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REACTION OF THE SULPHYDRYL GROUPS OF LOBSTER-MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE WITH ORGANIC MERCURIALS

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SUMMARY

1. Crystalline lobster-muscle glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) possesses 7.3 ± 0.3 cysteine residues per mole of enzyme (140 000 g), *i.e.* approx. 2 cysteine residues per polypeptide chain, which are extremely reactive toward organic mercurials; 8 of the 20 cysteine residues of the enzyme are totally unreactive toward organic mercurials.

2. Enzyme carboxymethylated at each of the 4 'active site' cysteine residues possesses 7.4 ± 0.3 sulphydryl groups which are extremely reactive toward *p*-mercuribenzoate and cannot be distinguished from native enzyme on the basis of these titrations.

3. Enzyme treated with iodosobenzoic acid and containing an intramolecular disulphide bridge at the 'active center' possesses 4.0 ± 0.3 sulphydryl groups which are extremely reactive toward *p*-mercuribenzoate, *i.e.* approx. 4 less than native enzyme.

4. Under normal conditions, lobster-muscle glyceraldehyde-3-phosphate dehydrogenase is 90–95% inactivated concomitant with the formation of 8 moles of mercaptide per mole of enzyme (140 000 g); inhibition takes place in a linear manner with respect to mercurial concentration.

5. In the presence of a large molar excess of NAD^+, the formation of 4 moles of mercaptide per mole of enzyme (140 000 g) has no inhibitory effect and must be attributed to reaction of a 'nonessential' sulphydryl group.

INTRODUCTION

The inhibitory effect of sulphydryl-specific reagents on the enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) is well documented (for review see [1])

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COLOWICK *et al.*[1]). This inhibitory effect is usually attributed to reaction of the particular reagent with the 'active site' cysteine residue of the enzyme, *i.e.* the cysteine residue acylated by substrate during catalysis[2–4]. This sulphydryl group is highly reactive toward a number of reagents, including iodoacetic acid[4], 2-acetamido-4-nitrophenol[5], *N*-(dimethylamino-3,5-dinitrophenyl)maleimide[6], and 5,5′-dithiobis-(2-nitrobenzoic acid)[7,8].

In conjunction with the X-ray crystallographic determination of the structure of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase, we have recently examined the reactivity of the sulphydryl groups of the enzyme toward several organic mercurials. Due in large part to a knowledge of the complete amino acid sequence of this enzyme[9], this study has provided additional insight into the role of the non-active site sulphydryl groups of glyceraldehyde-3-phosphate dehydrogenase.

EXPERIMENTAL

Materials

D-Glyceraldehyde-3-phosphate (barium salt of the diacetal), NAD^+ and iodoacetic acid were purchased from Koch–Light Labs. Organic mercurials and iodosobenzoic acid were purchased from Sigma and Sephadex G-25 from Pharmacia.

Enzyme purification and characterisation

Glyceraldehyde-3-phosphate dehydrogenase was prepared from lobster tail muscle according to the method described by ALLISON AND KAPLAN[10]. The enzyme was stored at 4° as a crystalline suspension in 75% $(NH_4)_2SO_4$. Lobster-muscle glyceraldehyde-3-phosphate dehydrogenase used in these studies exhibited a ratio of $A_{280\ m\mu}$ to $A_{260\ m\mu}$ of 1.07 ± 0.03. The molecular weight was taken as 140 000 (refs. 9, 11), and an extinction coefficient ($E\ {}^{0.1\%}_{1\ cm}$ at 280 mμ) of 1.00 ± 0.05 was determined from dry weight measurements.

Enzyme assays

Assays were performed on a Cary Model 14 recording spectrophotometer at room temperature. The assay mixture contained 0.25 mM glyceraldehyde 3-phosphate, 0.75 mM NAD^+, 3 mM arsenate and 1 mM EDTA and was brought to a volume of 3.0 ml with 0.05 M pyrophosphate buffer (pH 8.4). The reaction was initiated by the addition of 0.03 mμmole of enzyme (in 10 μl) to the assay mixture. Specific activity was calculated from the change in $A_{340\ m\mu}$ which occurred during the 15–45-sec interval after addition of the enzyme. The specific activities of several enzyme preparations varied between 110 and 120 μmoles of NADH formed per min per mg of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase.

