Metal regulation of metallothionein participation in redox reactions

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Abstract Like glutathione or dithiothreitol, metallothionein effects the formation of pancreatic ribonuclease A from its S-sulfonated derivative catalyzed by protein disulfide isomerase. EDTA increases the yield of ribonuclease A activity recovery with metallothionein but does not affect the reaction with glutathione or dithiothreitol. EDTA also increases the reactivity of thiol groups in metallothionein with 5,5′-dithiobis-(2-nitrobenzoic acid) by chelation of zinc ions. It is suggested that the thiol groups in metallothionein form a part of the pool of cellular thiols in the regulation of cellular redox reactions and their availability is modulated by zinc chelation.

Key words: Metallothionein; Redox reaction; Redox regulation; Protein disulfide isomerase; Zinc; Chelation

1. Introduction

Metallothionein (MT) is a protein ubiquitously distributed from single-cell organism to human tissues [1], and is unique among metalloproteins for its high content of cysteine residues constituting one third of its amino acid residues. The 20 cysteine residues bind seven zinc atoms and each metal atom binds four cysteine residues forming a tetrahedral structure [2]. MT binds zinc with a high stability constant of 10^{13} M^{-1} at pH 7 [3]. The ubiquitous occurrence and highly conserved structure suggest important roles of MT in living cells. It has been postulated that MT functions as a metal reservoir providing zinc where and when needed and as a scavenger of heavy metals such as cadmium and mercury, and of free hydroxyl radicals [2]. Recently, Maret and Vallee [3] proposed that the zinc-sulfur cluster of MT is sensitive to changes of the cellular redox state. They have provided evidence of the release and transfer of zinc from MT to zinc-depleted sorbitol dehydrogenase modulated by the glutathione disulfide (GSSG)/glutathione (GSH) couple, and suggested that a change of the redox state of the cell could serve as a driving force and a signal for zinc dissemination from MT [4]. Furthermore, groups of reagents have been identified to drive MT/Zn enzymes equilibrium in the direction of zinc transfer, among them the cellular redox state is a determining factor [3]. Glutathione mediates zinc transfer from Zn enzymes to thionein, whereas glutathione disulfide oxidizes MT with enhanced release and transfer of zinc to the respective apoenzymes in a thermodynamically unfavorable direction [5].

In this communication, the reverse process that zinc regulates MT contribution to redox reactions has been demonstrated. EDTA increases the formation of native ribonuclease A from its inactive S-sulfonated derivative catalyzed by protein disulfide isomerase (PDI) in the presence of MT instead of GSH or dithiothreitol (DTT) [6], suggesting metal regulation of the above reaction.

2. Materials and methods

2.1. Materials

GSH, GSSG and DTT were from Serva. Bovine pancreatic ribonuclease A (RNase A), Zincon and yeast RNA were from Sigma. Guanidine hydrochloride is from ICN and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) from Fluka. $Na_2S_4O_6$ was prepared according to Gilman [7]. Other materials were local products of analytical grade. Deionized water with resistivity of ≥18 MΩ/cm was obtained using a Milli-Q water system for buffer preparations, which were then passed through a Chelex-100 column (Sigma) to remove adventitious metal contaminants [5]. Unless otherwise specified, all solutions were flushed with nitrogen before use to reduce air oxidation.

2.2. Preparations

Zn_7-MT was prepared from rabbit liver according to the method described by Vasak [8] and Pan et al. [9]. To ensure that Zn is the only metal bound to MT, the preparation was first converted to its apoform with HCl in the presence of DTT and then reconstituted with Zn to result in Zn_7-MT [10]. It has 20 sulfhydryl groups as determined with DTNB [11] and seven zinc atoms per molecule determined by atomic absorption spectrophotometry, indicating good purity of the preparation [5,8]. PDI was prepared from bovine liver with a specific activity greater than 800 units/g and assayed as described by Lambert and Freedman [12].

S-sulfonated RNase A was prepared by incubation of 6 M guanidine hydrochloride denatured RNase A with 50-fold excess of Na_2SO_3 and $Na_2S_4O_6$ at 37°C for 1 h followed by thorough dialysis against 50 mM NH_4HCO_3 and lyophilization [6].

