Role of thioltransferases on the modulation of rat liver S-adenosylmethionine synthetase activity by glutathione

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Abstract Rat liver S-adenosylmethionine synthetase, high- and low-M_r forms, are regulated in vitro by the GSH/GSSG ratio at pH 8. The inhibition and oxidation constants for both forms have been calculated in the presence of thioltransferases. The mechanism of the reaction appeared to involve the formation of intramolecular disulfides. Increases of 3- to 4-fold in the oxidation constants for both S-adenosylmethionine synthetase isoenzymes in the presence of protein disulfide isomerase suggested the possibility of a thiol-disulfide exchange regulatory mechanism for this enzyme in vivo. The significance of these results is discussed on the light of the data available relating glutathione changes and modulation of enzyme activities, either in vivo and in vitro.

Key words: S-adenosylmethionine synthetase; Glutathione; Oxidation constant; Thioltransferase

1. Introduction

Structure and function of most cysteine-containing proteins depend on the oxidation state of the protein sulfhydryl groups. Cellular redox buffers exert important effects on the oxidation–reduction state of these thiol groups by the process of thiol-disulfide exchange [1]. Among them, glutathione, the most important cellular thiol [2], exists in the reduced (GSH) and oxidized (GSSG) forms, and the relationships between these two states has been suggested to act either enhancing or diminishing the activities of a variety of biological processes such as enzyme catalysis, protein synthesis, and receptor binding [3–7]. Oxidation–reduction and thiol-disulfide exchange reactions from normal metabolism or toxicological perturbations can cause the redistribution between some or all glutathione forms, GSH, GSSG and mixed disulfides [8]. Activation or inactivation of several enzymes by alterations in the GSH/GSSG ratio have been shown to occur in vitro [4,9,10], among them rat liver AdoMet synthetase has been shown to be inhibited by GSSG and this effect modulated in the presence of GSH [11].

Rat liver AdoMet synthetase, high- (tetramer) and low-M_r (dimer) forms, present 10 sulfhydryl groups per subunit [12,13]. The importance of these groups on the structure and activity of the enzyme has been proven by several experiments. First, the use of site-directed mutagenesis allowed the production of cysteine mutants that showed the importance that Cys^{69} has in the oligomeric state [14]. Second, inhibition of the enzyme activity has been obtained in vitro using thiol modifying agents such as N-ethylmaleimide [15,16], *p*-chloromercuribenzoate [17], nitric oxide [18] and GSSG [11]. Third, the effect of nitric oxide has been shown to be reverted in the presence of reducing agents [18], while the inhibition with GSSG can be modulated in the presence of GSH [11]. Finally, in vivo experiments using buthionine sulfoximine (BSO)[19] or carbon tetrachloride (CCl_4) [20] also relate modulation of AdoMet synthetase activity with changes in glutathione levels. These data adquired more importance when considering that the product of AdoMet synthetase is the main methyl group donor for the transmethylation reactions, and the connection that exists between hepatic AdoMet metabolism and glutathione synthesis [21]. However, in vitro K_i and $R_{0.5}$ (ratio GSH/GSSG at which 50% of the effect in protein function is observed) values obtained for AdoMet synthetase and many of the systems tested until the moment are outside the range expected for the in vivo occurence of a thiol-disulfide exchange regulatory mechanism [11], and hence, either it has no in vivo significance or the presence of a catalyst is needed in order to modify these parameters, keeping the system under non-equilibrium conditions. Therefore, the objective of this work has been to determine if modifications in K_i and $R_{0.5}$ for AdoMet synthetase can be produced in the presence of two thioltransferases of different redox potentials but structurally related, PDI and Trx. The significance of the results obtained has been further discussed.

