ACTIVATION OF PORCINE HEART MITOCHONDRIAL MALATE DEHYDROGENASE BY ZERO VALENCE SULFUR AND RHODANESE

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Incubation of malate dehydrogenase with thiosulfate and rhodanese lead to an increase of dehydrogenasic activity. Selenosulfate, elemental sulfur and elemental selenium were shown similarly able to activate this protein. The activation is limited to the presence of SH groups on the protein. Experiments with ^{35}S demonstrated the direct transfer of zero valence sulfur from rhodanese to malate dehydrogenase. It is proposed that this activation could be a mechanism of enzyme activity modulation in vivo.

Since the discovery of the "labile" sulfur into non-heme iron proteins, a number of zero valence sulfur bearing proteins have been described. This zero valence sulfur is often present into proteins as persulfide group (R-S-SH). Among this group of proteins rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) is a very interesting one in view of its ability to carry sulfur at zero oxidation state from thiosulfate to an appropriate nucleophilic acceptor. It was previously suggested (1) that this enzyme may partecipate to the intramitochondrial biosynthesis of non-heme iron proteins. Recently rhadanese was also shown able to reactivate a mitochondrial protein, namely succinate dehydrogenase (2).

In the present paper we report the activation of mitochondrial malate dehydrogenase (MDH) by zero valence sulfur mediated by rhodanese.

MATERIALS AND METHODS Potassium sulfite was purchased from Fisher (Springfield, N.J., U.S.A.). Other chemicals were from Merck (Darmstadt, Germany). NADH was obtained from Boehringer (Mannheim, Germany). Sodium thiosulfate labeled only in the outer sulfur was purchased from the Radiochemical Center (Amersham, U.K.). Its specific activity was 1.3 Ci M^{-1}.

Colloidal sulfur and selenium were prepared by the methods of Scandurra et al. (3, 4). Selenosulfate was prepared according to Klebanov and Ostapkevich (5). Since diluted aqueous solutions of this compound are stable only in strongly alkaline medium or in the presence of sul-

fite the latter was always present in our experiments in the stoichiometric ratio of 1:2.5 with respect to selenosulfate.

Porcine heart malate dehydrogenase (mitochondrial) was either from commercial source (Boehringer) or purified as a by product of mitochondrial aspartate amino transferase (6). After the CM-Sephadex chromatography (step n°3 in ref. 6) the MDH is first precipitated with 80% ammonium sulfate and dialyzed against water then against 10 mM 2-amino, 2-methyl 1,3 propandiol-HCl buffer pH 8.5. The protein solution is poured on a QAE-cellulose column equilibrated with the same buffer. MDH does not bind to the column and is eluted with the front of the solvent. Both commercial and purified MDH showed the presence of two components with different molecular weight. These two species were separated by gel filtration on a Sephadex G-200 column equilibrated with 0.01 M Tris-HCl buffer pH 7.4 containing 0.2 M NaCl and 1 mM mercaptoethanol. Both species showed MDH activity though at different extent as previously described by Consiglio et al. (7). MDH activity was followed spectrophotometrically (8).

Rhodanese was purified from beef kidney according to Cannella et al. (9). Its activity was determined by the method of Sorbo (10). Sulfur-free rhodanese was prepared by treatment of the protein with excess cyanide followed by precipitation with ammonium sulfate. The enzyme was then dissolved in a small volume of 0.05 M sodium acetate. Labeled Rho-

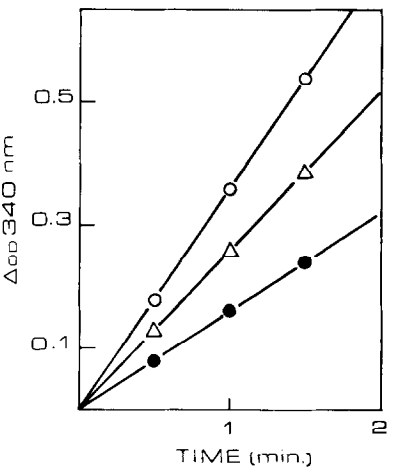

Figure 1

Activation of MDH by thiosulfate and rhodanese

●—● MDH (10^{-7} M) alone.

△—△ MDH (10^{-7} M) preincubated with 10^{-4} M $Na_2S_2O_3$

○—○ MDH (10^{-7} M) preincubated with 10^{-4} M $Na_2S_2O_3$ and 10^{-7} M rhodanese

For further experimental details see text.

danese was prepared by adding to the above solution a 30-fold molar excess of ^{35}S-sodium thiosulfate. The enzyme was allowed to crystallize by slow addition of 3.6 M ammonium sulfate previously adjusted to pH 7.9 with 1 M ammonia. The crystals were centrifuged and washed with 1.8 M ammonium sulfate at the same pH as above. Then they were dissolved in 0.01 M phosphate buffer pH 7.6 and filtered through a Sephadex G-50 fine column (1 x 35 cm) to remove excess thiosulfate.