2.3. Formation of native RNase A from its S-sulfonated derivatives

Formation of native RNase A from its S-sulfonated derivative was carried out according to Yu [6]. S-sulfonated RNase A at 7.3 μM in 0.1 M phosphate buffer, pH 7.4, containing different concentrations of EDTA, was incubated with MT, DTT or GSH at different ratios of -SH to $-SSO_3$ in S-sulfonated RNase A, in the presence or absence of 1.3 μM PDI (calculated as the protomer), at 4°C until the final state was reached. The RNase A thus formed was assayed by measurement of the hydrolysis of yeast RNA [13].

Number of thiol groups of MT after incubation with different concentrations of EDTA at 4°C for 40 h and the rate of reaction with DTNB were determined by measurements in a stopped-flow apparatus (Applied Photophysics, SF.17 MV) [11,14].

2.4. Determinations

All protein concentrations were determined spectrophotometrically, at 220 nm with the molar extinction coefficient of $\varepsilon_{220} = 48\,200$ M^{-1} cm^{-1} for apo-MT [8], and at 280 nm with the following absorption coefficients ($A^{0.1\%}_{1cm}$): 0.9 for PDI [15], 0.695 for native RNase A [16],

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Abbreviations: MT, metallothionein; GSH, reduced glutathione; GSSG, oxidized glutathione; RNase A, ribonuclease A; PDI, protein disulfide isomerase; DTT, dithiothreitol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid)

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0.535 for S-sulfonated RNase A. The $A_{1cm}^{0.1\%}$ for S-sulfonated RNase A was checked by protein concentration determination [17] with bovine serum albumin as a standard.

3. Results

3.1. Effects of MT on the formation of RNase A from its S-sulfonated derivatives

As shown in Fig. 1, MT, like GSH or DTT, does effect the formation of RNase A from its S-sulfonated derivatives in the presence of PDI and 4 mM EDTA. The yield of native RNase A increases to a maximum of 60% with increasing molar ratios of -SH/-SSO_3 to 1.2–1.4 for all three reagents, that is 3.5–4.1 μM for MT, 70–82 μM for GSH and 35–41 μM for DTT. Further increase in concentration of the reductant decreases the activity recovery of RNase A, sharply for the strong reductant DTT, but only slightly for MT and GSH. Presumably, DTT at high concentrations prevents the formation of disulfide bonds of RNase A. It appears that because of the chelation of zinc by 4 mM EDTA, the cysteine thiol groups of MT are all made available for the reduction of some of the -SSO_3 groups of S-sulfonated RNase A to free thiols, which then react with other S-sulfonates to form native disulfides in the presence of PDI.

3.2. Effect of EDTA on the formation of native RNase A from its S-sulfonated derivatives by MT

In order to examine whether the reducing power of MT is mediated by the release of zinc, EDTA at different concentrations was used to chelate zinc ions to different extents. As shown in Fig. 2, both the rate and the extent of RNase A formation increase with the increase of EDTA concentrations so that at a fixed MT concentration, the curve of RNase A activity recovery with increasing EDTA concentrations shifts to the left. Fig. 3 compares the effects of EDTA on the formation of RNase A by MT and by GSH. EDTA at higher concentrations decreases the concentration of MT required for the maximal activity recovery (Fig. 3A), but has no effect on the formation of RNase A by GSH (Fig. 3B) and DTT (data not shown). The above indicates that removal of zinc from the zinc/sulfur clusters by EDTA results in the release of thiol groups of MT so as to participate in redox reactions.

