Multiple Functions of Thioredoxins

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Reduced thioredoxins from microbial and plant cells, both of cytoplasmic or chloroplast origin, are interchangeable in stimulating such diverse enzyme activities as ribonucleoside diphosphate reductase *(E. coli)*, PAPS sulfotransferase *(Synechococcus)*, and fructose-1,6-bis-phosphatase (from spinach) *in vitro*. It is suggested that reduced thioredoxins are unspecific, multifunctional cellular proteins while in contrast the oxidized froms require specific enzymes for their reduction.

Introduction

Thioredoxins are heat-stable proteins consisting of little more than 100 amino acids which have been identified as part of a hydrogen transfer chain between NADPH and enzymatic ribonucleotide reduction in bacteria [1, 2], yeast [3], plant [4], and animal cells [5–7]. Originally considered specific reductants for DNA precursor biosynthesis, thioredoxins were also shown to be involved in assimilatory sulfate reduction in yeast and in *Escherichia coli* [8, 9]. More recently it was found that calf liver or *E. coli* thioredoxin catalyse unspecific reduction of disulfide bonds in mammalian proteins such as insulin or gonadotropin [6, 10], and that a factor, ARP_b (isolated from spinach leaves but also from liver), which is necessary for the light-induced activation of chloroplast enzymes, is identical with thioredoxin [11, 12]. These observations seem to suggest a more common function for thioredoxin in all organisms; however, they could not as yet be generalized because cross-reactivity experiments were often performed on a merely qualitative basis and under widely differing experimental conditions.

We here report quantitative data. It is demonstrated that thioredoxins of bacterial and plant origin which were purified by different activity criteria are freely interchangeable and active in three entirely different enzyme systems *in vitro*, *viz.* the ribonucleoside diphosphate reductase (EC 1.17.4.1) of *E. coli*, the 3′-phosphoadenosine 5′-phosphosulfate (PAPS) sulfotransferase [9, 13, 23] of the blue-green alga, *Synechococcus* 6301, and the fructose-1,6-bis-phosphatase (EC 3.1.3.11) of spinach.

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Materials and Methods

The thioredoxin preparations used in this study were obtained by previously published procedures. The proteins from *E. coli* [1] and from the green alga, *Scenedesmus obliquus* [4] were purified to homogeneity by following their ribonucleotide reductase activity. Pure thioredoxin from *Synechococcus* 6301 [13] and thioredoxin from spinach leaves ($\approx 50\%$ pure) [14] were isolated with the PAPS sulfotransferase assay. The enzymes mentioned above were prepared and assayed as described [15, 16, 22], thioredoxin samples being added as indicated in Fig. 1 and Table I.

Standard assays of *E. coli* ribonucleotide reductase contained 24 μg enzyme, 10 μCi [5-^{3}H]CDP (spec. activity, 0.23 Ci/mmol), 2.0 mM dithiothreitol, 15 mM Mg^{2+}, 0.8 mM EDTA, and 0.115 mM thymidine triphosphate as an allosteric effector in a total volume of 0.30 ml Tris-HCl buffer (pH 7.5). The mixtures were incubated for 30 min at 30°, and the amount of deoxyribonucleotide formed (as dCMP) was determined after thin layer chromatography on PEI cellulose and radioactivity scanning [4].

PAPS sulfotransferase activity was analysed by determination of acid-volatile radioactivity released from [^{35}S]PAPS in the presence of dithioerythritol and thioredoxin [13]. Assays contained, in a total volume of 1.00 ml Tris-HCl buffer (pH 8.0), 280 μg *Synechococcus* sulfotransferase, 0.05 μmol [^{35}S]-PAPS (spec. activity, 1720 cpm/nmol), 10 mM dithioerythritol, and 10 mM Mg^{2+}. The mixtures were

incubated under nitrogen for 1 h at 37°. $^{35}SO_2$ was liberated by acidification with HCl, trapped in 1 M triethanolamine and determined as described [22].

Fructose-1,6-bis-phosphatase from spinach was determined by incubating 15 μg enzyme with 6 mM fructose-1,6-bis-phosphate, 4 mM dithiothreitol, and 1 mM Mg^{2+} in a total volume of 0.55 ml Tris-HCl buffer (pH 7.9). After 15 min at 30° inorganic phosphate was determined colorimetrically [16].

Results

In one series of experiments the thioredoxin preparations were tested under identical assay conditions as substrates for [^{3}H]cytidine diphosphate (CDP) reduction catalysed by purified *E. coli* ribonucleotide reductase (Fig. 1). These parallel experiments eliminate the need for activity corrections which are very difficult in measuring enzymatic ribonucleotide reduction. It is seen that the pure thioredoxins of microbial origin all effectively stimulate CDP reduction over the low control value in presence of dithiothreitol. (The enzyme cannot be assayed in complete absence of a dithiol.) An apparent K_m value of 0.5×10^{-6} M is obtained for *E. coli* thioredoxin (*i. e.*, the homologous protein), and a ten-fold higher value for the *Synechococcus* and *Scenedesmus* thioredoxins ($K_m = 5 \times 10^{-6}$ M). The K_m for dithiothreitol is as high as 3.6×10^{-2} M. The heat-stable protein which was isolated from *Synechococcus* as a necessary cofactor for the sulfate transfer step of PAPS reduction [13] is thus identified as thioredoxin on the basis of its properties and enzyme activity. Likewise it is clear that the spinach protein, although not available in homogenous form, possesses good thioredoxin activity when assayed under optimized conditions, in contrast to previous experiments in which only low activity towards the *E. coli* enzyme was noted [11].

