The Reactivity of the Sulfhydryl Groups of Lobster Muscle Glyceraldehyde 3-Phosphate Dehydrogenase*

Paul M. Wassarman† and Jean P. Major

ABSTRACT: Crystalline glyceraldehyde 3-phosphate dehydrogenase from lobster muscle was found to contain 19.5 ± 0.5 cysteine residues/140,000 g of enzyme. This estimate is based on titrations of native and modified forms of the enzyme in 8 M urea with 5,5′-dithiobis-(2-nitrobenzoic acid). Under nondenaturing conditions, in the presence of a 40-fold molar excess of 5,5′-dithiobis-(2-nitrobenzoic acid), all of the SH groups react within approximately 15 min; 4.5 ± 0.5 of these, *i.e.*, 1 SH group/polypeptide chain, react immediately. Disulfide formation at the four, exceedingly reactive, SH groups results in complete inactivation of the enzyme. Carboxymethylation of the "active site" cysteine residues reduces the number of SH groups reactive toward 5,5′-dithiobis(2-nitrobenzoic acid) to 4.5 ± 0.5; approximately 12 SH groups are totally unreactive. Enzyme treated with iodosobenzoic acid, resulting in the formation of an intramolecular disulfide bond at the "active center" (Davidson, B. E., Sajgò, M., Noller, H. F., and Harris, J. I. (1967), *Nature 216*, 1181), has 11.0 ± 0.5 SH groups which react with 5,5′-dithiobis(2-nitrobenzoic acid).

The steps which lead to the unmasking of all the SH groups of lobster muscle glyceraldehyde 3-phosphate dehydrogenase upon reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) are discussed.

It is well established that glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) is an authentic "sulfhydryl" enzyme (for review, see Velick and Furfine, 1963). In addition, this enzyme possesses the rare attribute among the dehydrogenases of crystallizing with its coenzyme, NAD^+, bound to the protein. Consequently, a great deal of interest in glyceraldehyde 3-phosphate dehydrogenase has centered on interactions which may occur between its SH groups and NAD^+ (for review, see Colowick *et al.*, 1966).

In the present study glyceraldehyde 3-phosphate dehydrogenase from lobster muscle was used because, (a) its complete primary structure has been elucidated (Davidson *et al.*, 1967) and (b) it crystallizes in a form amenable to X-ray crystallographic analysis (Watson and Banaszak, 1964). These two conditions enhance the prospect that information obtained from a chemical study, such as the one described, will provide some additional insight into the relationship between the enzyme's structure and function.

Materials and Methods

Lobster muscle glyceraldehyde 3-phosphate dehydrogenase was prepared from lobster tail muscle according to the method described by Allison and Kaplan (1964b). The tails, removed from live lobsters, were frozen at −20° for approximately 16 hr. All of the steps involved in purification of the enzyme were carried out at 4° in the presence of 1 mM EDTA and 1 mM 2-mercaptoethanol. Each kilogram of wet muscle yielded approximately 600 mg of crystalline enzyme. Lobster muscle glyceraldehyde 3-phosphate dehydrogenase used in these studies exhibited a ratio of absorbance at 280 mμ to absorbance at 260 mμ of 1.07 ± 0.03, even after prolonged storage in 75% $(NH_4)_2SO_4$; this value corresponds to 4 moles of bound NAD^+ per mole of enzyme (Ferdinand, 1964; Murdock and Koeppe, 1964). The molecular weight of the enzyme was taken as 140,000 (Harrington and Karr, 1965; Davidson *et al.*, 1967). Based on dry weight measurements, an extinction coefficient ($E_{1\text{ cm}}^{0.1\%}$ at 280 mμ) of 1.00 ± 0.05 was obtained.

Enzymatic activity was assayed in the presence of 0.25 mM glyceraldehyde 3-phosphate (Koch-Light Laboratories; prepared from the barium salt of the diacetal by treatment with Dowex 50 resin and assayed in the presence of excess NAD^+), 0.75 mM NAD^+ (Koch-Light Laboratories), 3 mM sodium aresnate, and 1 mM EDTA, brought to a volume of 3.0 ml with 0.05 M pyrophosphate buffer (pH 8.4) at room temperature. The reaction was initiated by addition of 0.03 mμmole of lobster muscle glyceraldehyde 3-phosphate dehydrogenase to the cuvet and the production of NADH was monitored at 340 mμ on a Cary Model 14 recording spectrophotometer. Specific activity was calculated from the change in absorbance at 340 mμ which occurred during the 15–45-sec interval after addition of lobster muscle glyceraldehyde 3-phosphate dehydrogenase. The specific activities of enzyme preparations used in the present study was consistently between 110 and 120 μmoles of NADH formed per min per mg of enzyme.

