THE ROLE OF SULPHYDRYL GROUPS IN α-GLYCEROPHOSPHATE DEHYDROGENASE (L-GLYCEROL-3-PHOSPHATE: NAD OXIDOREDUCTASE 1.1.1.8) ACTIVITY

By

MARIANNA TELEGDI and T. KELETI

INSTITUTE OF BIOCHEMISTRY, HUNGARIAN ACADEMY OF SCIENCES, BUDAPEST

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The α-glycerophosphate dehydrogenase isolated from rabbit muscle contains 9 to 11 SH-groups per protein molecule. Blocking the SH-groups with PCMB the reaction catalyzed by the enzyme is inhibited in both directions. Blocking of 6 to 7 SH-groups is required for complete inactivation. The inhibition of enzymatic activity is due to the blocking of SH-groups, it is instantaneous and can be reverted by cysteine. This process is followed by a time-consuming reaction, the alteration of the steric structure, revertible by cysteine only partly and resulting in a decreased ability to be reactivated. Under certain circumstances the enzyme is undergoing spontaneous inactivation, accompanied by the disappearance of some of its free, titratable SH-groups. This inactivation cannot be reverted by cysteine.

The role of the SH-groups of α-glycerophosphate dehydrogenase in enzymatic catalysis has been studied by several authors [1, 2, 3, 4].

By the use of our new method [5] for the isolation of the enzyme in crystalline form, an enzyme preparation with a specific activity about 8 times higher than that of the best ones described in the literature [1, 6] can be produced. It was therefore reasonable to examine the role of SH-groups in the activity in this highly active enzyme preparation.

Methods

The enzyme was prepared by our method [5] from rabbit skeletal muscle. Enzyme preparations of maximum activity were used.

The number of SH-groups was determined by the spectrophotometric method of BOYER [7]. The PCMB preparation used was of 94 per cent purity, as determined from the molar extinction coefficient of the solution [7].

Enzymatic activity was assayed by the optical test of *Warburg*, using a reaction mixture already described [5]. As ingredients, α-glycerophosphate (Rhône-Poulenc), dihydroxyacetone phosphate (Sigma), NAD^+ and NADH (Boehringer) were used. The measurements were carried out in a *Hilger (Uvispek)* spectrophotometer.

The tryptic digestibility of the protein was controlled by the method of SZABOLCSI and SZÖRÉNYI [8], in 0.1 M phosphate buffer, pH 7.0, at room temperature, using 1×10^{-4} $[TU]^{Hb}_{ml}$ trypsin concentration in a 2 mg per ml α-glycerophosphate dehydrogenase solution. The trypsin was a three times recrystallized Tripure-Novo preparation. Proteolytic activity was checked by *Anson*'s test.

Results

Effect of SH-group blocking on enzymatic activity

In the α-glycerophosphate dehydrogenase molecule 9 to 11 SH-groups can be found by titration with PCMB. It has no inaccessible SH-groups, as after denaturation in 8 M urea 9 to 11 SH-groups react with PCMB.

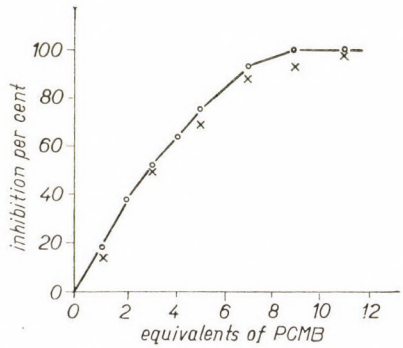

Fig. 1. Effect of PCMB on enzymatic activity: o—o, α-glycerophosphate as substrate; *x*—*x*, dihydroxyacetone phosphate as substrate. Enzymatic activity was measured by *Warburg*'s optical test using a reaction mixture described previously [5]. Protein concentration, 0.5 μg per ml

All of the SH-groups react with PCMB within 30 seconds, thus they show an identical reactivity.

