilation in chloroplasts. In this mechanism, ferredoxin, an iron–sulfur protein that functions as an acceptor in photosynthetic electron transport, activates key regulatory enzymes of chloroplasts such as fructose 1,6-bisphosphatase (Fru-P_2ase) – the enzyme that catalyzes the hydrolytic cleavage of fructose 1,6-bisphosphate to fructose 6-phosphate and P_i.

Enzyme activation by the ferredoxin-linked mechanism depends not only on reduced ferredoxin, but also on a 'protein factor' [2] that was shown to consist of two components (named assimilation regulatory protein 'a', ARP_a, and assimilation regulatory protein 'b', ARP_b) [3]. Whereas both ARP_a and ARP_b were required for enzyme activation by reduced ferredoxin, only ARP_b, the component found later in the major types of living cells [4], was required for activation by the nonphysiological sulfhydryl reagent dithiothreitol (cf. ref. [2]). Enzymes of CO_2 assimilation other than Fru-P_2ase were also shown to be activated by the ARP_b/dithiothreitol system.

Evidence was recently presented [5] that one of these regulatory proteins (ARP_b) is thioredoxin – an electron transport protein of low molecular weight that functions in ribonucleotide reduction via oxidation–reduction of an active (structurally defined) disulfide bridge [6,7]. Homogeneous preparations of thioredoxin from *Escherichia coli* replaced ARP_b in the activation of Fru-P_2ase either photochemically by reduced ferredoxin and ARP_a or chemically by dithiothreitol [5]. These findings suggested that chloroplast ARP_b is a thioredoxin and that, on the basis of its ability to catalyze the reduction of ARP_b by reduced ferredoxin ARP_a is a ferredoxin–thioredoxin reductase.

Since ARP_b isolated from nonphotosynthetic sources, such as bacteria and liver, was not identified, we have undertaken the identification of one of these proteins. We now report evidence that a highly purified preparation of rabbit liver ARP_b is identical with thioredoxin. The liver ARP_b interacted with liver NADPH-thioredoxin reductase in the reduction of the disulfide form of insulin and with *E. coli* ribonucleotide reductase in the reduction of ribonucleotides to deoxyribonucleotides. Like authentic mammalian thioredoxin, the liver ARP_b also activated certain of the thioredoxin-dependent enzymes of chloroplasts, viz., Fru-P_2ase and NADP-malate dehydrogenase [8,9], but not others, viz., NADP-glyceraldehyde 3-phosphate dehydrogenase [5].

2. Materials and methods

NADPH, dithiothreitol, oxalacetic acid, CDP, ATP, fructose 1,6-bisphosphate and 3-phosphoglycerate were from Sigma. [^{3}H]CDP was from Schwartz/Mann. Bovine insulin was from Vitrum, Sweden. Homogeneous thioredoxin from calf liver and thymus was prepared by the method of Engström et al. [10]. NADP-thioredoxin reductase was a highly purified preparation from rat liver (Luthman, M. and Holmgren, A. to be published). Assays of thioredoxin by thioredoxin reductase in the reduction of insulin with NADPH were performed as described [10,12]. Ribonucleotide reductase (proteins B1 and B2) [11] were pure preparations from *E. coli* B3 obtained from the laboratory of Dr Peter Reichard. Assays of ribonucleotide reductase were performed as described [13]. Previously described methods of preparation and assay were also used for chloroplast Fru-P_2ase [3], NADP-glyceraldehyde 3-phosphate dehydrogenase [3] and NADP-malate dehydrogenase [9].

For the experiments with thioredoxin reductase and ribonucleotide reductase, highly purified preparations of ARP_b from spinach chloroplasts and rabbit liver [3,4] were prepared in Berkeley, lyophilized in 0.5% NH_4HCO_3 and air-shipped to Stockholm. Samples were reconstituted with this buffer and assayed. For experiments with chloroplast Fru-P_2ase, NADP-malate dehydrogenase and NADP-glyceraldehyde 3-phosphate dehydrogenase, calf thymus thioredoxin was prepared, lyophilized and air-shipped to Berkeley where it was reconstituted as described for the ARP_b proteins.

3. Results and discussion

The idea that ARP_b from both photosynthetic and nonphotosynthetic sources might be related to thioredoxin led (i) to the demonstration that *E. coli* thioredoxin fully replaced chloroplast ARP_b in the activation of Fru-P_2ase [5] and (ii) to the present investigation on the activity of spinach ARP_b (henceforth called chloroplast thioredoxin) and liver ARP_b preparations in different thioredoxin assays systems.

