Thionein can serve as a reducing agent for the methionine sulfoxide reductases

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It has been generally accepted, primarily from studies on methionine sulfoxide reductase (Msr) A, that the biological reducing agent for the members of the Msr family is reduced thioredoxin (Trx), although high levels of DTT can be used as the reductant *in vitro*. Preliminary experiments using both human recombinant MsrB2 (hMsrB2) and MsrB3 (hMsrB3) showed that although DTT can function *in vitro* as the reducing agent, Trx works very poorly, prompting a more careful comparison of the ability of DTT and Trx to function as reducing agents with the various members of the Msr family. *Escherichia coli* MsrA and MsrB and bovine MsrA efficiently use either Trx or DTT as reducing agents. In contrast, hMsrB2 and hMsrB3 show <10% of the activity with Trx as compared with DTT, raising the possibility that, in animal cells, Trx may not be the direct hydrogen donor or that there may be a Trx-independent reducing system required for MsrB2 and MsrB3 activity. A heat-stable protein has been detected in bovine liver that, in the presence of EDTA, can support the Msr reaction in the absence of either Trx or DTT. This protein has been identified as a zinc-containing metallothionein (Zn-MT). The results indicate that thionein (T), which is formed when the zinc is removed from Zn-MT, can function as a reducing system for the Msr proteins because of its high content of cysteine residues and that Trx can reduce oxidized T.

metallothioneins | reducing systems

The methionine sulfoxide reductases (Msrs) are a family of proteins that reduce methionine sulfoxide [Met(o)] to methionine (Met) (1). The oxidation of Met to Met(o) results in the formation of two epimers of Met(o), called Met-S(o) and Met-R(o). In *Escherichia coli*, there are at least six members of this family that differ in their substrate specificity and location within the bacterial cell (2, 3). The protein that was first isolated and studied in greatest detail was *E. coli* MsrA (eMsrA) (4–8), which specifically reduces Met-S(o), whether in peptide linkage or as a free amino acid (8, 9). MsrB proteins specifically reduce the Met-R(o) in proteins but have very weak activity with free Met-R(o) (10, 11). In addition, in *E. coli*, there are specific Msr reductases that reduce free Met-S(o) and Met-R(o) (2) as well as membrane-associated activities that can reduce both epimers of Met(o), whether free or peptide-linked (3).

The situation in animal cells is somewhat different. It appears that there is one gene that codes for MsrA (12, 13) but three separate genes that code for MsrB proteins (14–16). MsrA localizes primarily in the mitochondria or cytoplasm (12, 13), whereas the three MsrB proteins have different subcellular localizations (16). MsrB1 (originally called Sel-x) is a selenoprotein that is primarily found in the cytoplasm and nucleus, MsrB2 (originally called CBS-1) is present mainly in the mitochondria, and MsrB3 is localized primarily in the endoplasmic reticulum and mitochondria. The biological reducing system for MsrA appears to be reduced thioredoxin (Trx) (4, 17), and Trx has also been assumed to be the biological reductant for the MsrB proteins. However, detailed studies comparing the reducing system requirement for the MsrA and MsrB proteins have

not been reported. Most *in vitro* studies have used DTT as the reducing agent for both MsrA and MsrB, because this agent works very well with the Msr family of proteins.

Recently, the human MsrB genes have been of interest to us as part of our studies on the role of the Msr system in protecting lens and retinal cells against oxidative damage (18–20). Using a colorimetric assay for Msr activity, based on the reduction of the individual epimers of 4-*N,N*-dimethylaminoazobenzene-4-sulfonyl (DABS)-Met(o), we were surprised to find that Trx serves very poorly as the reducing system for human recombinant MsrB2 (hMsrB2) and MsrB3 (hMsrB3). This result prompted us to examine in more detail the reducing requirement for different members of the Msr family and to search for a reducing system in mammalian cells that could function in place of Trx. A heat-stable factor in bovine liver was detected that, in the presence of EDTA, could serve as the reducing system for several of the Msr proteins. This factor was purified and identified as a zinc-containing metallothionein (Zn-MT). The results indicate that loss of zinc from Zn-MT after treatment with EDTA yields cysteine-rich thionein (T), which can provide the reducing power for the Msr enzymes. The possible significance of this finding relative to the redox state of the cell, oxidative damage, and the role of the Msr system will be discussed.