The changes of enzymatic activity as a function of SH-group blocking has been studied. Enzymatic activity is instantaneously inhibited after the addition of the reagent. The effect of SH-group blocking on the reaction in both directions, *i.e.* α-glycerophosphate oxidation and dihydroxyacetone phosphate reduction, has been examined *(Fig. 1)*.

Fig. 1 shows that complete inhibition is achieved only when all of the SH-groups are blocked. The diminution of enzymatic activity proceeds, however, not linearly with the loss of the number of free SH-groups. This suggests that they are not equivalent with respect to enzymatic activity. Blocking of the first three SH-groups results in a 50 to 60 per cent inhibition, while upon the reaction of further SH-groups with PCMB a slower increase of inhibition occurs. Assuming that the mercaptide formed dissociates, the blocking of 6 or 7 SH-groups is necessary for complete inactivation, as shown by the extrapolation, to 100 per cent inhibition of the nearly linear part of the curve.

The inhibition due to PCMB blocking does not change with time. This is valid for the reactions in both directions.

Reactivation by cysteine

If cysteine is added to the mixture within 25 to 30 minutes following the addition of PCMB, *i.e.* the development of the inhibition, enzymatic activity is completely restored, independently of the extent of inhibition. Obviously, some latent secondary alterations take place during this period, as incubating the enzyme with PCMB for more than 25—30 minutes the extent of the reversibility caused by cysteine decreases. The loss of the ability

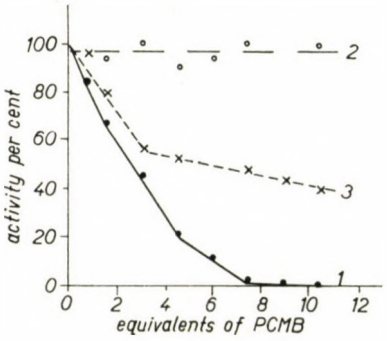

Fig. 2. Reversion by cysteine of inhibition due to PCMB: 1, enzyme + PCMB; 2, enzyme + PCMB + cysteine, within 30 minutes; 3, enzyme + PCMB and after 2 hours of incubation + cysteine. Enzymatic activity was assayed as described previously [5]. Protein concentration, 0.8 μg per ml

of the enzyme to be reactivated becomes detectable in the second half hour of incubation and is stabilized after about one hour of incubation. This residual ability of the enzyme to be reactivated remains unchanged even on incubation for 4 hours *(Fig. 2)*.

Fig. 2 shows that after incubating the enzyme with 3 or less equivalents of PCMB for a longer period, the addition of cysteine caused only a slight increase in activity; in several cases there was no reactivation at all. Adding more than 3 equivalents of PCMB, about 40 to 50 per cent of the original activity can be recovered by means of cysteine and there is no difference in this respect between enzymes containing 4 to 10 mercaptide linkages.

Reactivation by cysteine is accomplished within 30 seconds. The excess of cysteine used (20 equivalents) removed the PCMB quantitatively from the protein, as shown by gel-filtration experiments.

The fact that inhibition due to PCMB takes place instantaneously and remains unchanged after 2 hours of incubation, while the ability to reactivate falls from 100 to about 50 per cent during this period, indicates that two different effects of PCMB are to be considered. An immediately occurring change of the enzyme molecule, manifesting itself in the loss of activity, is

followed by a further, time-consuming alteration reflected in a diminished reversibility.

It is obvious that at least the latter phenomenon is due to the damage of the steric structure.

Tryptic digestibility of the protein

In order to obtain information on the possibility of steric alterations, the tryptic digestibility of enzymes containing 3 and 10 mercaptide linkages has been examined. Under the experimental conditions applied, the control (native) protein was not digested at a measurable rate in the first 30 minutes *(Table I)*.