Carboxymethylation of lobster muscle glyceraldehyde 3-phosphate dehydrogenase was carried out at 4° in 0.05 M Tris buffer (pH 7.5), using a fourfold molar excess of recrystallized iodoacetic acid (Koch-Light

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Laboratories). Oxidation of lobster muscle glyceraldehyde 3-phosphate dehydrogenase was performed at 4° in 0.1 M phosphate buffer (pH 7.0) using a fourfold molar excess of iodosobenzoic acid (Sigma Chemical Co.; dissolved in 1 M Tris buffer (pH 8.2) just prior to use). The extent of carboxymethylation or oxidation of lobster muscle glyceraldehyde 3-phosphate dehydrogenase was estimated by following the loss of dehydrogenase activity with time.

Titrations of the SH groups of lobster muscle glyceraldehyde 3-phosphate dehydrogenase with 5,5′-dithiobis(2-nitrobenzoic acid) were carried out according to the method described by Ellman (1959). A stock solution of 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma Chemical Co.) in 0.1 M phosphate buffer (pH 7.0) was prepared just prior to use. Lobster muscle glyceraldehyde 3-phosphate dehydrogenase (0.42 or 0.84 mg per ml) in 0.1 M phosphate buffer (1 mM EDTA) (pH 8.0) was incubated with excess 5,5′-dithiobis(2-nitrobenzoic acid) in a 3-ml cuvet at room temperature. The course of the reaction was followed at 412 mμ on a Unicam SP800 recording spectrophotometer. A molar extinction coefficient ($E_{412\,m\mu}$) of 13,600 M^{-1} cm^{-1} for the anion of thionitronbenzoic acid was used to determine the number of moles of SH group reacted (Ellman, 1959). The absorbance of a blank, containing buffer and 5,5′-dithiobis(2-nitrobenzoic acid) (plus urea when required), was continually subtracted from that of the reaction mixture.

Results

Cysteine Content of Lobster Muscle Glyceraldehyde 3-Phosphate Dehydrogenase. In an attempt to determine accurately the number of cysteine residues per mole of enzyme, advantage has been taken of the observation that specific cysteine residues of lobster muscle glyceraldehyde 3-phosphate dehydrogenase can be modified by iodoacetic acid and iodosobenzoic acid.[1] Spectrophotometric titrations of the SH groups of native, carboxymethylated, and oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase were carried out in 8 M urea with excess 5,5′-dithiobis(2-nitrobenzoic acid); the results of these experiments are summarized in Figure 1. Under these conditions 19.5 ± 0.5, 15.5 ± 0.5, and 11.5 ± 0.5 SH groups of native, carboxymethylated, and oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase, respectively, were reacted with 5,5′-dithiobis(2-nitrobenzoic acid) in approximately 15 sec. These results strongly suggest that there are 20 cysteine resides/140,000 g of lobster muscle glyceraldehyde 3-phosphate dehydrogenase, or 5/ polypeptide chain, in agreement with Davidson *et al.* (1967).

[1] 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 disulfide bridge is formed between cysteine residues 148 and 152 of lobster muscle glyceraldehyde 3-phosphate dehydrogenase (Davidson *et al.*, 1967).