Optical readings were done with a Beckman DB-GT spectrophotometer equipped with a W&W strip recorder mod. 1100.

Standard reactivation experiments were conducted as follows. MDH ($2-6 \times 10^{-7}$ M) in 0.1 M K_2HPO_4 containing 10^{-4} M EDTA was incubated with thiosulfate ($10^{-3}-10^{-6}$ M) in the presence or absence of rhodanese ($10^{-7} - 10^{-6}$ M). After a variable incubation time (5' to hours) a small aliquot (5-10 µl) of the incubation mixture was assayed for MDH activity.

TABLE I

Activation of MDH by Thiosulfate and Selenosulfate
(For experimental details see text)

	Additions		% Activity		
	Activator	Rhodanese			
	none	-		100	
	thiosulfate (10^{-3}M)	-		155	
	thiosulfate (10^{-3}M)	+		194	
	none	-	100	100	
	thiosulfate (10^{-4}M)	-	162	- -	
	thiosulfate (10^{-4}M)	+	225	161	
*	none	-		100	
*	thiosulfate (10^{-4}M)	-		150	
*	thiosulfate (10^{-4}M)	+		183	
	none	-	100	100	100
	thiosulfate (10^{-6}M)	-	92	100	136
	thiosulfate (10^{-6}M)	+	146	148	150
*	none	-		100	
*	thiosulfate (10^{-6}M)	-		120	
*	thiosulfate (10^{-6}M)	+		140	
	none	-	100	100	
	selenosulfate (10^{-3}M)	-	154	107	
	selenosulfate (10^{-3}M)	+	245	130	
	none	-		100	
	selenosulfate (10^{-5}M)	-		145	
	selenosulfate (10^{-5}M)	+		145	

* Experiments done with MDH purified on Sephadex G-200 (light component)

RESULTS

Activation of MDH by thiosulfate in the presence of rhodanese. The specific activity of MDH increases after incubation of the enzyme in the presence of 10^{-6} to 10^{-3} M thiosulfate and rhodanese (see fig. 1 and table I). In some conditions the activity is increased by thiosulfate alone, but always less than in the presence of rhodanese. The extent of this activation depends on the specific activity of MDH (see below). The concentration of thiosulfate used has a limited influence while the time of incubation show no influence in the present experimental conditions. It should be pointed out that the minimum effective concentration of thiosulfate and rhodanese are in the same range of MDH concentration. Selenosulfate was found even more active than thiosulfate in activating MDH. Both components isolated from the Sephadex G-200 column showed the ability to be reactivated. Control experiments demonstrated that rhodanese alone has no NADH oxidase activity nor MDH activating ability.

Controlled aging of MDH. MDH in solution loses activity with time also when stored at 4 °C. The protein tends to aggregate and to precipitate in a relatively short time (few days). The aging of MDH solutions were studied at low protein concentration in 0.1 M phosphate buffer pH 7.5 in the presence and absence of CN^-. The loss of enzymatic activity is much faster in the presence of cyanide. A decrease in the reactivation ability parallels the loss of enzymatic activity. Below a given activity MDH is not more activated by thiosulfate. It is possible to reconstitute, at least partially, activity of aged protein samples by addition of 10^{-4} M dithiotreitol followed by dialysis against buffer containing 10^{-4} M EDTA.

Activation of MDH by elemental sulfur or selenium. MDH was incubated in the presence of colloidal sulfur or selenium and rhodanese. In this case also an increase of specific activity was seen which is higher when rhodanese is present in the incubation mixture (Table II).

Transfer of ^{35}S from rhodanese to MDH. In order to demonstrate the direct interaction between MDH and rhodanese the transfer of radioactive sulfur from the latter protein to the former was studied. Crystalline ^{35}S-rhodanese (80 nanomoles) were incubated for 30' at room temperature with MDH (70 nanomoles) which had been previously tested for the

TABLE II

Activation of MDH by elemental sulfur or selenium
(For experimental details see text)

Additions			
	Activator	Rhodanese	% Activity
	none	-	100
	colloidal S ($2\ 10^{-4}$)	-	171
	colloidal S ($2\ 10^{-4}$)	+	171
	none	-	100
	colloidal S (10^{-4})	-	35
	colloidal S (10^{-4})	+	127
	none	-	100
	colloidal S ($5\ 10^{-5}$)	-	135
	colloidal S ($5\ 10^{-5}$)	+	174
	none	-	100
	colloidal S ($2\ 10^{-5}$)	-	118
	colloidal S ($2\ 10^{-5}$)	+	136
	none	-	100
	colloidal Se (10^{-5})	-	160
	colloidal Se (10^{-5})	+	206
	none	-	100
	colloidal Se (10^{-5})	-	200
	colloidal Se (10^{-5})	+	200
*	none	-	100
*	colloidal Se (10^{-5})	-	44
*	colloidal Se (10^{-5})	+	145

* Experiment done with MDH purified on Sephadex G-200 (light component)

ability of activation by thiosulfate-rhodanese. Then the mixture was chromatografed on a column of Sephadex G-75 (1 x 35 cm). Sufficiently small fractions were collected and the enzyme activities and radioactivity of each tube were immediately tested. The results are reported in fig. 2. The two enzyme activities are well separed due to the difference in molecular weight of the two proteins. The radioactivity/volume profile clearly shows that a certain amount of isotopic sulfur has been transferred from rhodanese to MDH.