Table I

Digestibility of α-glycerophosphate dehydrogenase

α-glycerophosphate dehydrogenase 2.5×10^{-2} μmoles/ml	PCMB	Time of preincubation with PCMB	Cysteine, 5 μmoles per ml	Rate of digestion, $\Delta E_{280}^{30'}$ mμ	α-glycerophosphate dehydrogenase activity
+	—	—	—	0.000	100
+	—	1—4 hr	—	0.000	100
+	3 equ	0—30 min	—	0.000	50
+	3 equ	0—30 min	+	0.000	100
+	3 equ	1—4 hr	—	0.062	55
+	3 equ	1—4 hr	+	0.057	50
+	10 equ	0—30 min	—	0.067	0
+	10 equ	0—30 min	+	0.000	100
+	10 equ	1—4 hr	—	0.150	0
+	10 equ	1—4 hr	+	0.062	48

Digestions were carried out in 0.1 M phosphate buffer, pH 7.0, at room temperature and trypsin concentration of $1 \times 10^{-4}[TU]_{ml}^{Hb}$ was used. Samples were taken from the reaction mixture every ten minutes, the protein was precipitated by the use of 6.6 per cent end concentration of trichloroacetic acid then centrifuged and the extent of digestion was determined by measuring specrophotometrically at 280 mμ in the supernatant the split peptides containing tyrosine and tryptophan. Digestion was linear for at least 30 minutes; calculations were therefore made from the data obtained in the first 30 minutes and these are shown in the *Table*. The enzymatic activity of α-glycerophosphate dehydrogenase was assayed by *Warburg*'s optical test [5] and is expressed in percentage of the original activity.

If the trypsin is added immediately after the formation of 3 mercaptide linkages, the protein is not digested like the native one. On the other hand, after several hours incubation with the same amount of PCMB, a well-detectable digestion occurs. An identical digestibility can be observed when, prior to the addition of trypsin, cysteine is added to the mixture at a concentration which does not affect the activity of trypsin, as shown by control experiments.

If digestion is started immediately after the addition of 10 equivalents of PCMB, in contrast to the enzyme containing 3 mercaptide linkages, a measurable digestibility is obtained. Adding cysteine prior to trypsin to the mixture, the enzyme, like the native one, is not digested.

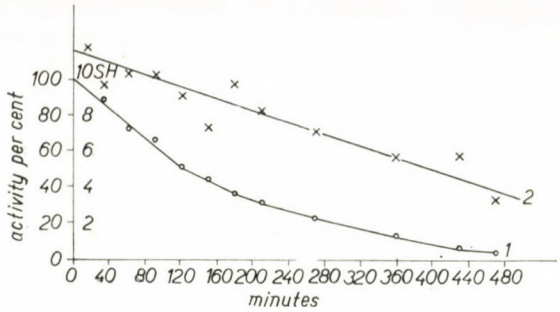

Fig. 3. Spontaneous inactivation of the enzyme: 1, enzymatic activity; 2, number of free SH-groups. The decrease in the number of SH-groups is shown only up to 8 hours of incubation. After 16—24—36 hours of incubation 3—4 free SH-groups are still detectable and these disappear only after incubating for 3—4 days. Enzymatic activity was measured in the usual way [5]. Protein concentration, 1 μg per ml. The number of free SH-groups was determined by spectrophotometric titration [7]

Incubating the enzyme with 10 equivalents of PCMB for several hours, a 2.5-fold increase in digestibility takes place as compared to that without preceding incubation. In this case, however, the addition of cysteine prior to trypsin diminishes the rate of digestion to a just measurable level.

Spontaneous inactivation

All the experiments described above were performed in phosphate buffer, pH 7. It has been observed that in glycine buffer, pH 10, in dilute solution, the enzyme is inactivated in a few hours. The change of the number of SH-groups during this process was examined *(Fig. 3)*.