Figure 1 shows the activity of chloroplast thioredoxin and liver ARP_b in the reduction of CDP to dCDP by *E. coli* ribonucleotide reductase using

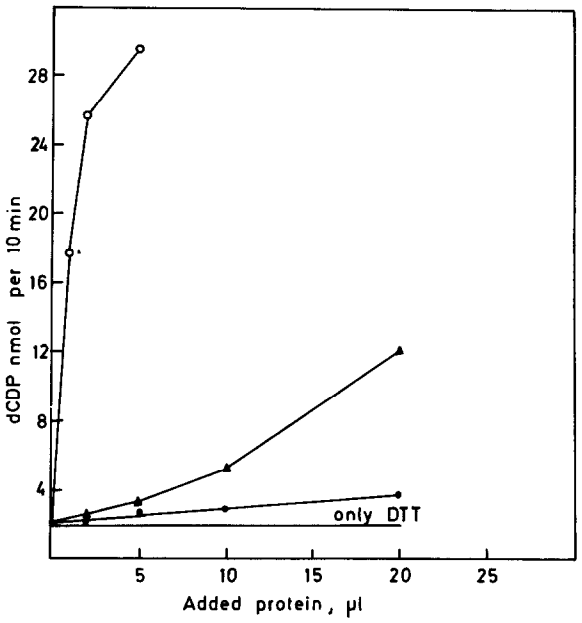

Fig.1. Reduction of CDP to dCDP by *E. coli* ribonucleotide reductase in the presence of 0.95 mM dithiothreitol (DTT) and various proteins as hydrogen donors. The conditions of the experiment were identical to procedures [11,13] using 15 µg *E. coli* ribonucleotide reductase. Thioredoxin from *E. coli* 1.50 mg/ml (o———o); ARP_b from rabbit liver 0.50 mg/ml (▲———▲); and thioredoxin from spinach chloroplasts 4.0 mg/ml (o———o). The synthesis of dCDP in the presence of only DTT 0.95 mM is shown by the continuous line.

dithiothreitol as electron donor [11,13]. Whereas the activity observed with chloroplast thioredoxin was low, rabbit liver ARP_b showed an activity similar to that observed with homogeneous calf liver thioredoxin (spec. act. approx. 30% that of native *E. coli* thioredoxin) [10]. Similar results were obtained in a second set of experiments in which dithiothreitol was replaced by 1 mM NADPH and 5 µg rat liver thioredoxin reductase (data not shown).

Another line of evidence for the identification of rabbit liver ARP_b came from the finding that it fully replaced other liver thioredoxins (calf and rat) in the NADPH-dependent reduction of insulin disulfides by liver thioredoxin reductase (fig.2). Chloroplast thioredoxin showed no activity in this system and only very low activity when its reduction was coupled to the oxidation of NADPH (added to 0.4 mM in the presence of 5 µg rat liver NADP-thioredoxin reductase

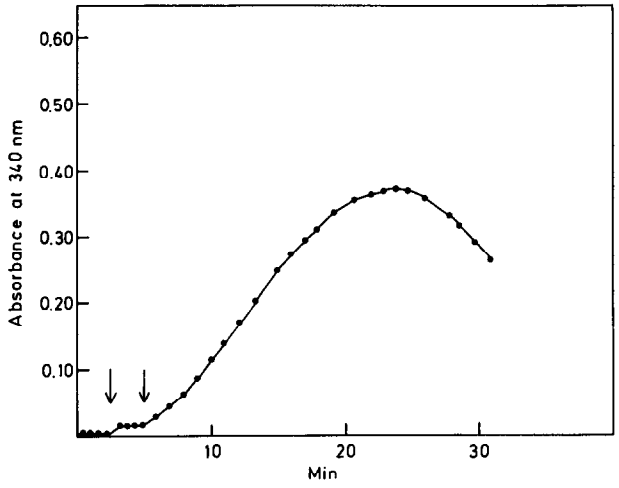

Fig.2. Reduction of disulfides in insulin by ARP_b protein from rabbit liver in the presence of NADPH and thioredoxin reductase from rat liver. Two cuvettes contained in final vol. 0.50 ml: 0.10 M potassium phosphate – 2 mM EDTA, pH 7.0, 0.4 mM NADPH. To one cuvette which was placed in the reference position of an automatic Zeiss PM QIII spectrometer was added 25 μl solution of ARP_b from liver (0.5 ml/ml) and to the other cuvette was added 25 μl water. After recording A_{340} of the cuvettes 5 μg thioredoxin reductase in 5 μl was added to both cuvettes and the reduction of ARP_b was followed. At the second arrow 250 μg bovine insulin in 25 μl was added to both cuvettes and the oxidation of NADPH resulting from disulfide reduction was recorded at 340 nm. The fall in A_{340} after 25 min was due to the precipitation of insulin-free peptide chains [12] in the cuvette containing ARP_b.

