Fe^{3+}-chelates mediate the oxidative modulation of cyanobacterial and chloroplast enzymes

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Fe^{3+}–EDTA and the Fe^{3+} complexes of naturally occurring compounds (ATP, ADP, GTP, oxalate, pyridoxal 5′-phosphate, P_i, PP_i) mediated the oxidative modulation of several, partially purified cyanobacterial and chloroplast enzymes. Cyanobacterial glucose-6-phosphate dehydrogenase deactivated by treatment with dithiothreitol + thioredoxin was reactivated in the presence of Fe^{3+}-chelates under aerobic conditions. Cyanobacterial fructose-1,6-bisphosphatase, spinach leaf NADP-glyceraldehyde-3-phosphate dehydrogenase and NADP-malate dehydrogenase activated by incubation with dithiothreitol + thioredoxin were deactivated in the presence of Fe^{3+}-chelates under aerobic conditions. Cyanobacterial isocitrate dehydrogenase and cyanophage AS-1-induced site-specific endonuclease, enzymes known to be devoid of redox properties, were not affected by the Fe^{3+} complexes. The possible role of iron-chelates in enzyme modulation is discussed.

Chloroplast *Cyanobacteria* *Enzyme modulation* *Fe^{3+}-chelate* *Redox protein* *Thioredoxin*

1. INTRODUCTION

Chelating compounds are widely used by biochemists to remove catalytically active metal ions from biological systems. In some cases, however, complex formation may endow metal ions with specific catalytic functions. Current research interest is focussed on the role of iron-chelates in the production of the OH˙ radical [1] and in lipid peroxidation [2]. We report here a novel model reaction of possible in vivo significance: the mediation by Fe^{3+}-chelates of the oxidative modulation of reduced cyanobacterial and chloroplast enzymes in the presence of O_2.

New models for the oxidative modulation of chloroplast and cyanobacterial enzymes are worthy of consideration because, in contrast to a host of information available on the mechanism of reductive (light-mediated) enzyme modulation (review [3,4]), little is known about the reverse, oxidative process. The ultimate source of oxidizing power involved in oxidative enzyme modulation appears to be molecular oxygen [5,6]. However, O_2 does not react directly with the modulated enzymes; therefore, systems able to mediate the effect of O_2 are being studied intensively [7–10].

Abbreviations: 2,6-DCPIP, 2,6-dichlorophenolindophenol; DTT, dithiothreitol; FBPase, fructose-1,6-bisphosphatase (EC 3.1.3.11); G6PDH, glucose-6-phosphate dehydrogenase (EC 1.6.4.2); NADP-GAPDH, NADP-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13); NADP-MDH, NADP-malate dehydrogenase (EC 1.1.1.12); ICDH, NADP-isocitrate dehydrogenase (EC 1.1.1.42); P_i, inorganic phosphate; PMS, phenazine methosulfate; PP_i, inorganic pyrophosphate

2. MATERIALS AND METHODS

Anabaena sp. PCC 7120 was grown axenically in Allen's medium [11] as in [12]. *Anacystis nidulans* 14625 (*Synechococcus* AN PCC 6301) was cultured as in [13]. Spinach leaves were purchased from a local market.

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The following enzymes were partially purified and assayed by procedures published elsewhere: G6PDH from *Anacystis* [14] and *Anabaena* [12]; FBPase [15], ICDH [16] and cyanophage AS-1-induced site-specific endonuclease [17] from *Anacystis*; NADP-GAPDH [18] and NADP-MDH [19] from spinach leaves. An ascorbate-sensitive cyanobacterial phosphatase [20] was assayed in crude extracts. All purified enzyme preparations were devoid of thioredoxins.

Thioredoxins were partially purified and characterized from *Anabaena* and *Anacystis* cells [12] as well as from spinach leaves [19].

The determination of thiol groups was carried out as in [21]. Oxidation of reduced 2,6-DCPIP, PMS and ascorbic acid was determined spectrophotometrically, by following the changes in absorbance at 600, 387 and 280 nm, respectively.

The iron content of the preparations was assayed by proton-induced X-ray elemental analysis (PIXE) [22].

Fe^{3+}–EDTA was prepared as in [23]. Other Fe^{3+}-chelates were prepared by dissolving $FeCl_3$ and a chelating compound, in different ratios, in distilled water [24]. The mixtures were gel-filtered on a Sephadex G-10 column before use.