Sulphydryl titrations

The reaction of *p*-mercuribenzoate with the sulphydryl groups of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase was followed spectrophotometrically on a Cary Model 14 at 250 mμ, according to the method described by BOYER[12]. *p*-Mercuribenzoate was dissolved in 0.05 M glycylglycine buffer (pH 8.0) just prior to use, and an estimate of the concentration of the mercurial was made at 232 mμ using a molar extinction coefficient of $1.69 \cdot 10^4$. Titrations were carried out in 0.1 M phosphate

buffer (pH 7.0) at room temperature with 3.0 μM enzyme. The change in absorption at 250 mμ was corrected for absorption due to enzyme and to *p*-mercuribenzoate itself. A molar extinction coefficient (at 250 mμ) of $7.6 \cdot 10^3$ was used to calculate the extent of mercaptide formation (determined by titration of known concentrations of reduced glutathione with *p*-mercuribenzoate).

Enzyme modifications

Bound NAD^+ was removed from the holoenzyme by treatment with a 1:1 mixture (by weight) of Sephadex G-25 (fine) and activated charcoal, in 0.1 M phosphate buffer (pH 7.0). The mixture was filtered on sintered glass and resulted in a 50–60% yield of NAD^+-free enzyme. The apoenzyme had a ratio of $A_{280\ m\mu}$ to $A_{260\ m\mu}$ of 1.92 ± 0.05, as compared to a ratio of 1.07 ± 0.03 for the holoenzyme. The apoenzyme was fully active under the conditions of assay but was considerably more labile than the holoenzyme even at 4°.

Carboxymethylation of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase was carried-out at 4° in either 0.05 M Tris buffer (pH 7.5) or in 0.1 M phosphate buffer (pH 7.0), using recrystallised iodoacetic acid. The extent of the reaction was estimated from the percent remaining enzymatic activity*.

Oxidation of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase was carried-out at 4° in 0.1 M phosphate buffer (pH 7.0), using iodosobenzoic acid (dissolved in 1 M Tris buffer (pH 8.2) just prior to use). The enzyme was incubated with a 4-fold molar excess of iodosobenzoic acid for at least 24 h; the oxidised enzyme retained less than 10% of its original activity*. Lobster-muscle glyceraldehyde-3-phosphate dehydrogenase incubated under identical conditions in the absence of iodosobenzoic acid retained full enzymatic activity.

RESULTS

Number of sulphydryl groups in lobster-muscle glyceraldehyde-3-phosphate dehydrogenase

A total of 20 sulphydryl groups were found per 140 000 g of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase by titrating the enzyme with 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of 8 M urea[8]. DAVIDSON *et al.*[9], in their report of the complete amino acid sequence of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase, identified the positions of 5 cysteine residues per polypeptide chain which accounts for 20 sulphydryl groups per 140 000 g of enzyme.

Titration of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase with p-mercuribenzoate

A time-course of the titration of native lobster-muscle glyceraldehyde-3-phosphate dehydrogenase with *p*-mercuribenzoate is shown in Fig. 1A. At pH 7.0, in the presence of a 40-fold molar excess of *p*-mercuribenzoate, a total of 11.7 ± 0.6

* It has been demonstrated that in the presence of a low molar excess of iodoacetic acid lobster-muscle glyceraldehyde-3-phosphate dehydrogenase is carboxymethylated exclusively at active site cysteine residue 148. In the presence of iodosobenzoic acid an intramolecular disulphide bridge is formed between cysteine residues 148 and 152 of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase[9].