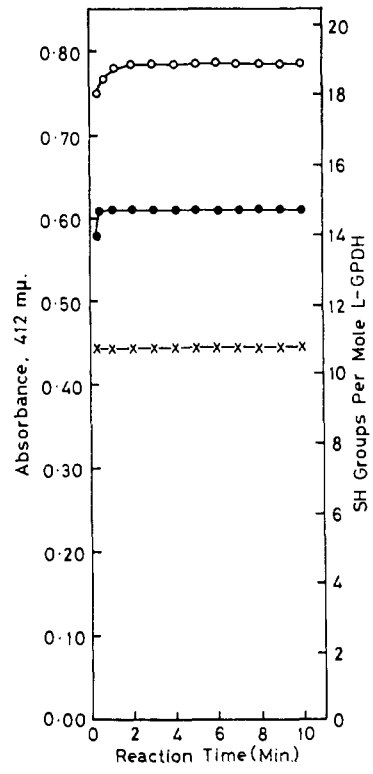


FIGURE 1: Reaction of lobster muscle glyceraldehyde 3-phosphate dehydrogenase with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of urea. Native (○), carboxymethylated (●), and oxidized (x) lobster muscle glyceraldehyde 3-phosphate dehydrogenase (0.42 mg/ml) were incubated for 15 min at room temperature in 0.1 M phosphate buffer (1 mM EDTA) (pH 8.0) brought to 8 M with urea. Spectrophotometric titrations were initiated by the addition of a 40-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) (120 μM) to the enzyme mixture. Absorbance values at 412 mμ were corrected for self-absorption by 5,5′-dithiobis(2-nitrobenzoic acid) and urea and were converted into moles of SH groups using a molar extinction coefficient of 13.6×10^3 (see Materials and Methods).

Titration of Native, Carboxymethylated, and Oxidized Lobster Muscle Glyceraldehyde 3-Phosphate Dehydrogenase with 5,5′-dithiobis(2-nitrobenzoic acid). The reactivity of the SH groups of native lobster muscle glyceraldehyde 3-phosphate dehydrogenase was first examined at various molar ratios of 5,5′-dithiobis(2-nitrobenzoic acid) to enzyme, in 0.1 M phosphate buffer (1 mM EDTA) (pH 8.0). It was found that, at very low concentrations of 5,5′-dithiobis(2-nitrobenzoic acid), the number of SH groups titrated per mole of lobster muscle glyceraldehyde 3-phosphate dehydrogenase was greater than could be accounted for by the amount of 5,5′-dithiobis(2-nitrobenzoic acid) incubated with the enzyme; these results are presented in Table I and are explained in the Discussion. Consequently, all subsequent experiments were performed in the presence of at least a 40-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) compared to lobster muscle glyceraldehyde 3-phosphate dehydrogenase.

In the presence of a 40- to 200-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid), 19.5 ± 0.5 SH groups were titrated per mole of native lobster muscle

TABLE I: Stoichiometry of the Reaction of 5,5′-Dithiobis(2-nitrobenzoic Acid) with Lobster Muscle Glyceraldehyde 3-Phosphate Dehydrogenase.[a]

Molar Excess 5,5′-Dithiobis-(2-nitrobenzoic Acid)	Moles of 5,5′-Dithiobis(2-nitrobenzoic Acid) Reacted/Mole of Lobster Muscle Glyceraldehyde 3-Phosphate Dehydrogenase	
	15 sec	15 min
2	1.2	3.5
4	1.8	7.4
6	2.2	10.1
40	4.3	19.2
100	4.3	19.4
200	4.5	19.5

[a] The sulfhydryl equivalents were calculated per tetramer of 140,000 molecular weight, using an extinction coefficient of 13.6×10^3 at 412 mμ for the thionitrobenzoate anion of 5,5′-dithiobis(2-nitrobenzoic acid) (see Materials and Methods).

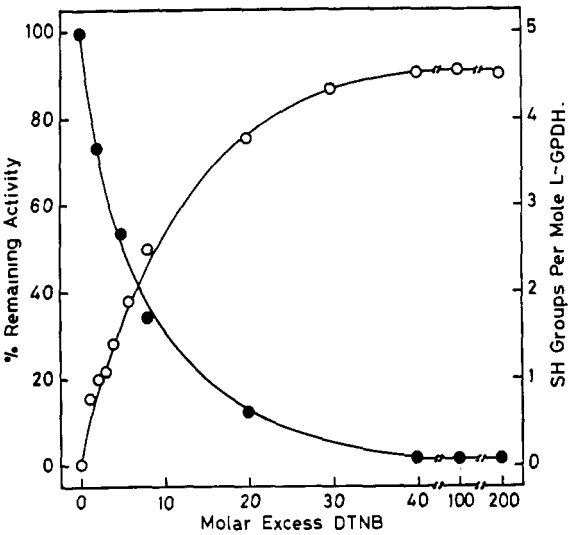


FIGURE 3: Effect of 5,5′-dithiobis(2-nitrobenzoic acid) on the enzymatic activity of lobster muscle glyceraldehyde 3-phosphate dehydrogenase. Native lobster muscle glyceraldehyde 3-phosphate dehydrogenase (0.42 mg/ml) was titrated with various molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) and the per cent remaining activity (●) was compared with the moles of SH groups reacted (○) after a 15-sec period. The reactions were carried out as described in Figure 2.