3. RESULTS

3.1. *Reactivation of reductively deactivated G6PDH in the presence of Fe^{3+}–EDTA*

Partially purified G6PDH preparations from *Anacystis* and *Anabaena* were deactivated under conditions known to mimick the reductive modulation of enzymes via the photosynthetic electron transport chain; i.e., the enzymes were incubated with the powerful, non-physiological reductant DTT + the natural electron carrier, thioredoxin, that actually reduces the enzymes [3]. Then, efforts were made to reactivate G6PDH under various conditions. Results of a typical deactivation/reactivation experiment, summarized in fig.1, show that: (i) the activity of purified G6PDH was not affected by 0.2 mM DTT in the absence of thioredoxin; (ii) 0.2 mM DTT + thioredoxin deactivated the enzyme; (iii) the deactivated G6PDH preparation was not reactivated by aeration; (iv) the deactivated enzyme was reactivated in the presence of Fe^{3+}–EDTA under aerobic, but not under anaerobic, conditions; (v) the reaction process mediated by Fe^{3+}–EDTA was reversible (cf. deactivation of G6PDH in the presence of 2 mM DTT).

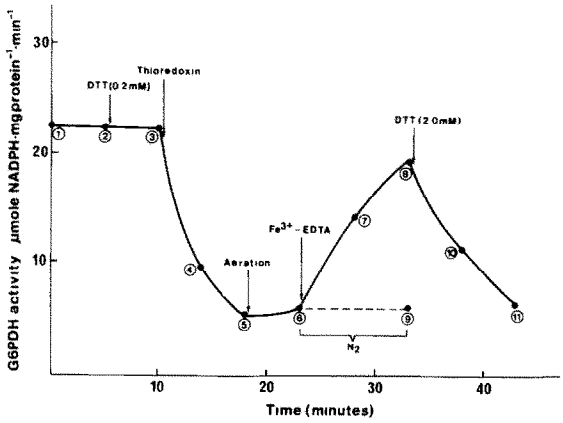

Fig.1. Mediation by Fe^{3+}–EDTA of the oxidative reactivation of reductively deactivated cyanobacterial glucose-6-phosphate dehydrogenase. A G6PDH preparation purified from *Anacystis* was subjected to a sequence of treatments marked with arrows in the figure. After each treatment an aliquot was withdrawn and its G6PDH activity measured. The numbers in the figure and in the text below refer to the enzyme activities determined after the following treatments: (1) Untreated sample withdrawn from a stock solution kept on ice. (2) Untreated sample after incubation for 5 min in 0.05 M Tris–HCl buffer at pH 7.5 at 25°C. (3) Sample incubated for 5 min with 0.2 mM DTT. (4,5) Samples incubated for 4 and 8 min, respectively, with 0.2 mM DTT + 80 $\mu g \cdot ml^{-1}$ *Anacystis* thioredoxin preparation. (6) Sample pretreated with DTT + thioredoxin and aerated for 5 min. (7,8) Samples pretreated with DTT + thioredoxin and aerated for 5 and 10 min, respectively, in the presence of 0.1 mM Fe^{3+}–EDTA. (9) Sample pretreated with DTT + thioredoxin and flushed with N_2 for 10 min in the presence of 0.1 mM Fe^{3+}–EDTA. (10,11) Samples aerobically reactivated in the presence of Fe^{3+}–EDTA and treated with 2 mM DTT for 5 and 10 min, respectively. The samples, and all ingredients added to them, were diluted 20-fold in the assay system.

Fe^{3+} or EDTA, when added separately to the enzyme preparations, were ineffective.

Results similar to those described for the *Anacystis* G6PDH were obtained with a purified G6PDH preparation from *Anabaena*.