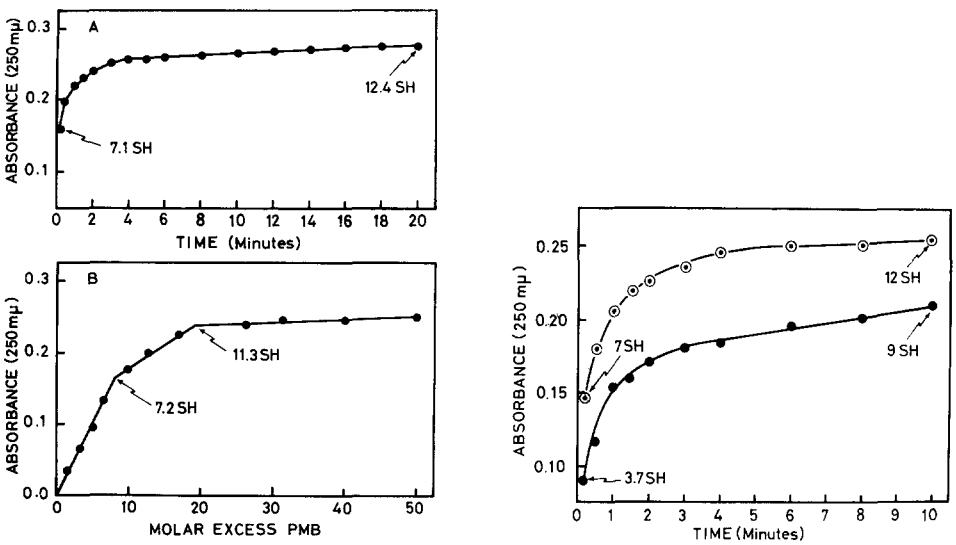


Fig. 1. A. Titration of the sulphydryl groups of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase with *p*-mercuribenzoate. Enzyme concn. 3.0 μM; 0.1 M phosphate buffer (pH 7.0); room temperature. A 40-fold molar excess of *p*-mercuribenzoate (120 μM) was added to the enzyme mixture, and mercaptide formation was followed at 250 mμ. $A_{250\ m\mu}$ was corrected for self-absorption by enzyme and *p*-mercuribenzoate and was converted to moles of sulphydryl groups using a molar extinction coefficient of $7.6 \cdot 10^3$ (see EXPERIMENTAL). B. Titration of the sulphydryl groups of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase with *p*-mercuribenzoate (PMB). Enzyme concn. 3.0 μM; 0.1 M phosphate buffer (pH 7.0); room temperature. Enzyme mixtures were incubated at various molar excess of *p*-mercuribenzoate, and the extent of mercaptide formation was determined at the end of 2 min. Calculations were carried out as described in A.

Fig. 2. Titration of native, carboxymethylated and oxidised lobster-muscle glyceraldehyde-3-phosphate dehydrogenase with *p*-mercuribenzoate. Native and carboxymethylated (○) and oxidised (●) lobster-muscle glyceraldehyde-3-phosphate dehydrogenase (see EXPERIMENTAL) were titrated with a 40-fold molar excess of *p*-mercuribenzoate (120 μM) in 0.1 M phosphate buffer (pH 7.0) at room temperature. Calculations were carried out as described in Fig. 1A.

sulphydryl groups were reacted per 140 000 g of enzyme (approx. 3 sulphydryl groups per polypeptide chain) in less than 10 min. Of these, 7.4 $\pm$ 0.3 sulphydryl groups (approx. 2 sulphydryl groups per polypeptide chain) were reacted with *p*-mercuribenzoate within 10 sec. A titration curve plotted as a function of *p*-mercuribenzoate concentration *versus* moles of mercaptide formed in 2 min is shown in Fig. 1B. Approx. 8 moles of mercaptide were formed per 140 000 g of enzyme in a nearly stoichiometric manner. Two sulphydryl groups per polypeptide chain were totally unreactive toward *p*-mercuribenzoate.

Titration of carboxymethylated and oxidised lobster-muscle glyceraldehyde-3-phosphate dehydrogenase with p-mercuribenzoate

Lobster-muscle glyceraldehyde-3-phosphate dehydrogenase carboxymethylated at each of its 4 'active site' cysteine residues was titrated with *p*-mercuribenzoate under conditions identical to those used for native enzyme. Enzyme treated with iodosobenzoic acid and containing an intramolecular disulphide bridge at the active center

TABLE I

STOICHIOMETRY OF THE REACTION OF *p*-MERCURIBENZOATE WITH LOBSTER-MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

These results are calculated from four individual titrations of each form of the enzyme. The moles of *p*-mercuribenzoate bound per 140 000 g of enzyme were determined 2 min after the addition of *p*-mercuribenzoate to the enzyme mixture. The titrations were carried out in 0.1 M phosphate buffer (pH 7.0) at room temperature using 3.0 μM enzyme. The carboxymethylated and oxidised forms of the enzyme retained less than 10% of their original enzymatic activity.