2. Materials and methods

2.1. Materials

Male Wistar rats (150 g) were from our own inbred colony. Glutathione, reduced and oxidized forms, phenylmethylsulfonyl fluoride, soybean tripsin inhibitor, benzamidine, methionine, adenosine 5′-triphosphate, dithiothreitol, 5,5′-dithio-bis-(2-nitrobenzoic acid), yeast ribonucleic acid type III, *E. coli* thioredoxin (2.04 U/mg) and the molecular weight standards for gel filtration chromatography were products of Sigma (St. Louis, MO). Ribonuclease A was from Boehringer Mannheim (Mannheim, Germany). DEAE-Sephacel, phenyl-Sepharose CL-4B, blue-Sepharose CL-6B, thiopropyl Sepharose 6B, CM-Sephadex C-50 and Sephadex G-25 were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). OptiPhase HiSafe 3 scintillation fluid and glass fiber filters were from E&G Wallac (Turku, Finland) and Whatman (Maidstone, UK), respectively. Biogel HTP, cation exchanger AG-50W-X4, Bio-Gel A 1.5 m, electrophoresis products and a protein assay kit were purchased from Bio-Rad (Hercules, CA). YM-30 ultrafiltration membranes and the Protein Pak 300 SW gel filtration column were obtained from AMICON (Beverly, MA) and Waters (Milford, MA), respectively. [2-^{3}H]ATP (21 Ci/mmol) and [^{35}S]GSH (50.9 Ci/mmol) were products of Amersham Corp. (Amersham, Bucks., UK) and DuPont New England Nuclear (Boston, MA), respectively. The rest of the buffers and reagents were the best quality commercially available.

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Abbreviations: GSH, glutathione reduced form; GSSG, glutathione oxidized form; AdoMet synthetase, S-adenosylmethionine synthetase; PDI, protein disulfide isomerase; Trx, thioredoxin; DTT, dithiothreitol; NO, nitric oxide

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Table 1
Protein disulfide isomerase and thioredoxin effects on rat liver AdoMet synthetase inhibition by GSSG

	K_i (mM)		
	Control	+PDI	+Trx
Low-M_r AdoMet synthetase	4.09 ± 0.17	2.46 ± 0.05	18.29 ± 0.06
High-M_r AdoMet synthetase	2.03 ± 0.16	0.93 ± 0.13	1.82 ± 0.66

Rat liver purified AdoMet synthetase dimeric and tetrameric forms were incubated in the presence of GSSG and either protein disulfide isomerase or thioredoxin as described under Section 2. The inhibition constants were calculated in the absence or presence of either PDI or thioredoxin. The table shows the results (± SD) of three independent experiments carried out in triplicate.

2.2. Purification of rat liver AdoMet synthetase, high- and low-M_r forms

AdoMet synthetase forms were purified from rat liver according to Pajares et al. [11], excluding the thiopropyl Sepharose 6B chromatography. This last step was omitted in order to avoid the presence of GSH in the protein preparations that could interfere with further experiments.

2.3. Determination of rat liver AdoMet synthetase activity

Measurements of enzyme activity were carried out as described by Cabrero et al. [22] in a final reaction volume of 250 μl, in the presence of 5 mM methionine and 5 mM [2-^{3}H]ATP (1 Ci/mol) for 30 min at 37°C. Whenever needed reducing agents were included or excluded from the reaction mixture.

2.4. Purification of rat liver protein disulfide isomerase

PDI was purified according to the method described by Carmichael et al. [23]. Briefly, rat liver cytosol was heated and fractionated using ammonium sulfate. The dialysate was subjected to chromatography on CM-Sephadex C-50, followed by ammonium sulfate precipitation and DEAE-Sephacel chromatography. The PDI preparations obtained showed an average activity of 3.5×10^{-3} ΔA/min/min.

2.5. Determination of protein disulfide isomerase activity

For this purpose the method of Freedman et al. [24] was used. Scrambled ribonuclease A was included as substrate and the PDI activity monitorized following the changes in A_{260}/A_{280}.