3.2. *Deactivation of reductively activated enzymes in the presence of Fe^{3+}–EDTA*

Chloroplast and cyanobacterial FBPases and

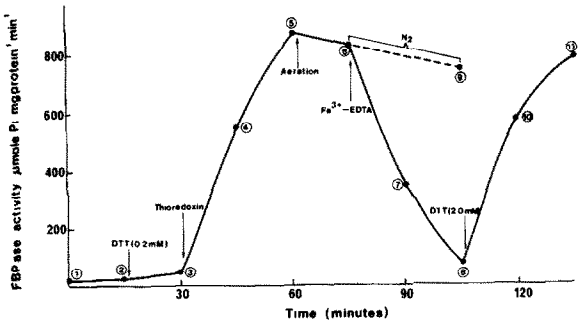

Fig.2. Mediation by Fe^{3+}–EDTA of the oxidative deactivation of reductively activated cyanobacterial fructose-1,6-bisphosphatase. The design of the experiment was identical with that described in the legend to fig.1.

NADP-GAPDHs, as well as the NADP-MDH of chloroplasts, are known to be activated by reductants and deactivated by oxidants [3,4]. It was, therefore, of interest to learn whether Fe^{3+}–EDTA would mediate the oxidative deactivation of the reduced forms of these enzymes. Results obtained with a purified FBPase preparation from *Anacystis* (fig.2) show that: (i) the enzyme was activated by incubation with 0.2 mM DTT + thioredoxin but not by incubation with DTT alone; (ii) the activated enzyme was only moderately affected by aeration; (iii) incubation of the activated enzyme with Fe^{3+}–EDTA resulted in a drastic decrease in enzyme activity under aerobic, but not under anaerobic conditions; (iv) the enzyme was reactivated by the addition of 2 mM DTT.

Fe^{3+} or EDTA, when added separately to the enzyme preparations, were ineffective.

Results similar to those described for the *Anacystis* FBPase were obtained with spinach NADP-GAPDH and NADP-MDH.

3.3. *Fe^{3+}–EDTA deactivates an ascorbate-sensitive cyanobacterial phosphatase*

Since all the redox-modulated enzymes dealt with in sections 3.1 and 3.2 are activated/deactivated either via the reduction/oxidation of some of their essential disulfide/sulfhydryl bonds/groups [3] or via thiol-disulfide exchange [4], the question arises as to whether only thiol enzymes are modulated by Fe^{3+}–EDTA in air. A cyanobacterial phosphatase which is activated by reduction with ascorbate + a protein factor, but not by the thioredoxin system [20], was chosen to study this problem. We found that the activated form of

Table 1

Mediation of the oxidative reactivation of glucose-6-phosphate dehydrogenase and oxidative deactivation of fructose-1,6-bisphosphatase from *Anacystis* by Fe^{3+}-chelates of naturally occurring compounds

Fe^{3+}-chelate of	Fe^{3+}/chelator ratio[b]	Percent aerobic reactivation of reductively deactivated G6PDH[a]	Percent aerobic deactivation of reductively activated FBPase[a]
Oxalate	1:200	37	63
ATP	1:20	54	64
ADP	1:20	34	53
CTP	1:20	23	76
GTP	1:200	62	–
Pyridoxal 5′-phosphate	1:20	26	80
Pyrophosphate (inorganic)	1:200	56	–

[a] For details, cf. legend to fig.1 and section 2

[b] The concentration of Fe^{3+} was 0.1 mM throughout the experiments. $FeCl_3$ dissolved in distilled H_2O was mixed with the chelating compounds in different ratios, such as 1:1, 1:20, 1:200 [24]. The mixtures were kept at 4°C for 2 h and gel-filtered on a Sephadex G-10 column. The chelate-containing fractions eluted by H_2O were pooled and concentrated by evaporation at room temperature. Results obtained at the optimum Fe^{3+}/chelator ratios are shown in the table

this enzyme was deactivated by Fe^{3+}–EDTA in the presence of air (not shown). Thus, the effect of Fe^{3+}–EDTA does not appear to be restricted to thiol enzymes. We found that even the reduced forms of miscellaneous, low-M_r redox compounds (glutathione, cysteine, ascorbic acid, PMS and 2,6-DCPIP) were oxidized in the presence of Fe^{3+}–EDTA under aerobic, but not under anaerobic, conditions. Fe^{3+}–EDTA can, therefore, mediate the oxidation by O_2 of a wide variety of redox compounds of suitable redox potential.

3.4. *Fe^{3+}–EDTA has no effect on enzymes that are not subject to redox modulation*

To determine whether Fe^{3+}–EDTA affects the activity of redox-modulated enzymes only (or at least preferentially), we studied the effect of Fe^{3+}–EDTA on two cyanobacterial enzymes which are devoid of redox properties: (i) the isocitrate dehydrogenase of *Anacystis* [16] and (ii) the site-specific endonuclease isolated from *Anacystis* cells infected by cyanophage AS-1 [17]. The activity of these two enzymes was not affected by Fe^{3+}–EDTA + O_2.