Molar excess p-mercuribenzoate	*Moles p-mercuribenzoate bound per mole enzyme*		
	Native	*Carboxymethylated*	*Oxidised*
2	2.0 ± 0.2	2.0 ± 0.2	2.0 ± 0.3
4	4.0 ± 0.2	4.0 ± 0.2	4.0 ± 0.3
6	6.0 ± 0.2	6.0 ± 0.2	4.7 ± 0.3
8	7.3 ± 0.3	7.4 ± 0.3	5.6 ± 0.3
10	8.3 ± 0.3	8.4 ± 0.4	6.4 ± 0.2
12	9.6 ± 0.4	9.6 ± 0.4	7.2 ± 0.3
16	11.3 ± 0.3	11.3 ± 0.3	8.1 ± 0.3
20	12.3 ± 0.4	12.2 ± 0.4	8.4 ± 0.2
25	12.5 ± 0.4	12.4 ± 0.3	8.7 ± 0.4

was also titrated with *p*-mercuribenzoate. The time-courses of these titrations are compared with those of native enzyme in Fig. 2. The results of several experiments are presented in Table I. It is apparent that eliminating reaction of the active site cysteine residues of the enzyme with *p*-mercuribenzoate does not alter the kinetics of reaction observed with native enzyme. On the other hand, formation of a disulphide bridge at the active center of the enzyme (*via* iodosobenzoic acid) results in the loss of approx. 4 highly reactive sulphydryl groups per 140 000 g of enzyme (1 sulphydryl group per polypeptide chain).

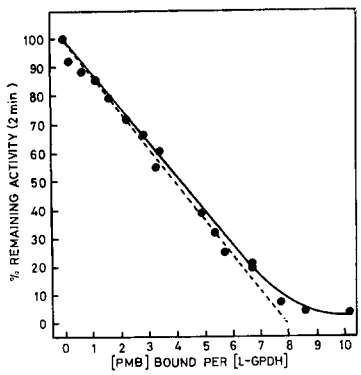
Effect of organic mercurials on the enzymatic activity of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase

The relationship between moles of mercaptide formed per mole of enzyme (140 000 g) and loss of enzymatic activity is shown in Fig. 3. Binding of 8 moles of *p*-mercuribenzoate was accompanied by a 90–95% loss of enzymatic activity; inactivation took place in a linear manner with respect to *p*-mercuribenzoate concentration. The inhibitory effect was reversed in the presence of 10 mM 2-mercaptoethanol.

Similar results were obtained in the presence of two other organic mercurials, *p*-mercuribenzenesulphonate and *o*-mercuribenzoate. An 8-fold molar excess of either of these mercurials was necessary to achieve greater than 90% inactivation of the enzyme (Fig. 4).

Effect of coenzyme on the inhibition of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase by p-mercuribenzoate

The effect of *p*-mercuribenzoate on the enzymatic activity of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase was examined in the presence of excess coenzyme, NAD^+. As seen in Fig. 5A, a 100-fold molar excess of NAD^+ protected the



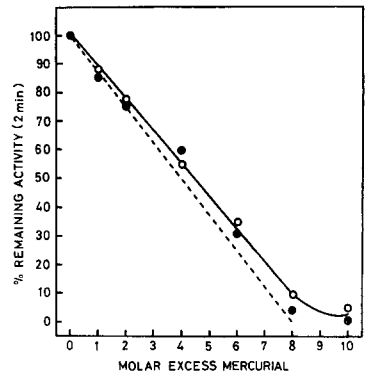


Fig. 3. Effect of *p*-mercuribenzoate(PMB) on the enzymatic activity of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase (L-GPDH). The reactions were carried out as described in Fig. 1B, and aliquots for activity measurements were removed and assayed directly.