In Table I are summarized the relative activities of five different thioredoxins in the reductase, sulfotransferase, and phosphatase reactions. These three enzymes have low, yet measurable activity in the presence of dithiothreitol as an unphysiologic dithiol

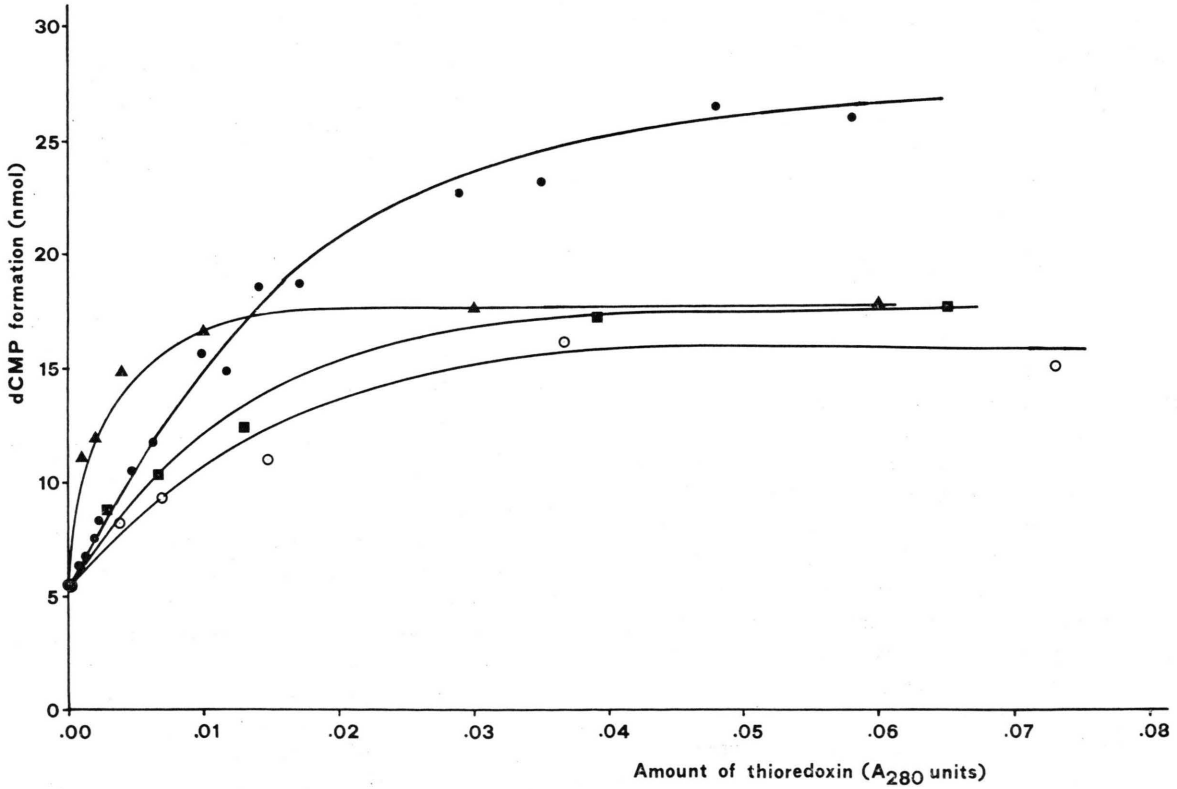

Fig. 1. Stimulation of *E. coli* ribonucleotide reductase-catalysed CDP reduction by thioredoxins. Addition to standard enzyme assays was made on a A_{280} unit basis (0.01 A unit $\approx$ 10 μg protein in case of the three pure microbial thioredoxins). ▲ *Escherichia coli*, ■ *Synechococcus* 6301, ● *Scenedesmus obliquus* (protein B), ○ spinach thioredoxin.

Table I. Stimulation of enzyme activities by thioredoxin from different sources

Source and molecular weight of thioredoxins		Ribonucleotide reductase		PAPS sulfotransferase		Fructose-bis-phosphatase	
amount added [μg]		0	4	0	94	0	12
		nmol	dCMP	nmol	SO_2	μmol	PO_4
no addition		5.4		1.0		0.12	
E. coli	11,700		14.9		11.1		0.36
Synechococcus	11,500		9.5		11.7		0.18
Scenedesmus, A	23,500		6.3		2.5		0.31
Scenedesmus, B	12,600		10.4		11.9		0.16
spinach [14]	16,000; 20,000		8.5		7.7		0.39

Identical amounts of each thioredoxin (adjusted to fall into the linear range of enzyme activity stimulation) were added to all assays of an enzyme as indicated; because of the very different assay methods it was not possible to use the same quantities of thioredoxin for all enzymes. It is not meaningful to calculate specific activities of the thioredoxins from these data because one deals with stimulations of enzyme activity (Fig. 1).