3.5. *Fe^{3+}-chelates of naturally occurring substances mediate the aerobic modulation of cyanobacterial enzymes*

Fe^{3+} complexes of ATP, ADP, GTP, oxalate, pyridoxal 5′-phosphate, P_i and PP_i mediated the oxidative modulation of redox-regulated cyanobacterial enzymes. These complexes partially reactivated the reduced G6PDH, and deactivated the reduced FBPase, upon aeration (table 1). The results show that the Fe^{3+}-chelates of several naturally occurring substances behave in the same way as Fe^{3+}–EDTA, a model compound.

3.6. *Do natural analogues of the Fe^{3+}–EDTA complex occur in* Anabaena *cells and spinach leaves?*

No unequivocal answer can be given to this question at this stage. However, published data [25] as well as the following observations do sug-

Table 2

Aerobic reactivation of reductively deactivated glucose-6-phosphate dehydrogenase and aerobic deactivation of reductively activated fructose-1,6-bisphosphatase and NADP-dependent malate dehydrogenase by deproteinized extracts from spinach leaves, supplemented with Fe^{3+}[a]

Preincubation of the enzyme preparation prior to enzyme assay with					Enzyme activity (μmol·mg protein^{-1}·min^{-1})		
					Anacystis		Spinach MDH
DTT[b]	Thioredoxin (70 μg·ml^{-1})	Spinach leaf extract[c]	Fe^{3+} (0.1 mM)	EDTA (0.1 mM)	G6PDH	FBPase	
–	–	–	–	–	33.7	1.5	11.0
+	–	–	–	–	33.9	1.8	13.0
+	+	–	–	–	3.2	148.3	155.0
+	+	+	–	–	5.9	61.2	99.0
+	+	–	+	–	5.5	96.0	106.0
+	+	–	–	+	4.7	131.7	102.0
+	+	+	+	–	17.3	2.5	41.9
+	+	+	–	+	7.4	37.2	95.0
+	+	+	+	+	21.8	11.9	25.0

[a] For details of enzyme purification, activation/deactivation and assays cf. legend to fig.1 and section 2

[b] DTT (0.2 mM) was used in the experiments with G6PDH and MDH and 0.05 mM DTT with FBPase

[c] Spinach leaves were homogenized in 50 mM Tris–HCl buffer at pH 7.5 (1:3, w/v). The slurry was centrifuged at 10000 × *g* for 10 min. The supernatant was placed in a boiling water bath for another 10 min, cooled quickly in ice and centrifuged at 10000 × *g* for 10 min. Ten-μl aliquots were used in the preincubation mixtures. Results similar to those described for spinach leaf extracts were also obtained with deproteinized extracts from *Anabaena*

gest that such complexes may well be formed in vivo:

(i) Iron is present in washed *Anabaena* cells in a form which is suitable for active complex formation. This was shown by the observation that after incubation of *Anabaena* crude extracts with EDTA, a substance could be isolated and purified from the slurry which: (a) had a chromatographic mobility identical with that of Fe^{3+}–EDTA (fig.3); (b) contained iron as the only major metal component (fig.4); (c) exhibited spectral properties in the visible range identical with those of Fe^{3+}–EDTA (absorption maximum at 254 nm; not shown) and (d) was active in oxidative enzyme modulation.