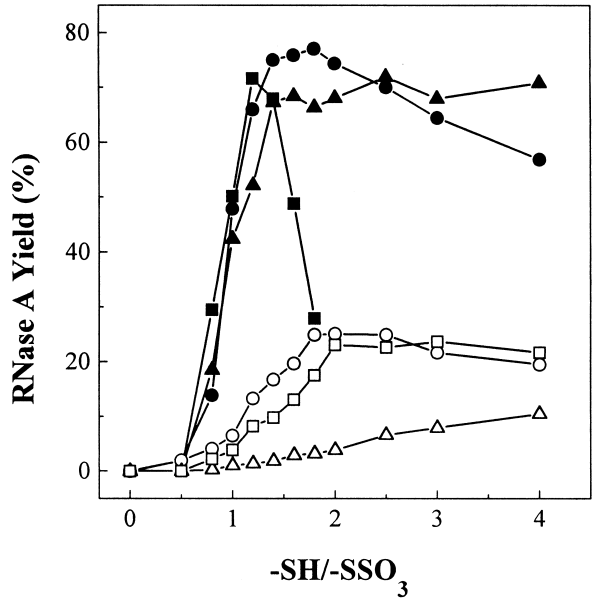

Fig. 1. Formation of native RNase A from its S-sulfonated derivative in the presence of different thiols. S-sulfonated RNase A at 7.3 μM was incubated with different concentrations of MT (●), GSH (▲), or DTT (■) as indicated in the presence (filled symbols) or absence (open symbols) of 1.3 μM PDI in 0.1 M phosphate buffer, pH 7.4, containing 4 mM EDTA. The activities of RNase A were then assayed after incubation at 4°C for 24 h.

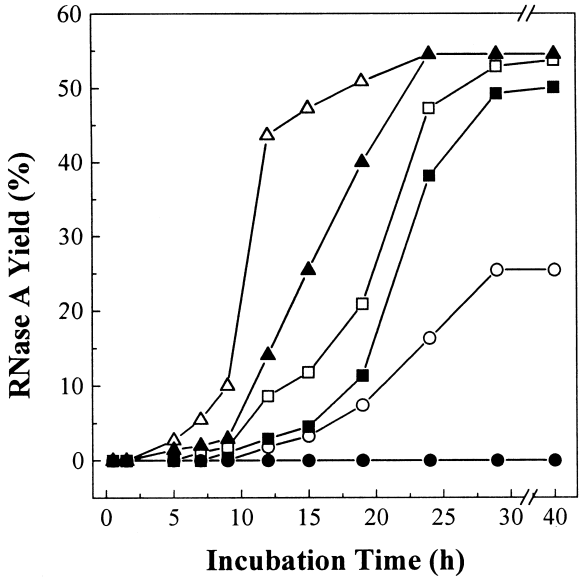

Fig. 2. Time courses of the formation of native RNase A from its S-sulfonated derivative by MT in the presence of different concentrations of EDTA. MT at 4.1 μM was incubated with 7.3 μM S-sulfonated RNase A (at a ratio of -SH/-SSO_3 of 1.4) and 1.3 μM PDI in 0.1 M phosphate buffer, pH 7.4, containing EDTA of 0 (●), 20 μM (○), 50 μM (■), 100 μM (□), 200 μM (▲) or 500 μM (△) at 4°C. Aliquots were taken for RNase A activity assay at different time intervals as indicated. The activity recovery of RNase A in the presence of PDI has been corrected for the activity from the corresponding reactions in its absence.

3.3. Effect of EDTA on the reactivity of thiol groups of MT

The suggestion that removal of zinc by EDTA from the zinc/sulfur clusters changes the reactivity of the thiol groups of MT has been directly inspected by following the kinetics of the reaction of MT toward DTNB with a stopped-flow spectrophotometer. As shown in Fig. 4, the thiol groups of MT fully coordinated with zinc are available for reaction with DTNB only slowly, taking over 20 min to reach completion (Fig. 4, curve 1). The rate increases with increasing EDTA concentration and at 30 μM EDTA, all thiol groups of MT at 4.1 μM react with DTNB instantly (Fig. 4, curve 4). The inset of Fig. 4 shows the initial phase of the reaction within the first 0.5 s. The course of the reaction with DTNB shows complicated kinetics suggesting that the multiple thiol groups may react with different rates. Curve fitting suggests at least four consecutive first order reactions. The rate constant for the first fast phase recorded in the first 0.5 s, $13.7 \pm 1.2\ s^{-1}$, is significantly greater than the pseudo-first order rate constants of $1.39 \pm 0.11\ s^{-1}$ and $4.15 \pm 0.07\ s^{-1}$ respectively for the reactions of GSH and DTT with DTNB (the reaction curves were not shown). From the amplitudes of the fast phase re-

action with DTNB, the percentages of free in total thiol groups in MT have been estimated to be 33.1 ± 6.6, 79.4 ± 2.0 and 95.4 ± 4.4 in the presence of EDTA at 10, 22 and 30 μM respectively.