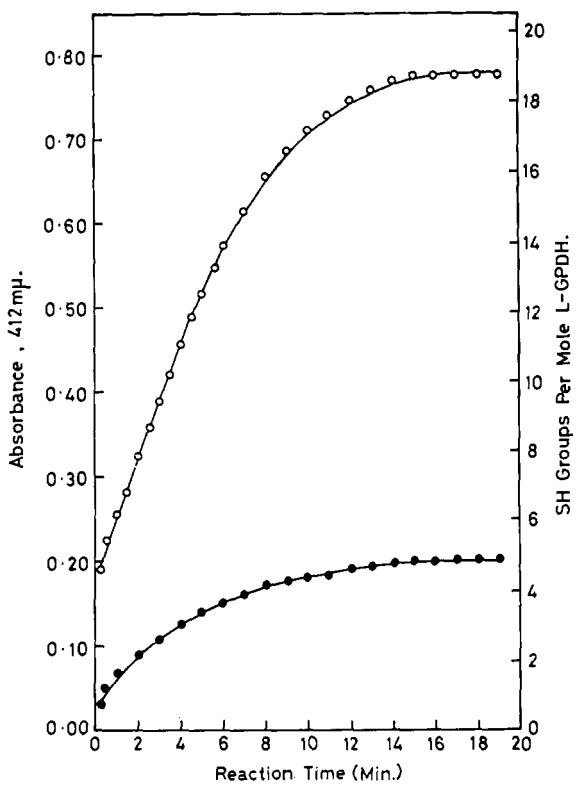


FIGURE 2: Reaction of lobster muscle glyceraldehyde 3-phosphate dehydrogenase with 5,5′-dithiobis(2-nitrobenzoic acid) under nondenaturing conditions. Native (○) and carboxymethylated (●) lobster muscle glyceraldehyde 3-phosphate dehydrogenase (0.42 mg/ml) were titrated with a 40-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) (120 μM) in 0.1 M phosphate buffer (1mM EDTA) (pH 8.0) at room temperature. Calculations were carried out as described in Figure 1.

glyceraldehyde 3-phosphate dehydrogenase within a 15-min period (Figure 2). The profile of the reactivity curve was characterized by an "initial burst" which corresponded to reaction of 4.5 ± 0.5 SH groups, only 10 sec after the addition of 5,5′-dithiobis(2-nitrobenzoic acid) to the enzyme mixture. Experiments carried out to correlate changes in enzymatic activity with the number of SH groups titrated showed that the inhibitory effect of 5,5′-dithiobis(2-nitrobenzoic acid) was attributable to reaction of these 4.5 ± 0.5 extremely reactive SH groups of lobster muscle glyceraldehyde 3-phosphate dehydrogenase (Figure 3).

Lobster muscle glyceraldehyde 3-phosphate dehydrogenase, carboxymethylated at each of its four "active site" cysteine residues, was titrated with a 40-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid). The profile of the reactivity curve of carboxymethylated lobster muscle glyceraldehyde 3-phosphate dehydrogenase is presented in Figure 2. It is apparent that by eliminating reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with the "active site" cysteine residues of lobster muscle glyceraldehyde 3-phosphate dehydrogenase (a) the "initial burst" of reaction is lost, and (b) only 4.5 ± 0.5 SH groups are free to react, as compared with 19.5 ± 0.5 in the unmodified enzyme.

The reactivity of the SH groups of oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase toward 5,5′-dithiobis(2-nitrobenzoic acid) was examined in a similar manner. Following a short lag period, during which little or no reaction took place, a total of 11.0 ± 0.5 SH groups were titrated per mole of enzyme within a 20-min period (Figure 4).