Results

Reduced Trx Is Not an Efficient Reducing Agent for hMsrB2 and hMsrB3. In the course of developing the DABS colorimetric assay for Msr activity (see *Materials and Methods*), it was confirmed that eMsrA and eMsrB could use either DTT or Trx to supply the reducing power, with similar or more activity observed with Trx *in vitro*. However, although both hMsrB2 and hMsrB3 could use DTT as the reducing agent, these proteins showed very little activity with Trx. Table 1 compares the activity of several recombinant Msr proteins using either DTT or Trx. It can be seen that eMsrA, bovine MsrA (bMsrA), and eMsrB are active with either DTT or Trx as the reducing system. In fact, eMsrB was much more active with Trx than with DTT. In contrast, hMsrB2 and hMsrB3 work very poorly with Trx, having <10% of the activity seen with DTT. One possibility was that the hMsrB proteins specifically required mammalian Trx and not the bacterial Trx that was used in these experiments. We therefore tested hMsrB3, as well as eMsrA and eMsrB, with mammalian Trx and mammalian Trx reductase. Reduced mammalian Trx, like the bacterial Trx, gave very poor activity with hMsrB3, but

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Abbreviations: Met(o), Met sulfoxide; Msr, Met sulfoxide reductase; Trx, thioredoxin; hMsr, human recombinant Msr; eMsr, *Escherichia coli* Msr; bMsr, bovine Msr; MT, metallothionein; Zn-MT, zinc-containing MT; DABS, 4-*N,N*-dimethylaminoazobenzene-4-sulfonyl; PAR, 4-(2-pyridylazo)resorcinol; T, thionein; T(o), oxidized T.

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Table 3. Trx stimulates the activity of MsrB3 in the presence of T(o)

No.	T(o)	Trx	NADPH	Trx reductase	DABS-Met, nmol
1	+	+	+	+	23.4
2	—	+	+	+	2.4
3	+	—	+	+	0.5
4	+	+	—	+	3.4
5	+	+	+	—	3.9

The incubations contained hMsrB3 (2.3 μ g) as described in *Materials and Methods*. Where indicated by plus sign, 8.3 nmol of T(o), 10 μ g of Trx, 2.4 μ g of Trx reductase, and 100 nmol of NADPH were added. In this experiment, with the amount of hMsrB3 used, 52.7 nmol of DABS-Met was formed in the presence of 15 mM DTT.

as shown in Table 3, T(o) could support hMsrB3 activity in the presence of the complete Trx reducing system (row 1) but not in the absence of Trx, Trx reductase, or NADPH (rows 3–5). As discussed previously, the Trx system alone showed very low activity (row 2). It is also apparent from the results in Table 3 that the free SH groups remaining in the T(o) cannot support the Msr reaction, indicating that the SH groups in T are not all equivalent with respect to their ability to function as a reducing agent for the Msr system. The results in Fig. 4 and Table 3 indicate that disulfide bonds in T(o) can be reduced by the Trx system. Thus, Trx may be one of the cellular agents that can enable T(o) to recycle and function as a metabolic reducing system.

In contrast to the results with hMsrB3, hMsrB2, which had low activity with either Trx or Zn-MT (see Table 2), was also not stimulated when both T(o) and the Trx reducing system were present (data not shown).