Fig. 4. Effect of organic mercurials on the enzymatic activity of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase. The reactions were carried out as described in Fig. 1B in the presence of *p*-mercuribenzenesulphonate (○) and *o*-mercuribenzoate (●). Aliquots for activity measurements were removed and assayed directly.

enzyme against the inhibitory effect of low concentrations of mercurial. Under these conditions the enzyme retained full activity in the presence of a 4-fold molar excess of *p*-mercuribenzoate; this is to be compared with a 50% loss of activity in the absence of excess NAD^+. Further addition of mercurial resulted in complete inactivation of the enzyme*.

Lobster-muscle glyceraldehyde-3-phosphate dehydrogenase which had been treated with a charcoal–Sephadex mixture to remove bound NAD^+ was also reacted with *p*-mercuribenzoate. A 4-fold molar excess of the mercurial completely inactivated the apoenzyme (Fig. 5B).

DISCUSSION

Preliminary X-ray crystallographic studies have shown that crystals of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase are orthorhombic with space group $P2_12_12_1$ and that there are four molecules of approx. 140 000 molecular weight in the unit cell[13]. Although these crystals are quite amenable to X-ray crystallographic analysis, further structural work on this enzyme has been limited by the inability to obtain single-site, isomorphous, heavy atom derivatives which are required to solve the phase problem (see refs. 14 or 15). It was in this connection that we undertook to examine the reactivity of the sulphydryl groups of the enzyme toward organic mercurials.

The primary structure of lobster-muscle glyceraldehyde-3-phosphate dehydro-

* Enzyme reacted with 4 moles of *p*-mercuribenzoate in the presence of a 100-fold molar excess of NAD^+ has been crystallised from $(NH_4)_2SO_4$ solutions. X-Ray diffraction photographs show that these crystals are completely isomorphous with the native enzyme, and intensity changes indicative of heavy atom binding are observed. Spectral studies of enzyme solutions prepared from these crystals confirm the presence of approx. 4 moles of *p*-mercuribenzoate per 140 000 g of enzyme (P. M. WASSARMAN AND H. C. WATSON, unpublished observations).

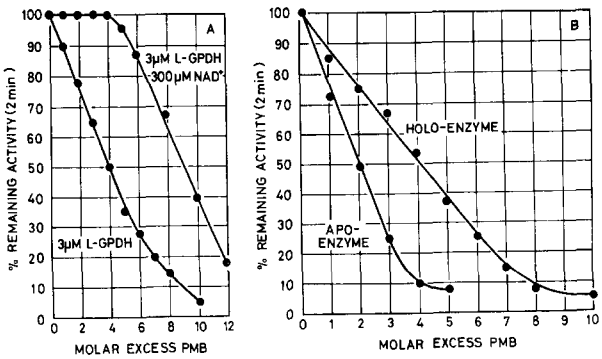


Fig. 5. A. Effect of NAD^+ on the kinetics of inhibition of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase (L-GPDH) by *p*-mercuribenzoate (PMB). Enzyme concn. 3.0 μM; 0.1 M phosphate buffer (pH 7.0); room temperature. Enzyme mixtures were incubated at various molar excess of *p*-mercuribenzoate in the presence and absence of excess NAD^+ (300 μM). At the end of 2 min, aliquots for activity measurements were removed and assayed directly. B. Effect of NAD^+ on the kinetics of inhibition of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase by *p*-mercuribenzoate (PMB). The reactions were carried out as described in Fig. 1B. At the end of 2 min, aliquots for activity measurements were removed and assayed directly.

genase includes 5 cysteine residues per polypeptide chain (molecular weight, 36 000), cysteine residue 148 being the active site sulphydryl group[9]. We have found that 2 of the 5 cysteine residues per polypeptide chain are highly reactive toward *p*-mercuribenzoate, while a third less reactive cysteine residue can be titrated in the presence of excess mercurial. The remaining 2 sulphydryl groups per polypeptide chain are totally unreactive toward *p*-mercuribenzoate and are presumeably inaccessible to the mercurial.