Titration of Native, Carboxymethylated, and Oxidized Lobster Muscle Glyceraldehyde 3-Phosphate Dehydro-

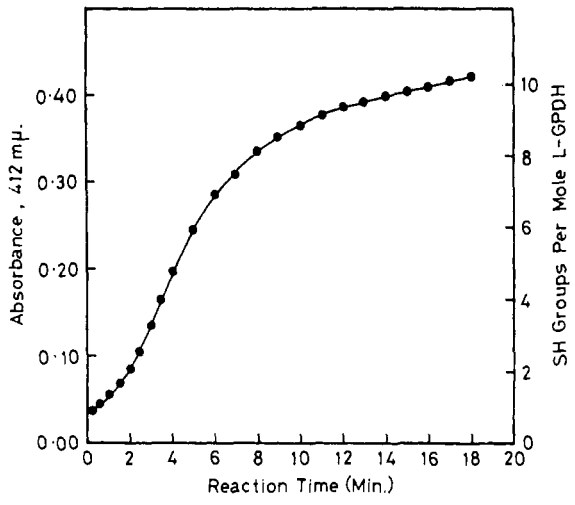


FIGURE 4: Reaction of oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase with 5,5′-dithiobis-(2-nitrobenzoic acid) under nondenaturing conditions. Iodosobenzoate treated lobster muscle glyceraldehyde 3-phosphate dehydrogenase (10 mg/ml) (see Materials and Methods) was dialyzed *vs.* 0.01 M phosphate buffer (pH 8.0), prior to use. The enzyme (0.42 mg/ml) was titrated with a 40-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) (120 μM) in 0.1 M phosphate buffer (1 mM EDTA) (pH 8.0) at room temperature. Calculations were carried out as described in Figure 1.

genase with 5,5′-Dithiobis(2-nitrobenzoic Acid) in the Presence of Urea. The effect of low concentrations of urea on the reactivity of the SH groups of native, carboxymethylated, and oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase was examined. The enzyme mixtures were incubated in the presence of urea (pH 8.0) at room temperature for 15 min, just prior to the addition of 5,5′-dithiobis(2-nitrobenzoic acid). A sample containing no urea was treated in an identical manner.

Low concentrations of urea increased significantly the rate of reaction of the SH groups of both native and oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase (Figures 5 and 6). However, no change was observed in the extent of the "initial burst" of reaction (4.5 ± 0.5 SH groups) between native lobster muscle glyceraldehyde 3-phosphate dehydrogenase and 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of up to 2 M urea. Low concentrations of urea (0.5 M) had no effect on the reactivity of the SH groups of carboxymethylated lobster muscle glyceraldehyde 3-phosphate dehydrogenase.

Discussion

The differential reactivity of the cysteine residues of a protein toward a particular chemical reagent is attributable to a number of factors, most important of which are the state of ionization and/or the accessibility of the SH groups (for review, see Boyer, 1959; Cecil, 1963; Webb, 1966). The properties which determine reactivity are conferred on the SH groups by their "local environment" and, therefore, depend upon the over-all three-dimensional structure of the protein. Consequently, a study of the reactivity of the SH groups of a protein can provide information of a structural nature (Guidotti, 1965, 1967), especially when the amino acid sequence of the protein is available to aid interpretation of the chemical data.

It is well established that SH groups participate directly in the reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase (Krimsky and Racker, 1955; Koeppe *et al.*, 1956; Harris *et al.*, 1963). The primary structure of lobster muscle glyceraldehyde 3-phosphate dehydrogenase, determined by Davidson *et al.* (1967), includes five cysteine residues per polypeptide chain of 333 amino acids, cysteine residue 148 being the "active site" SH group. Titrations of native, carboxymethylated, and oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase with 5,5′-dithiobis(2-nitrobenzoic acid) in 8 M urea, confirm that there are 20 cysteine residues/140,000 g of enzyme.