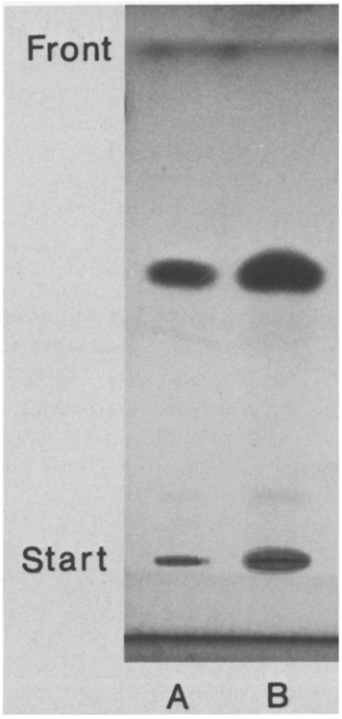

Fig.3. Silica gel chromatography of a redox substance purified from *Anabaena* crude extracts supplemented with EDTA (A) and of authentic Fe^{3+}–EDTA (B). EDTA (20 mM) was added to 10000 × *g* supernatants (25 mg protein·ml^{-1}) from *Anabaena* cells. After precipitation of alcohol-insoluble substances with 75% ethanol, the alcohol-soluble fraction was chromatographed on Sephadex G-10 (in 20 mM Tris–HCl buffer, pH 7.5), DE-52 cellulose (0–0.5 M NaCl gradient), and once again on Sephadex G-10 (in H_2O) columns. The 'active' fractions, able to mediate the oxidative modulation of enzymes by O_2, were established in each step by using the G6PDH reactivation assay described in section 3.1. The fractions, obtained in the third step, were pooled and 10-μl aliquots were subjected to thin-layer chromatography on silica gel. Solvent system: ethanol/H_2O/ethyl acetate/acetic acid (3:2:1:0.5, v/v), at pH 2.5–3.0. Running time: 2 h 30 min. The spots were cut out and eluted in H_2O. The eluted substance was active in the enzyme modulation assays. The negative of a photo taken in UV light is shown.

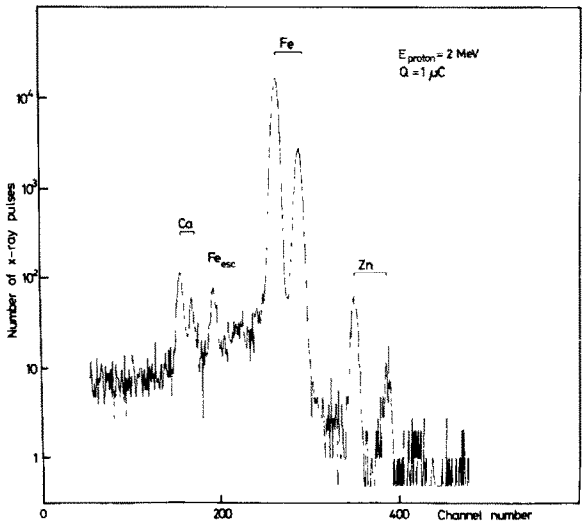

Fig.4. X-ray spectrum of the substance purified from EDTA-treated *Anabaena* cells (cf. fig.3). Excitation by 2 MeV protons of $Q = 1\,\mu C$ charge; measurement by Si(Li) detector (Canberra 7300). Ratio of the number of pulses Fe/Zn ~310.

(ii) Supernatants of boiled, crude extracts from spinach leaves or *Anabaena* cells tended to reactivate the partially purified, reductively deactivated cyanobacterial G6PDH and considerably deactivated the reductively activated FBPase. Both effects were substantially increased when, before the reactivation assay, Fe^{3+} was added to the boiled extracts (table 2).

4. DISCUSSION

A number of iron-containing compounds are involved in the redox processes of the cell. Some of them have specific functions in light-dependent enzyme modulation. Ferredoxins, which transfer electrons from the photosynthetic electron

transport chain via a ferredoxin-dependent thioredoxin reductase to thioredoxins [3], contain iron in the form of iron-sulfur clusters. Ferralterin, a recently discovered iron-containing protein, is able to link the photosynthetic electron transport directly to reductive enzyme modulation [26].

This paper is the first report on oxidative enzyme modulation via low-M_r iron complexes, i.e., via non-protein-bound iron. A remarkable feature of enzyme modulation via Fe^{3+}-chelates is that the reaction is powerful enough to alter enzyme activity, and, at the same time, mild enough not to destroy the enzyme. It is important to stress that (i) the changes in enzyme activities, evoked by reactions mediated by Fe^{3+}-chelates were found to be reversible; (ii) enzymes not subject to redox modulation were not affected by the iron complexes; (c) enzymes known to be activated by oxidants (e.g., G6PDH) were activated and those known to be deactivated by oxidation (e.g., FBPase, NADP-GAPDH, NADP-MDH) were deactivated by Fe^{3+}-chelates, when exposed to air. These properties are compatible with a physiological function of iron-chelates in oxidative enzyme modulation.

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