As seen in *Fig. 3*, the rate of inactivation was approximately proportional to the disappearance of SH-groups, as in the case of blocking with PCMB. About 6 to 7 SH-groups are lost before complete inactivation is achieved. The remaining 3 to 4 SH-groups can be detected even after prolonged incubation. This inactivation process takes place between pH 8.5 and 10.0, whether glycine, Tris or veronal buffer is used, and in this pH range the rate of inactivation depends upon the concentration of the protein. In a dilute solution the enzyme is more rapidly inactivated than in a concentrated one.

The phosphate ion stabilizes the enzyme; in its presence no inactivation occurs even at pH 10.

By this method, as shown in *Fig. 3*, differences in reactivity can be established among the SH-groups of the enzyme, demonstrated by the existence of 3 to 4 not or only extremely slowly oxidizable SH-groups.

It has been investigated which of the SH-groups exhibiting different reactivity towards oxidation combine first with PCMB. Increasing amounts of PCMB were added to the enzyme in glycine buffer, pH 10, and all the samples were incubated until inactivation had been achieved. The number of remaining SH-groups was then determined *(Table II)*.

Table II

SH-groups during spontaneous inactivation

Number of mercaptide linkages formed before incubation	Total number of mercaptide linkages measured after inactivation with excess PCMB	Number of SH-groups not oxidizable during incubation
0.0	3.4	3.4
0.7	4.2	3.5
0.8	4.4	3.6
1.2	5.2	4.0
1.3	5.0	3.7
1.8	5.3	3.5
1.9	4.7	3.8
2.1	6.1	4.0
2.2	5.6	3.4
3.0	6.5	3.5
3.2	7.1	3.9
3.5	7.5	4.0
4.0	8.2	4.2
4.4	7.9	3.5
5.0	8.7	3.7
5.3	9.0	3.7
6.3	10.0	3.7
7.3	10.8	3.5

The experiment was carried out as follows: 1. the number of SH-groups of the protein to be examined was determined. 2. The solution was divided into 10—11 portions, then 0—7 equivalents of PCMB were added to the tubes and the number of developed mercaptide linkages was measured (column I). 3. The mixtures were incubated at room temperature until the enzymatic activity of the control sample, containing no mercaptide linkages, fell to 0 (about 7—9 hours). Calculating from 38 experimental data the oxidation of 7 SH-groups, as a round number, is required for complete inactivation to occur. Mixtures containing 6—7 mercaptide linkages are naturally practically inactive already at the beginning of the experiment. It was found that the protein containing 1—6 mercaptide linkages is inactivated much faster than the native one, and the rate of inactivation increases with the number of mercaptide linkages.

Nevertheless, each sample was incubated for the same period, *i. e.* up to the inactivation of the native, mercaptide-free sample, in order to achieve a complete oxidation of SH-groups. 4. Excess PCMB was added to each mixture, then the total number of mercaptide linkages *i. e.* the number of not oxidized SH-groups was determined (column II). 5. Subtracting the number of mercaptide linkages formed at the beginning of the experiment from the number of SH-groups titrated with PCMB after incubation, the number of not-oxidizable SH-groups is obtained (column III). The above data are taken from six identical experiments.

It is seen that whatever the amount of PCMB added before the inactivation (up to 7 equivalents) (*Table II*, column 1), the number of SH-groups resistant to oxidation remains constant (*Table II*, column 3). In all cases — as a round number — 4 not oxidizable SH-groups can be detected.

This proves that those SH-groups react first with PCMB which are sensitive also to oxidation, *i.e.* the reactivity of SH-groups is different. This difference in reactivity cannot, however, be detected during blocking with PCMB since even the least reactive SH-groups form mercaptide within 30 seconds.

The effect of cysteine on the inactivation process has been examined. It was found that cysteine in a 200-fold excess protected the protein from inactivation. However, had the process already been accomplished, in contrast to the enzyme blocked with PCMB, no reactivation was obtained with a much greater excess of cysteine.

Discussion

Our investigations have shown that blocking of about 6 or 7 of the total 9 to 11 SH-groups is required for the complete inhibition of the reaction, in both directions, catalyzed by the enzyme.