The exceedingly high rate of reaction of "active site" cysteine residue 148 toward 5,5′-dithiobis(2-nitrobenzoic acid) is consistent with its reactivity toward a number of other SH group reagents; among these reagents are iodoacetic acid (Davidson *et al.*, 1967; Trentham, 1968), 2-acetamido-4-nitrophenol (Kirtley and Koshland, 1966), *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (Gold and Segal, 1964), and β-(2-furyl)acryloyl phosphate (Malhotra and Bernhard, 1968). The high reactivity of this particular SH group is probably due to its nucleophilic character, attested to by the abnormally low p*K*, 5.4, which it has been assigned (Boross and Cseke, 1967). The low p*K* of cysteine residue 148, however, is markedly dependent upon the presence of NAD^+; Behme and Cordes (1967) estimated that the p*K* of this residue is 8.1 in the apoenzyme. Indirect evidence for the effect of coenzyme on the nucleophilicity of cysteine residue 148 comes from inhibition studies of lobster muscle glyceraldehyde 3-phosphate dehydrogenase. Carboxymethylation of the "active site" SH group by iodoacetic acid takes place at a much faster rate in the presence, rather than in the absence, of bound NAD^+ (Trentham, 1968; H. C. Watson and P. M. Wassarman, unpublished observations), quite the opposite effect of that observed with other dehydrogenases (Li and Vallee, 1965; Rabin *et al.*, 1968). The increased nucleophilicity of cysteine residue 148 may be due to the direct interaction of this residue with the coenzyme. However, it is more likely that the binding of NAD^+ alters the conformation of the "active center" of the enzyme, such that (an) amino acid side chain(s), now juxtaposed to cysteine residue 148, can either draw a proton away from its sulfur atom or change its local electric field. It is apparent that the p*K* of a cysteine residue is greatly influenced by neighboring ionic groups, a positively charged field lowering the p*K* (Edsall and Wyman, 1958). This may be particularly pertinent to the reactivity of the "active site" SH group of glyceraldehyde 3-phosphate dehydrogenase, since it has been shown that at alkaline pH migration of acetyl groups from *S*-acetylcysteine ("active site" SH group) to a specific lysine residue takes place, to yield *N*-acetyl enzyme (Polgar, 1966; Harris and Polgar, 1965). In the case of lobster muscle glyceraldehyde

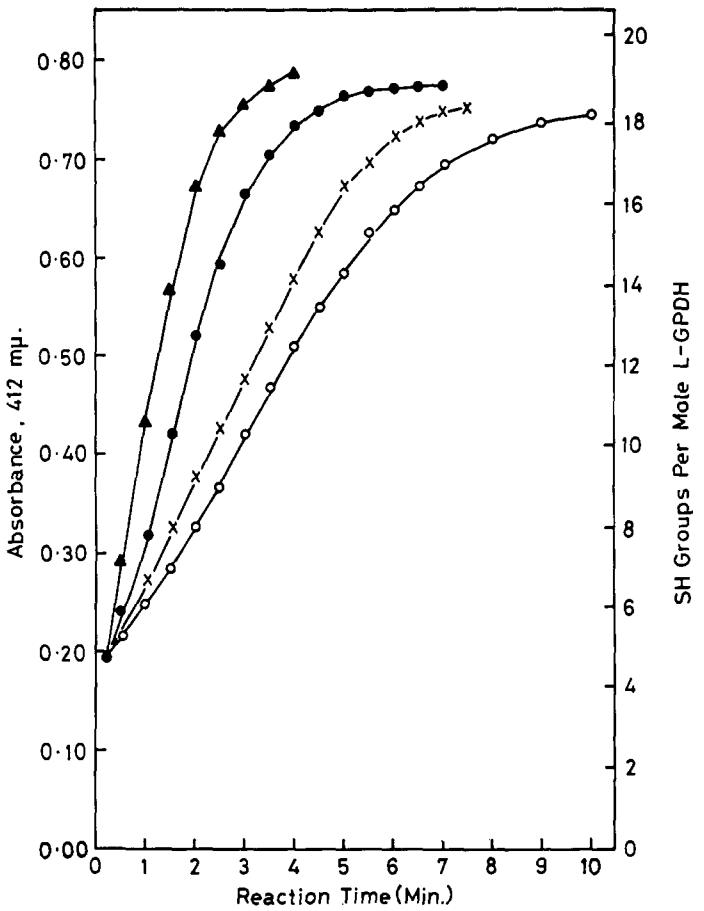


FIGURE 5: Reaction of native lobster muscle glyceraldehyde 3-phosphate dehydrogenase with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of urea. Lobster muscle glyceraldehyde 3-phosphate dehydrogenase (0.42 mg/ml) was incubated for 15 min in 0.1 M phosphate buffer (1 mM EDTA (pH 8.0) at room temperature in the presence of no urea (○), 0.2 M (x), 0.5 M (●), and 1.0 M (▲) urea. The enzyme was then titrated with a 40-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) (120 μM) at room temperature. Calculations were carried out as described in Figure 1.