In the presence of a low molar excess of iodoacetic acid, lobster-muscle glyceraldehyde-3-phosphate dehydrogenase is carboxymethylated exclusively at cysteine residue 148, *i.e.* the active site[9]. X-Ray diffraction studies have shown that the enzyme does not undergo a measureable conformational change concomitant with the carboxymethylation of this residue[16]. A comparison of the kinetics of the reaction of *p*-mercuribenzoate with the sulphydryl groups of native and carboxymethylated enzyme has revealed no significant difference (Fig. 2; Table I). The carboxymethylated enzyme (with less than 5% of its original enzymatic activity remaining) possesses 3 titratable sulphydryl groups per polypeptide chain; 2 of these groups are extremely reactive toward *p*-mercuribenzoate. The inability to distinguish between the two forms of the enzyme on the basis of these sulphydryl titrations suggests that cysteine residue 148 is not one of the 3 reactive sulphydryl groups present in native lobster-muscle glyceraldehyde-3-phosphate dehydrogenase. This result, unexpected in view of the reactivity of this residue toward several other sulphydryl reagents (for discussion see ref. 8), accounts for the failure of glyceraldehyde-3-phosphate and acetylphosphate to protect the rabbit-muscle enzyme against inhibition by *p*-mercuribenzoate[17,18]. We concur, therefore, with MURDOCK AND KOEPPE[18] who suggested that the inhibitory effect of *p*-mercuribenzoate on glyceraldehyde-3-phosphate dehydrogenase was attributable to reaction of the mercurial with groups other than the cysteine residue involved in acyl enzyme formation.

Oxidation of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase with

iodosobenzoic acid results in the formation of an intramolecular disulphide bridge at the active center of the enzyme between cysteine residues 148 and 152 (ref. 9). Titrations of oxidised lobster-muscle glyceraldehyde-3-phosphate dehydrogenase with *p*-mercuribenzoate indicate that one of the 2 highly reactive cysteine residues present in the native enzyme is lost as a consequence of disulphide bridge formation. The differential reactivity of the sulphydryl groups of carboxymethylated and oxidised enzyme suggests that the missing group is cysteine residue 152. This interpretation is open to criticism, however, since it has been demonstrated in previous studies that the oxidised form of lobster-muscle enzyme undergoes a conformational change[8]. Alternatively, reaction of *p*-mercuribenzoate with active center cysteine residue 152 can account most readily for the effects of mercaptide formation on the catalytic and physical properties of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase. In this regard, it is interesting to note that comparative studies of glyceraldehyde-3-phosphate dehydrogenase from a variety of organisms have revealed that only the 2 sulphydryl groups at the active center of the enzyme have been conserved during evolution[19,20]. This may indicate some fundamental catalytic or structural role for cysteine residue 152 which is abolished as a result of reaction with organic mercurials; on the other hand, the inhibitory effect of these reagents may be due merely to the proximity of this residue to the active site.

The effect of organic mercurials on the enzymatic activity of lobster-muscle glyceraldehyde-3-phosphate dehydrogenase has been examined. Under normal conditions (pH 7.0, 3.0 μM enzyme) the binding of 8 moles of *p*-mercuribenzoate per 140 000 g of enzyme (2 moles per polypeptide chain) causes 95% inactivation of the enzyme; loss of enzymatic activity takes place in a linear manner with respect to mercaptide formation. In the presence of excess NAD^+, however, the binding of 4 moles of *p*-mercuribenzoate per mole of enzyme (1 mole per polypeptide chain) has no effect on enzymatic activity. These results indicate that reaction of a single cysteine residue per polypeptide chain is actually responsible for the inhibitory effect of *p*-mercuribenzoate on lobster-muscle glyceraldehyde-3-phosphate dehydrogenase; the presence of NAD^+ apparently lowers the reactivity of this residue relative to some other "nonessential" sulphydryl group. The ability of NAD^+ to protect against inhibition by sulphydryl reagents has been noted for several other dehydrogenases[21–23]. We suggest, therefore, that the effect of organic mercurials on lobster-muscle glyceraldehyde-3-phosphate dehydrogenase, *i.e.* loss of enzymatic activity, release of bound coenzyme[24,25] and accompanying change in conformation[26–28], is attributable to reaction of cysteine residue 152 at the active center of the enzyme.

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