Table 1
Effectiveness of liver ARP_b and different thioredoxins in the activation of chloroplast enzymes

Treatment	Fru-P_2ase	NADP-malate dehydrogenase	NADP-glyceraldehyde 3-P dehydrogenase
	(nmol released/min)	(nmol NADPH oxidized/min)	
Chloroplast thioredoxin	39	10	81
Calf thymus thioredoxin	12	15	22
Rabbit liver ARP_b	12	5	17
Control	4	1	16

For Fru-P_2ase, the reaction mixture (in 0.5 ml) contained 8 μg of the pure enzyme and the following (in μmol): Tris–HCl buffer, pH 7.9, 50; $MgCl_2$, 0.5; dithiothreitol, 2.5; sodium fructose 1,6-bisphosphate, 3. Chloroplast thioredoxin (13 μg), calf thymus thioredoxin (20 μg) and liver ARP_b (20 μg) were added as indicated. The reaction was started by adding fructose 1,6-bisphosphate and was continued for 30 min. Temp. 22°C. The reaction was stopped by adding a 2 ml Fiske-SubbaRow reagent mixture [1] used to analyze P_i

For NADP-malate dehydrogenase, partly purified enzyme from spinach chloroplasts (260 μg) was preincubated in 0.1 ml solution containing (in μmol) Tris buffer, pH 7.9, 10; dithiothreitol 1; and as indicated, chloroplast thioredoxin, (13 μg), calf thymus thioredoxin (15 μg) and liver ARP_b (20 μg). After 5 min preincubation, the mixture was injected into a 1 cm cuvette of 1 ml capacity that contained (in μmol): Tris–HCl buffer, pH 7.9, 100; and NADPH, 0.25. The reaction was started by the addition of 2.5 μmol oxalacetic acid. ΔA_{340} was followed with a Cary 14 spectrophotometer. Temp. 22°C

For NADP-glyceraldehyde 3-phosphate dehydrogenase, highly purified spinach chloroplast enzyme (15 μg) was preincubated in 0.1 ml of a solution containing (in μmol): Tricine buffer, pH 8.4, 10; dithiothreitol, 1; and as indicated, chloroplast thioredoxin (25 μg), calf thymus thioredoxin (20 μg) and liver ARP_b (22 μg). After 5 min preincubation, the mixture was injected into a 1 cm cuvette of 1 ml capacity that contained 3-phosphoglycerate phosphokinase (3 μg) and the following (in μmol): Tricine buffer, pH 8.4, 40; dithiothreitol, 1.5; $MgCl_2$, 10; sodium 3-phosphoglycerate, 5; ATP, 5; and NADPH, 0.12. ΔA was followed with a Cary 14 spectrophotometer. Temp. 22°C

in 0.5 ml 0.1 M potassium phosphate buffer, pH 7.0, under the conditions in fig.2).

When tested with chloroplast enzymes known to be activated by reduced chloroplast thioredoxin, liver ARP_b (and authentic calf thymus thioredoxin) showed inconsistent behavior: the preparation was active with Fru-P_2ase and NADP-malate dehydrogenase, but was inactive with NADP-glyceraldehyde 3-phosphate dehydrogenase (table 1). Furthermore, although the liver ARP_b activated chloroplast Fru-P_2ase in the presence of dithiothreitol as shown in table 1, the same ARP_b preparation was totally inactive in the photochemical system with ferredoxin and ferredoxin–thioredoxin reductase (ARP_a) (data not shown). This observation indicates that chloroplast Fru-P_2ase can interact with liver ARP_b, but that ferredoxin–thioredoxin reductase cannot.

The interchangeability of mammalian and chloroplast thioredoxins thus appears to depend on the specificity of the test enzymes. While chloroplast thioredoxin showed low or no activity with the bacterial and mammalian test enzymes, mammalian thioredoxin showed good activity with some (but not all) of the chloroplast enzymes. A similar type of specificity is known for the ferredoxins – e.g., when tested with ferredoxin-dependent enzymes from nonphotosynthetic sources, chloroplast ferredoxin is active in certain assays [14] but is totally inactive in others [15].

To recapitulate, the present results provide firm evidence for the identification of rabbit liver ARP_b as thioredoxin. The data also indicate that, based on its behavior in the insulin and ribonucleotide reductase assays, chloroplast thioredoxin is of a specialized type that is perhaps characteristic of thioredoxins associated with photosynthetic electron transport.

A final noteworthy point concerns the possible general role that thioredoxin may exercise in enzyme regulation. Thioredoxin was shown recently to catalyze the reduction of disulfide bonds in insulin in the presence of either dithiothreitol, dihydrolipoic acid or the fully reduced form of ribonuclease (Holmgren, A., unpublished results). This general disulfide/dithiol oxidoreductase activity of thioredoxin may be of importance in the regulation of enzymes through oxidation–reduction of SH/S–S groups or through the formation of a stable complex between the enzyme and thioredoxin as was shown with a DNA polymerase [16]. In principle thioredoxin may be regarded as the physiological equivalent of dithiothreitol which is known to promote and stabilize the activity of many different enzymes. The search for enzymes whose activity is controlled by thioredoxin should be directed by the known stability properties of enzymes and their activation or deactivation by dithiothreitol.

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