Discussion

Until the present studies, it has been assumed that Trx was the biological reducing system in cells for all of the Msr proteins. The initial experiments using eMsrA indicated that Trx was the biological reducing agent (4, 17), in agreement with earlier experiments (23). In those experiments, it was shown that Met(o) could support growth of a Met-requiring strain but not if the organism was also Trx deficient, indicating that Trx is necessary for the conversion of free Met(o) to Met in *E. coli*. The present experiments are in agreement with these earlier results. It appears that MsrA from both bacterial and mammalian sources utilizes Trx very efficiently, as does MsrB from *E. coli*. However, the studies reported here show that hMsrB2 and hMsrB3 (and, presumably, MsrB proteins from other mammalian sources) use Trx very poorly. As noted above, hMsrB1 was not tested in the present studies because of our inability to overexpress and purify the protein from *E. coli*, but Trx may work well with this protein. Recently, Kim and Gladyshev (22) postulated that, in MsrB1, a cysteine was required in addition to selenocysteine for Trx to function. In contrast, with MsrB2 and MsrB3, only the active-site cysteine was required, and Trx was thought to directly reduce the sulfenic acid intermediate on the enzyme (22). It seems clear from the low activity using Trx with both MsrB2 and MsrB3 that this reaction is not efficient, which raises the possibility that Trx may not be the direct biological reducing system for MsrB2 and MsrB3. The ability of a heated bovine liver extract to support Msr activity with hMsrB3 in the absence of an exogenous reducing system provides evidence that animal cells contain a factor that, in the presence of EDTA, can substitute for Trx in this reaction. The identification of Zn-MT as the active factor was based on the heat stability, purification characteristics, absorption spectra at neutral and acidic pH values, gel analysis, metal determination, and molecular weight analyses. The role of EDTA appears to be to release the zinc from the Zn-MT to form

T, the apoform of MT, which can function as a reducing agent because of its high content of cysteines. In support of this conclusion, it was shown that T, prepared by acid treatment (see *Materials and Methods*), could function as a reducing agent in the Msr system without EDTA. It is known that T is a small protein having ≈ 60 amino acids and a molecular mass in the range of 6–7 kDa. Of the total amino acids, approximately one-third are cysteines, which could make this protein an important cellular source of sulfhydryl groups. For many years, it was felt that MT's primary function was to scavenge free radicals and/or detoxify metals. However, in 1998, Maret and Vallee, in a seminal study (24), postulated that the zinc-sulfur clusters in MT also acted as a sensor for the redox state of the cell. Oxidation of Zn-MT resulted in release of the zinc so it could be mobilized within the cell, whereas under reducing conditions, T would efficiently bind zinc. Thus, the major role of MT may be to control cellular zinc mobilization as a function of the redox state of the cell. However, there does not appear to be much information on other possible functions of T, the reduced apoform of MT, in addition to its critical role in binding zinc. In one report similar to our studies, it was reported that Zn-MT in the presence of EDTA can reactivate the S-sulfonated (inactive) form of ribonuclease (25). These researchers also suggested that the thiol groups in T are part of the pool of cellular thiols that can regulate redox reactions in a mechanism that is modulated by zinc chelation. Our results on the ability of T to supply the reducing system for some of the Msr proteins support this conclusion and link the MT proteins to another cellular antioxidant system.

Although the results indicate that T can supply the reducing system for all of the Msr enzymes tested, with the exception of hMsrB2, it is clear that the Trx system is the preferred reducing system for MsrA and eMsrB. If there is an important reducing role of T, it is with hMsrB3. One of the unexplained findings in this study was the failure of T (or Zn-MT and EDTA) to stimulate hMsrB2. As mentioned, all of the other Msr proteins that were tested showed significant activity with Zn-MT in the presence of EDTA. Because MsrB2 and MsrB3 are both zinc proteins, they are thought to have similar reaction mechanisms (16, 22), which makes the lack of activity of T (the active agent) with hMsrB2 puzzling. One possibility is that different sulfhydryls on T react with hMsrB3 and hMsrB2. Thus, the active sulfhydryls in T that can interact with hMsrB3 cannot reduce the oxidized hMsrB2 intermediate. Because, in our hands, hMsrB2 shows only weak activity with the Trx system, we are uncertain as to what may be the normal reducing system for this enzyme.

Because both MT-1 and MT-2 gave similar results in supporting Msr activity in the presence of EDTA, we have assumed that T derived from other MTs, such as MT-3 (found in the brain and reported to have growth inhibitory activity) and MT-4 (26, 27), would behave in a similar fashion. The electrospray MS analysis did not show the presence of MT-3 in our samples. However, it is possible that slight structural differences in the MTs might be important, and it will be necessary to test the individual MT species for their ability to provide a reducing system for the Msr enzymes.