4. Discussion

The formation of RNase A from its S-sulfonated derivative by MT in the presence of PDI and 4 mM EDTA indicates that the thiol groups of MT are available for reduction of the S-sulfonated groups followed by exchange reactions to form the native disulfides of RNase A catalyzed by PDI. But unlike the cases with GSH or DTT, both the yield and the rate of activity recovery of RNase A by MT increase with increasing concentration of EDTA, suggesting regulation by EDTA through the chelation of zinc ions in MT to make the thiol groups available for reaction. This is in complete accord with the reaction of MT with DTNB at different concentrations of EDTA, which increases the availability of thiol groups in MT by chelation of zinc. With EDTA in excess, the rate constant of the fastest reacting thiol groups of MT with DTNB is greater than that of GSH and even greater than that of DTT, showing the high reactivity of free thiol groups in MT after zinc removal by chelation with EDTA. The concentration of EDTA (30 μM) required for making almost all of the thiol groups fast reacting (28.7 μM zinc in 4.1 μM MT) is in accord with that required for the maximal formation of RNase A.

Although the potential action of thionein was considered to be physiologically essential [18,19], and the possibility that MT, by virtue of its high cysteine content, functions in sulfur metabolism or control of the intracellular redox potential has been mentioned before [20], no evidence has been presented so far. It has not been previously shown whether the release of zinc from MT controls the redox state of other thiol proteins, although the reactivity of thionein toward DTNB as a function of increasing concentration of zinc has been reported by Jacob et al. [5]. The present results in vitro demonstrated the novel idea for the modulation in cellular redox reactions by Zn-regulated availability of thiol groups in MT.