DISCUSSION The physiological role of some otherwise well known protein is very obscure. The problem is more interesting when the protein under investigation is widely distributed among the living beings. One of

these proteins is certainly rhodanese which is present in relatively large amounts in plants (11) and animal tissues (12). This protein is found in the mitochondrial matrix (13). In vitro it carries zero valence sulfur from a donor (thiosulfate) to a nucleophilic acceptor like cyanide. It was previously suggested that the natural acceptors could be the intramitochondrial labile sulfur bearing proteins (1) which need it to bild up their prosthetic group. A further possibility is that some proteins may change their physiological activity when they bind labile sulfur. The activities of 1,-6-fructose bisphosphatase (14) and of pepsin (15) are increased by formation of mixed disulfides with one of their -SH residues. The chemical ground of this possibility is the presence of a large number of essential -SH residues in mitochondrial proteins. In the present work mitochondrial pig heart malate dehydrogenase was chosen in view of its availability as a pure isolated protein. It has a number of essential -SH groups which make the protein very sensitive toward heavy metals or -SH reagents.

Freshly prepared solutions of pure mitochondrial MDH show an increase in specific activity when they are incubated in the presence of

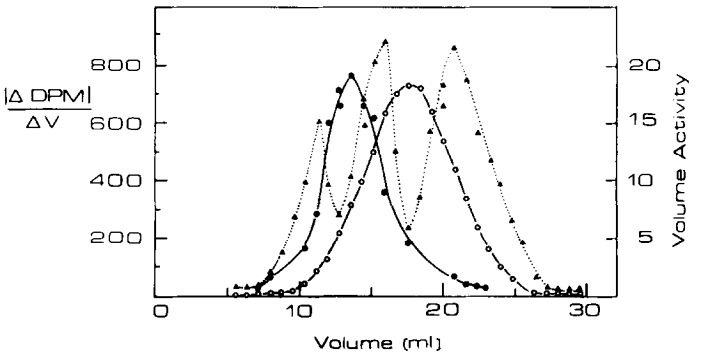

Figure 2

Gel filtration of MDH and ^{35}S-rhodanese

Solid circles: MDH activity, open circles: rhodanese activity, solid triangles: radioactivity pattern reported as the first-derivative curve, 0.80 ml having been used as the increment in volume.
MDH (70 nanomoles) was incubated 1 hour at room temperature with rhodanese (80 nanomoles); the mixture was then chromatographed on a column (1 x 35 cm) of Sephadex G-75. Fractions of 0.8 ml were collected and in each the enzyme activities and radioactivity were assayed.

thiosulfate and rhodanese (fig. 1). It appears that the activation is due to the binding of zero valence sulfur on malate dehydrogenase. In fact a comparable effect is obtained when MDH is incubated in the presence of colloidal sulfur instead of thiosulfate. Selenosulfate and colloidal selenium, which are known to bind rhodanese much like sulfur (16), also activate MDH (see Table II). This activation is in fact related to the -SH groups of MDH. Aging of this protein is reflected by loss of activity and of ability to be activated in the presence of thiosulfate-rhodanese. Incubation with cyanide speeds up the aging of protein samples. The involvement of -SH groups in the aging process is also suggested by the possibility of MDH reactivation by dithiothreitol.

A more conclusive evidence for the involvement of zero valence sulfur in the activation mechanism comes from the experiment with labeled rhodanese. After incubating ^{35}S-rhodanese with MDH it was found that a significant amount of radioactivity was eluted from the Sephadex column with the MDH activity (fig. 2). This indicate that zero valence sulfur has been transferred from rhodanese to MDH. Little can be said at the moment on the nature of binding of this sulfur on MDH, but the mandatory presence of free -SH groups for the reactivation seems to indicate the formation of persulfide groups. It is interesting to note that thiosulfate is available *in vivo* at the concentrations required *in vitro* for activation of MDH (17). Thus the activity of this enzyme and maybe others could be regulated inside the cell via rhodanese in the presence of inorganic ions.

In this context it should be recalled the dramatic effect exherted by selenium on mitochondria (13) which in some way could be related with this activation phaenomena.

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