The *in vivo* significance of the present results is clearly not known. Certainly, there is no chelating agent, like EDTA, that is present in the cell to convert the MT to T. However, Yang *et al.* (28) have reported that as much as 50% of the total MT in mammalian tissues is present as T. Thus, the high concentration of T in tissues is consistent with a possible role of T as a cellular reducing agent, especially if there are mechanisms to regenerate T from T(o), as shown here with Trx. The heat step should have destroyed any T in our liver preparations, although, as shown in Fig. 1, there was a slight activity in the heated S-100 in the absence of EDTA that could have been due to T that was not destroyed by the heat step.

Fig. 5. Postulated role of Trx and MT in supplying the reducing requirement for the Msr enzymes.

Because oxidative stress is believed to release zinc and other metals from MT, one can postulate a reaction sequence, summarized in Fig. 5, in which cells, under oxidative stress, mobilize zinc from Zn-MT for use for the hundreds of zinc-containing proteins. The loss of the zinc from MT as a result of oxidation would yield T(o). As postulated in Fig. 5, T(o) can be reduced to T by the Trx system, and evidence for this reaction is shown in Table 3 and Fig. 4. T can serve as a cellular reducing agent and reduce the oxidized Msr intermediates, either an enzyme-bound disulfide or sulfenic acid (6, 7, 21, 22). At present, we do not know how many of the cysteines in T can function to reduce the oxidized Msr proteins, but we have evidence that more than one cysteine on T is functional. Trx may be only one of the possible cellular reducing systems that can reduce T(o). It is known that oxidized glutathione can oxidize MT and cause the release of Zn from Zn-MT and that reduced glutathione can reduce T(o), which can bind zinc. Of interest were the findings that certain selenium compounds, such as selenocystamine, can accelerate these reactions (29, 30). Preliminary experiments in our system have indicated that selenocystamine can be reduced to selenocystamine by T and that selenocystamine can supply the reducing system for both hMsrB2 and hMsrB3. Further studies are required to determine whether the interaction between the Msr system and T has physiological relevance, especially because both may play an important role in protecting cells against oxidative damage. In addition, the possibility should be considered that T may be playing an important role as a cellular reductant for other systems.

Materials and Methods

Met(o), DABS chloride (DABS-Cl), PAR, and other chemicals, including rabbit liver Zn-MT, were purchased from Sigma-Aldrich, unless specified otherwise. DABS-Met-S-(o) and DABS-Met-R-(o) were prepared by derivatizing the amino group of the Met-R-(o) or Met-S-(o) epimers (31) with DABS-Cl (32). Trx and Trx reductase (*E. coli*) were overexpressed and purified from *E. coli*, and human Trx was purchased from Sigma. Rat Trx reductase (TR3) was a generous gift from Vadim Gladyshev (University of Nebraska, Lincoln). Clones for

bMsrA, eMsrA, eMsrB, and hMsrB2, the latter generously provided by Todd Lowther (Wake Forest University School of Medicine, Winston-Salem, NC), were overexpressed in *E. coli*, and the respective proteins were purified as described in refs. 11, 33, and 34. The hMsrB3 cDNA from the human lens was amplified by PCR, cloned into a pET vector, and overexpressed in BL21 *E. coli* cells. The harvested cells were suspended in 1/100 volume of original culture by using 50 mM Tris (pH 7.4). After sonication and centrifugation at $10,000 \times g$, the supernatant was fractionated on a Sephadex G-75 column. Active fractions were combined, and protein purity (>80%) was confirmed by SDS/PAGE.

Purification of an Active Factor from Bovine Liver. Fresh bovine liver was homogenized in three volumes of 50 mM Tris (pH 7.4) and centrifuged at $10,000 \times g$ for 30 min and then at $100,000 \times g$ for 16 h (S-100). The S-100 fraction was heated at 80°C for 5 min and centrifuged to remove precipitated proteins (heated S-100). Once the active material was suspected to be a MT, further purification followed an established method for MT (35). Using a Bio-Rad DuoFlow HPLC system, the heated S-100 was placed on a sizing column (Superdex 75 HR 10/30) followed by DE-52 anion-exchange chromatography. The fractions were routinely monitored at 240 and 280 nm. As reported in ref. 35, two distinct peaks of activity were eluted from the DE-52 column that corresponded to Zn-MT-1 and Zn-MT-2, as described in *Results*.