(control values) and are characteristically stimulated by thioredoxin. The data were determined under conditions where enzyme stimulation was linearly dependent upon added thioredoxin, and they are strictly comparable for each enzyme in the vertical columns. Although the mechanism of activation is different in each case (see below) stimulation is observed in any thioredoxin/enzyme combination. The highest effect is always found in the homologous systems, *i. e.* *E. coli* thioredoxin/ribonucleotide reductase, *Synechococcus* thioredoxin/PAPS sulfotransferase, and spinach thioredoxin/fructose-bis-phosphatase, but other combinations (*e. g.* those with *E. coli* thioredoxin) closely resemble the homologous mixtures. An especially interesting feature is the differential activity of thioredoxins A and B from *Scenedesmus* [17]: Like chloroplast thioredoxin from spinach, protein A is most active in stimulating the chloroplast enzyme, fructose-bis-phosphatase, but least active towards ribonucleotide reductase, while protein B behaves in the opposite way and resembles the *Synechococcus* factor in activity. Algal thioredoxin A and the spinach protein also have in common a significantly higher molecular weight than all other known thioredoxins, including species B from *Scenedesmus*, which are of cytoplasmic origin. These properties allow the preliminary assignment of thioredoxins A and B as chloroplast and cytoplasmic proteins, respectively; unfortunately it is not possible to prepare intact chloroplasts from the green algae to unambiguously prove the nature of fraction A.

Discussion

The ubiquitous occurrence of thioredoxins in microorganisms, plants, and animals is now well documented. Their basically similar protein structure is apparently overlaid by specific differences in molecular size and composition. The results described above, together with those previously reported [9, 11, 12, 19] confirm that the two essential cysteine residues make these polypeptides a rather versatile class of cellular components in bacteria and plants: They act as hydrogen donors for both the non-heme-iron containing and the coenzyme B12-dependent ribonucleotide reductases [1, 2], they appear to serve as an acceptor of sulfate groups, forming a thiosulfate intermediate [9, 13], and they reduce, and thereby activate, regulatory chloroplast enzymes [11], presumably by protein disulfide-thiol exchange reactions. The most important known function of mammalian thioredoxins is in ribonucleotide reduction, but an activation of liver deoxycytidylate kinase has also been noted [19, 20]. (In contrast, we could not observe a stimulatory effect of thioredoxins upon the same enzyme isolated from *E. coli*.) Thioredoxin levels remain unaffected during liver regeneration [21] or during the algal cell cycle [18] where ribonucleotide reductase activities change strongly, indicating that the compounds' synthesis and intracellular supply are not exclusively coupled with deoxyribonucleotide biosynthesis.

If thioredoxins function in more biochemical reactions in the same cell than previously anticipated, an exchangeability of the reduced proteins from different species appears as a necessary consequence. It is unreasonable to assume that all thioredoxin-dependent enzymes (of otherwise entirely different nature) of an organism could possess the same high structure specificity for their common substrate or cofactor, which is a macromolecule, but they will

have to exhibit some broad affinity for thioredoxin molecules; this must then also apply to heterologous thioredoxin/enzyme mixtures. The observed differences in apparent K_m values and stimulatory activities (Table I) among homologous and heterologous combinations are in complete agreement with that expectation. Although intracellular compartmentation and functional specialisation appear likely at least for the plant thioredoxins, *in vitro* cross-reactivities even remain among thioredoxins and enzymes of cytoplasmic and chloroplast origin. Further studies on the distribution and multiplicity of thioredoxin in green algae [17] and in leaves [24] are currently in progress in our own and in other laboratories.

These considerations finally lead to an understanding of why thioredoxin reductases (EC 1.6.4.5), which act upon *oxidized* thioredoxin, are usually species-specific. For example, NADPH-dependent thioredoxin reductase of *E. coli* does not reduce *Scenedesmus*, *Synechococcus*, or any other microbial or mammalian thioredoxin whereas ribonucleotide reductase of the same organism readily accepts all these thioredoxins as substrates. While thioredoxin and ribonucleotide reductase of the green algae are similar to the bacterial proteins [4, 18], *Scenedesmus* does not contain a thioredoxin reductase of the NADPH/flavoprotein type found in *E. coli* or yeast, and a corresponding, light-independent plant enzyme remains to be identified. These surprising differences become plausible if we assume that thioredoxin reductases have specifically developped in each organism for the only purpose to regenerate reduced thioredoxin and to maintain an intracellular level of that multifunctional protein.

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