The concentration of GSH in the cell varies in the range of

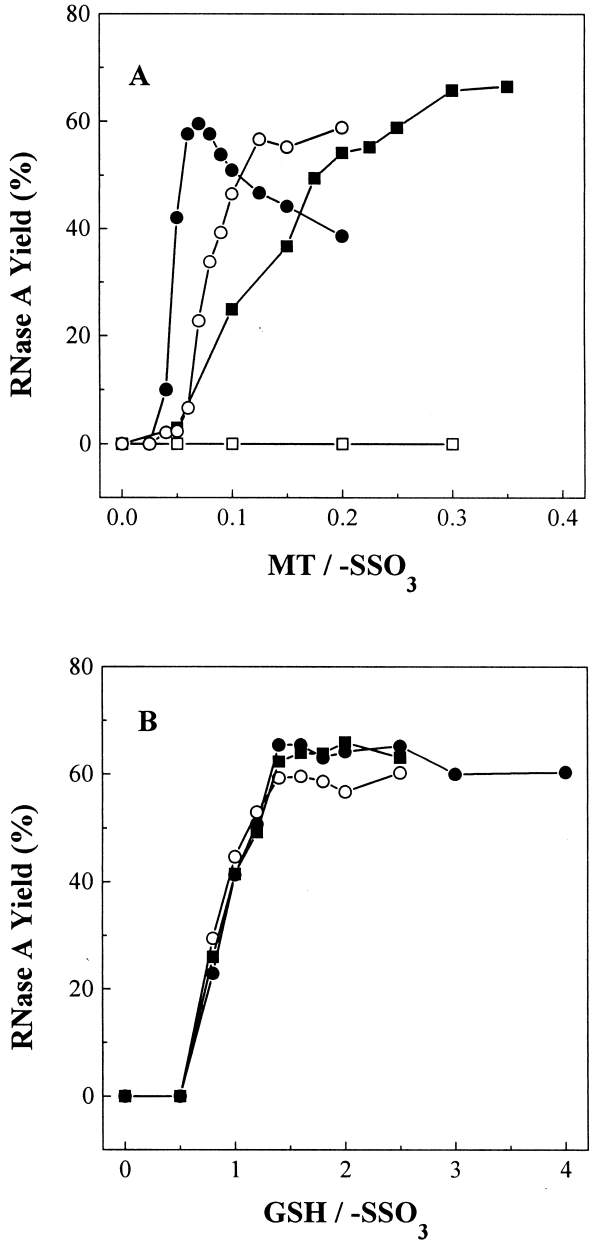

Fig. 3. Formation of native RNase A from its S-sulfonated derivative by MT or GSH at different concentrations of EDTA. The experimental conditions were as described in the legend to Fig. 1 except the reactions by MT (A) or GSH (B) were carried out in the presence of different concentrations of EDTA of 4 mM (●), 20 μM (○), 10 μM (■) or 0 μM (□).

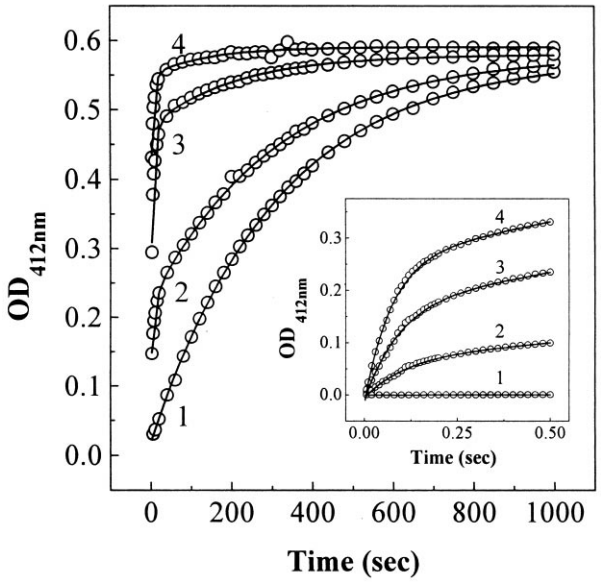

Fig. 4. Reactions between MT and DTNB in the presence of different concentrations of EDTA. MT of 4.1 μM was incubated with different concentrations of EDTA for 40 h at 4°C, and then mixed with 0.71 mM DTNB in the stopped-flow spectrophotometer with flushing N_2 to record the absorbance changes at 412 nm in 0.5 s and 1000 s. Each reaction curve was fitted with consecutive first order reactions. The amplitudes of the fast phases correspond to the reaction between free thiol groups and DTNB. The concentrations of EDTA were 0 μM, 10 μM, 22 μM and 30 μM for curves 1, 2, 3 and 4 respectively. The inset shows the initial phases of the reactions within 0.5 s. The open circles were from the experimental data and the curves are fittings for four phase first order reactions.

0.1–10 mM [4,21]. The redox ratio of GSH/GSSG in the cell under steady-state conditions has been determined to be 30:1 to 100:1 [22]. The concentration of thioredoxin in the cell has been estimated to be at the level of 6–7 μM in eukaryotic cells [23] and 40 μM in *Escherichia coli* [24]. The concentration of MT could change from 13 μM without induction [25,26] to 2.5 mM in response to stress [8]. Therefore, compared to the concentrations of GSH, GSSG and thioredoxin in the cell and considering the high reactivity of the free thiol groups in MT, it is reasonable to suggest that MT may make a significant contribution to the redox state in the cell.

Moreover, selenium compounds have been shown to be potential enhancers of zinc transfer from MT to apoenzymes [27]. Zinc-chelating agents, such as Tris, citrate or glutathione mediate zinc transfer from zinc enzymes to thionein [5]. It is possible that MT functions not only in zinc transfer modulated by redox reactions, but is also as a regulator of the cellular redox reactions by the reverse mechanism.

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