2.6. Effect of PDI or thioredoxin on the inhibition of AdoMet synthetase by GSSG

PDI or thioredoxin were preincubated with 4 mM GSH for 10–15 min at room temperature. The reduced proteins (10 μl) were then added to a reaction mixture containing either of the AdoMet synthetase forms (30 μg) and different concentrations of GSSG, ranging from 0 to 30 mM. AdoMet synthetase activity was then assayed as described by Pajares et al. [11]. The ratio AdoMet synthetase/PDI or Trx used was 50:1 (mol/mol).

2.7. Effect of PDI or thioredoxin on the modulation of AdoMet synthetase activity by the GSH/GSSG ratio

PDI or Trx were preincubated with GSH as described above. The assay was carried out in the presence of GSH/GSSG ratios ranging from 1 to 500 at a GSH concentration of 10 mM, using AdoMet synthetase/PDI or Trx ratios of 50:1 (mol/mol), as described by Pajares et al. [11].

2.8. Determination of protein concentration

The protein concentration of the samples was measured using the Bio-Rad assay kit based on the method of Bradford [25] and BSA as the standard.

2.9. Preparation of [^{35}S]GSSG

[^{35}S]GSSG was prepared from [^{35}S]GSH as described previously [11]. GSH levels were measured by the method of Hissin et al. [26].

2.10. Determination of the formation of mixed disulfides

For this purpose reaction mixtures were prepared as for the GSSG inhibition assays and the GSH/GSSG modulation of AdoMet synthetase, but including either [^{35}S]GSSG, [^{35}S]GSH/GSSG or GSH/[^{35}S]GSSG. The specific activity used was 1 μCi/nmol in each case. The reaction was carried out in a final volume of 160 μl for 30 min at 37°C. Samples were divided in two fractions of 100 and 60 μl each and the reaction stopped by addition of 1 ml of 10% TCA. One type of sample was spotted onto glass-fiber filters and after two washes with 10% TCA the filters were counted. The other type of sample was centrifuged and the supernatants discarded. Pellets were then prepared for electrophoresis as described by Laemmly [27]. Gels were fixed and dried before their autorradiography.

3. Results and discussion

Rat liver AdoMet synthetase, high- and low-M_r forms, has been shown to be inhibited in vitro by GSSG [11]. This effect was modulated in the presence of GSH and this thiol-disulfide exchange reaction led to the formation of intramolecular disulfides [11]. Unfortunately, the K_i and $R_{0.5}$ values calculated for both enzyme forms lie outside the normal cellular ranges determined by several authors (micromolar concentrations of GSSG and GSH/GSSG ratios of 300)[28,29]. In fact, it has been shown that GSSG concentration would only reach millimolar levels under severe oxidative stress [30], or in compartments such as the endoplasmic reticulum [31]. The same is true for GSH/GSSG ratios about 1–2, that are in the range for the calculated $R_{0.5}$ for AdoMet synthetase isoenzymes. On the light of these data the nonenzymatic formation of any type of disulfides seemed quite unlikely. Experiments under mild conditions indicated a possible significance for a thiol-disulfide exchange mechanims for the regulation of AdoMet synthetase in vivo [19,20]. Therefore, the possible involvement of a catalyst to place these K_i and $R_{0.5}$ values under limits of physiological importance has been explored. In order to solve

Table 2
Protein disulfide isomerase and thioredoxin effects on the modulation of rat liver AdoMet synthetase activity by the GSH/GSSG ratio

	$R_{0.5}$		
	Control	+PDI	+Trx
Low-M_r AdoMet synthetase	2.86 ± 0.25	8.81 ± 0.2	1.21 ± 0.12
High-M_r AdoMet synthetase	1.39 ± 0.24	5.75 ± 0.02	1.20 ± 0.1

Purified rat liver AdoMet synthetase tetramer and dimer forms were incubated in the presence of several GSH/GSSG ratios in the absence or presence of either protein disulfide isomerase or thioredoxin.
$R_{0.5}$ values were calculated and the assays were carried out as described under Section 2.
The values shown in the table were obtained from three independent experiments carried out in triplicate.