Preparation of T and T(o) and Assay of T(o) Reduction by Trx. T and T(o) were prepared from Zn-MT by modification of the procedure described in ref. 36. Briefly, purified Zn-MT was dialyzed against 10 mM HCl (pH 2.0) containing 150 mM NaCl for 12 h at 4°C . The protein after dialysis is reduced, metal-free T and appears stable when left at pH 2.0. To study the activity of T in the Msr system, T was neutralized and added to the reaction mixtures immediately before the incubations were initiated. To oxidize T, 0.75 volumes of 50 mM Tris base were added to the T sample to bring the pH to 8.5. Under these conditions, $\approx 50\%$ of the sulfhydryls will become oxidized after 4 h at room temperature or 2 h at 37°C . With longer incubations, the T(o)

started to precipitate. The assay for free sulfhydryl groups used 5,5'-dithiobis(2-nitrobenzoic acid) as described in ref. 37.

To study the reduction of T(o) by Trx, the reaction mixtures contained (in a total volume of 1 ml) 100 μ M NADPH, 26 μ g of Trx, 6 μ g of TrxB, and 28 μ g of partially oxidized T (see above). The oxidation of NADPH was followed at 340 nm at room temperature.

Analysis of Zinc Content. Zinc was quantitatively determined in the MT preparations by using the PAR reagent (38, 39). The samples (100 μ l) were incubated with 10 mM *N*-ethylmaleimide for 1 h at room temperature. PAR (100 nmol) was then added, and the sample was diluted with water to 1 ml. The Zn-PAR complex was measured at 500 nm. Ten nanomoles of zinc gave a reading of 0.720 at 500 nm. A complete metals analysis of the purified MT preparation was performed by Joseph Caruso (University of Cincinnati, Cincinnati) by using an Agilent 7500 inductively coupled plasma mass spectrometer (Agilent Technologies, Palo Alto, CA). Molecular weight determinations of the purified protein were performed by Peter Yau (University of Illinois, Urbana-Champaign) by using electrospray MS.

Colorimetric Assay for Msr Activity Based on the Reduction of DABS-Met(o). The reaction mixture (200 μ l) to measure Msr activity contained 100 mM Tris-Cl (pH 7.4), 100 nmol of the indicated DABS-Met(o) epimer, either 15 mM DTT or the Trx regenerating system (10 μ g of Trx/2.4 μ g of Trx reductase/500 μ M NADPH), and Msr enzyme as indicated. When the liver fractions [Zn-MT, T, or T(o)] were tested, DTT was omitted, and the Trx reducing system and 5 mM EDTA were added where indicated. Incubations were for 60 min at 37°C unless noted

otherwise. In experiments using T(o), the incubations did not contain EDTA. The quantitation of DABS-Met formed used a slight modification of an extraction procedure previously described by Etienne *et al.* (40) for the reduction of sulindac to sulindac sulfide by MsrA. The reactions were stopped by the addition of 200 μ l of 1 M sodium acetate (pH 6.0), followed by the addition of 100 μ l of acetonitrile and 1 ml of benzene. After thorough shaking and centrifugation, the optical density of the benzene layer was read at 436 nm. One hundred nanomoles of the product, DABS-L-Met, carried through this procedure gave an optical density reading of 1.7, whereas 100 nmol of the substrate, either the R or S epimer of DABS-Met(o), read <0.04. Under these conditions, the reaction was proportional to Msr concentration until >75% of the substrate was reduced. Unless indicated otherwise, the results are presented as nmol of DABS-Met formed in 60 min. As little as 2 nmol of product could be measured. This assay could be used with purified preparations of MsrA and MsrB as well as crude extracts of mammalian tissues. Bacterial extracts, in the presence of a reducing system, destroyed the substrate, and further studies are needed to adapt the assay to bacterial extracts.

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