It should be mentioned that VAN EYS *et al.* [1] working with an enzyme isolated by their own procedure, found that only 1 SH-group must be blocked for the complete inhibition of dihydroxyacetone phosphate reduction. The explanation of this contradiction might be that the isolation procedure of VAN EYS *et al.* is much more complicated than ours and their crystalline enzyme is less active than our preparation. It might thus be assumed that the enzyme protein isolated by them is damaged to some extent and the sterically loosened enzyme molecule is ruined by the addition of 1 equivalent of PCMB, or most of the SH-groups had oxidized in air and this partially inactive enzyme was completely inactivated by 1 equivalent of PCMB.

The activity of the enzyme isolated by us surpasses several times that of the best preparation described in the literature. We therefore suppose that the properties of this enzyme may better approach the native one than those of the enzymes so far isolated.

An important problem is how PCMB inactivates the enzyme. This inactivation may be due either to the elimination of free SH-groups, or to some secondary steric alteration induced by the reagent [*cf.* 9] or to a combination of these two effects.

Summarizing our data, the following may be stated.

As 50 per cent inhibition immediately occurs when the enzyme is treated with 3 equivalents of PCMB while its digestibility is similar to the native enzyme, it can be concluded that the inhibition is a direct consequence of the blocking of SH-groups. In the course of incubation with PCMB, however, digestibility increases, *i.e.* the primary effect is followed by a slow steric change. The cysteine removes PCMB from the protein but cannot completely reconstitute the altered steric structure.

On the addition of 10 equivalents of PCMB some steric alterations instantaneously appear which can be reverted by the immediate removal of the reagent. During prolonged incubation, however, profound damage to the structure takes place, revertible only in part by cysteine.

In our opinion, the peculiar fact that enzyme samples incubated with different amounts of PCMB for prolonged periods can be reactivated to the same degree and their digestibility is also similar, allows to attribute identical structural features to these enzyme molecules. In other words, the native enzyme may turn into a relatively stabile enzyme possessing about 50 per cent of the original activity.

Our experimental observation that the enzyme inactivated by oxidation cannot be reactivated by cysteine indicates the formation during inactivation of stronger bonds than the mercaptide linkage, or the occurrence of more profound steric changes than that which is caused by PCMB-blocking. The study of the free SH-groups during inactivation revealed that the SH-groups of the enzyme are of different reactivity; about 4 SH-groups are not or only slowly oxidized and these very residues react with PCMB at the least speed. These SH-groups do not seem to participate in the maintenance of enzymatic activity, since the blocking of 6 to 7 SH-groups with PCMB or their oxidation at pH 10 is sufficient for complete inhibition to develop.

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LITERATURE

1. VAN EYS, J., NUENKE, B. J., PATTERSON, M. F., JR.: J. biol. Chem. **234,** 2308 (1959).
2. ANKEL, H., BÜCHER, T., CZOK, R.: Biochem. Z. **332,** 315 (1960).
3. NAGRADOVA, N. K.: Биохимия **24,** 336 (1959).
4. MARTINENKO, F. P.: Укр. Биохим. Журн. **33,** 476 (1961).
5. TELEGDI, M.: Acta physiol. Acad. Sci. hung. **25,** 177 (1964).
6. BEISENHERZ, G., BOLTZE, H. J., BÜCHER, T., CZOK, R., GARBADE, K. H., MEYER-ARENDT, E., PFLEIDERER, G.: Z. Naturforsch. **8b,** 555 (1953).
7. BOYER, P. D.: J. Amer. chem. Soc. **76,** 4331 (1954).
8. SZABOLCSI, G., SZÖRÉNYI, E. T.: Acta physiol. Acad. Sci. hung. **9,** 293 (1956).
9. SZABOLCSI, G., BISZKU, E.: Biochim. Biophys. Acta (Amst.) **48,** 335 (1961).

Marianna TELEGDI, Tamás KELETI
MTA Biokémiai Intézete, Budapest XI., Karolina út 29.