Table 3
Calculation of rat liver AdoMet synthetase oxidation constant (K_{ox})

	K_{ox} (mM)		
	Control	+PDI	+Trx
Low-M_r AdoMet synthetase	28.6	88.1	12.1
High-M_r AdoMet synthetase	13.9	57.5	12.02

Calculations were based on the production of intramolecular disulfides in both AdoMet synthetase forms, tetramer and dimer. Thus, $K_{ox} = R$ [GSH]. The $R_{0.5}$ values used for the determination of K_{ox} were those of Table 2 and the GSH concentration was 10 mM.

this question two structurally related proteins known to catalyze thiol-disulfide exchange reactions in vitro were tested, protein disulfide isomerase (PDI) and thioredoxin (Trx) [32,33].

PDI and Trx have similar active site domains, but they differ in the number of such domains per subunit (two for PDI and one for Trx), in their in vivo localization (PDI is located in the endoplasmic reticulum and Trx is cytoplasmic), in their M_r and oligomeric state (PDI is a dimer of 57 kDa subunits, and Trx a monomer about 12 kDa), and also in their oxidation–reduction potential (PDI has a more oxidizing character (K_{ox} of 42–60 μM), while Trx shows more reducing potential (K_{ox} of 10–16 M)) [34–38]. For our purposes, several experimental conditions were tested and, finally, preincubation of PDI or Trx with GSH was found necessary for any effect to be detected. Moreover, several ratios AdoMet synthetase/PDI or Trx were also used. Ratios between 10:1 and 50:1 (mol/mol) were found to give the best results (i.e., 3- to 4-fold changes in K_{ox} for PDI), and hence, in order for our studies to have catalytical significance the lowest ratio was used for further experiments. Moreover, the total glutathione concentration was high enough when compared to the PDI concentration as to account for the redox potential of the mixture.

Experiments performed in the presence of PDI or Trx did not modify the extent of inhibition produced by GSSG (data not shown). However, changes in the K_i values were observed in the presence of either of the thioltransferases. PDI was able to reduce these values about 2-fold, while Trx effects were different depending on the AdoMet synthetase isoenzyme used (Table 1). Such contradictory results may be explained on the light of the differences in the accessibility to sulfhydryl groups in the two AdoMet synthetase oligomeric forms for thioltransferases that differ in size. In addition, differences in the behavior of these two related proteins have been observed already in the reduction of small compounds, such as dehydroascorbate [39,40].

Modulation of the GSSG inhibition by GSH was also obtained in the presence of either PDI or Trx. Again opposite effects were obtained. PDI increased the $R_{0.5}$ for both AdoMet synthetase forms 3- to 4-fold, while Trx decreased this ratio about 2-fold in both cases (Table 2). To the best of our knowledge no former description of such changes has been made in this and other systems. In fact, only accelerations in the thiol-disulfide exchange reactions have been previously reported [41,42].

The mechanism of the reaction was also explored using [^{35}S]GSH or [^{35}S]GSSG in order to determine if mixed disulfides were produced. This type of disulfides have been mostly observed under oxidative stress, corresponding to K_{mix} of approximately 1 [43]. However, K_{mix} as high as 27 have been reported [44], and hence this possibility should be tested. For this purpose apropriate mixtures containing different ratios GSH/GSSG and including either of the labelled compounds were used. No incorporation of radiactivity was obtained in either of the AdoMet synthetase forms (data not shown), suggesting that the oxidation reaction involved the formation of intramolecular disulfides. This process would be of the type:

$$P(SH)_2 + GSSG \rightleftarrows P(SS) + 2\ GSH$$

Therefore, the equilibrium constant for this type of reaction would be:

$$K_{eq} = \frac{[P(SS)][GSH]^2}{[P(SH)_2][GSSG]}$$

and hence, $K_{ox} = R$ [GSH] [33]. Using this expression it is possible to calculate estimated values of the K_{ox} for both, high- and low-M_r AdoMet synthetases, at the GSH concentration used in the assay (10 mM), and making the assumption of the equivalent reactivity of all the protein sulfhydryl groups (Table 3). The values obtained for the controls and Trx containing experiments were similar to those described in the literature for other in vitro thiol-disulfide regulated en-

Table 4
Calculation of the fraction of reduced AdoMet synthetase (f_{red}), for tetrameric and dimeric forms

	f_{red} (%)			
	Normal	Fasted	MOS	SOS
Control				
Low-M_r AdoMet synthetase	99.14	96.92	91.29	9.4
High-M_r AdoMet synthetase	99.58	98.47	95.57	17.75
+PDI				
Low-M_r AdoMet synthetase	97.39	91.08	77.29	3.29
High-M_r AdoMet synthetase	98.28	93.99	83.91	4.96
+Trx				
Low-M_r AdoMet synthetase	99.63	98.67	96.12	19.86
High-M_r AdoMet synthetase	99.6	98.68	96.14	19.97

The calculations were made assuming a mechanism for this oxidation that involved the formation of intramolecular disulfides, and thus: $\% f_{red} = (R\ [GSH]\ 100)/(K_{ox} + R\ [GSH])$. The cellular R [GSH] used were as follows: normal, 3300 mM; fasted, 900 mM; mild oxidative stress (MOS), 300 mM; and severe oxidative stress (SOS), 3 mM [46].
Oxidation constant values were those shown in Table 3.

zymes, such as in [1]. Equilibrium constants for thiol-disulfide exchange reactions depend on the differences on stability between the two disulfides and two thiols involved [28]. The higher the values of K_{ox} the more stable intramolecular disulfides will be [45]. Changes in the glutathione status would be expected to vary significantly the oxidation state of the protein only if the oxidation potential of the protein falls within the R [GSH] range maintained by the cellular redox buffer [28]. In our case, only the estimates of K_{ox} obtained in the presence of PDI are close to R [GSH] values determined in vivo under mild to severe oxidative stress [46,4], thus giving some physiological importance to the data.

Assuming that the only active AdoMet synthetase species are those in the reduced state it is possible to calculate the percentage of these protein fraction (f_{red}).

$$\% f_{red} = \frac{R\,[GSH]\,100}{K_{ox} + R\,[GSH]}$$

Hepatic GSH concentration values and GSH/GSSG ratios have been obtained by several authors under diverse conditions. Normal, fasted, mild and severe oxidative stress have been evaluated and, the R [GSH] values published were 3300, 900, 300 and 3 mM, respectively [46]. Using these data and the calculated K_{ox} for high- and low-M_r AdoMet synthetases presented in Table 3, the f_{red} percentage under each condition has been deduced (Table 4). In all the cases a strong decrease in the f_{red} percentage is observed under severe oxidative stress, but in the presence of PDI a reduction is already calculated under mild oxidative stress. In fact, the data in the presence of PDI indicate the presence of about a 20% of oxidated AdoMet synthetase under mild stress conditions, while under severe stress 95% of both enzyme forms would be in the oxidated-inactive state. Similar effects have only been suggested from the in vitro data for hydroxymethylglutaryl coenzyme A reductase [3]. Such a result was suggested by the in vivo experiments using BSO [19] and carbon tetrachloride [20], where mild reductions in the GSH concentration (about a 30% decrease) led to a 60% diminution in total AdoMet synthetase activity. Even when these in vivo measurements included the contribution of both, high- and low-M_r AdoMet synthetases, it would be possible to infer an estimate of the K_{ox} value, using the equation for f_{red}, and assuming again that only reduced species are active. This value would be around 450 mM, still about 5-fold higher than that observed in vitro in the presence of PDI (Table 3). Analysis of these data cannot forget the differences produced in proteins upon isolation, as well as the cellular location and origin of the thioltransferases available for these in vitro studies. Altogether may account for most of the differences observed when comparing in vitro and in vivo results, leading to under- or overestimations of the K_{ox} values. Finally, the data presented in this paper suggested that it is possible to regulate rat liver AdoMet synthetase by a thiol-disulfide exchange mechanism involving a PDI-like thioltransferase that remains to be identified.

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