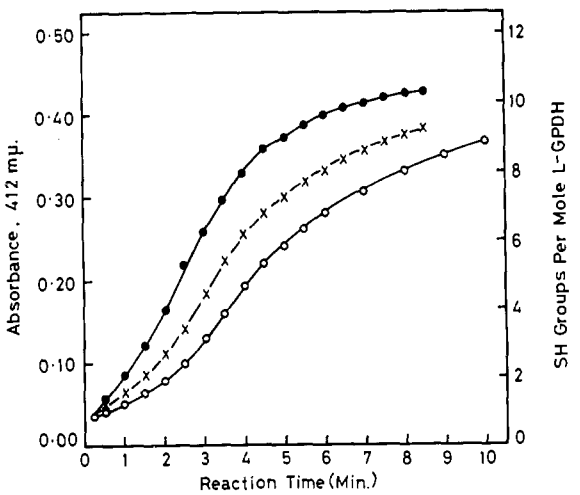
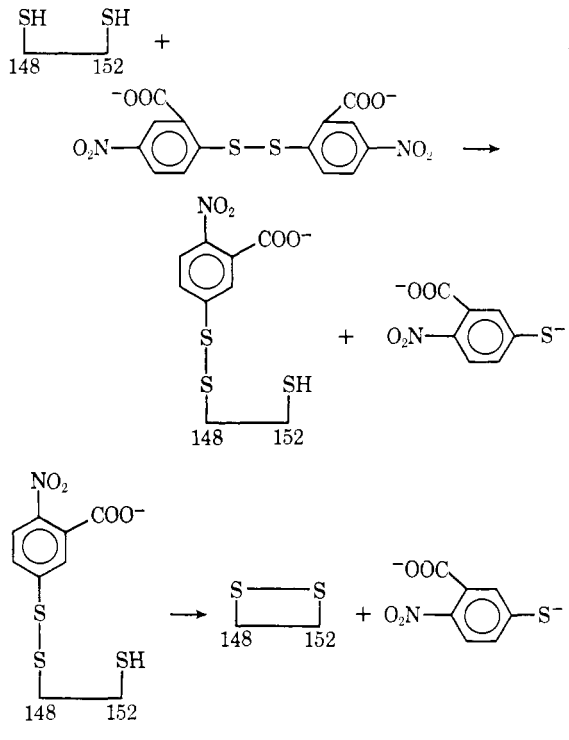


FIGURE 6: Reaction of oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase with 5,5′-dithiobis-(2-nitrobenzoic acid) in the presence of urea. Iodosobenzoate treated lobster muscle glyceraldehyde 3-phosphate dehydrogenase (10 mg/ml) (see Materials and Methods) was dialyzed *vs.* 0.01 M phosphate buffer (pH 8.0) prior to use. The enzyme (0.42 mg/ml) was incubated for 15 min in 0.1 M phosphate buffer (1 mM EDTA) (pH 8.0) at room temperature in the presence of no urea (○), 0.2 M (x), and 0.5 M (●) urea. The enzyme was then titrated with a 40-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) (120 μM) at room temperature. Calculations were carried out as described in Figure 1.

3-phosphate dehydrogenase the transfer occurs between cysteine residue 148 and lysine residue 182 (Davidson *et al.*, 1967). Such evidence indicates the close proximity of the two reactive residues and could account for a cationic environment at the "active center" of lobster muscle glyceraldehyde 3-phosphate dehydrogenase. With regard to the occurrence of a conformational change at the "active center" it is important to note that significant changes in the physical and catalytic properties of the enzyme accompany coenzyme binding (Elödi and Szabolcsi, 1959; Park *et al.*, 1961; Krimsky and Racker, 1963; Listowsky *et al.*, 1965). Crystallographic studies of lobster muscle glyceraldehyde 3-phosphate dehydrogenase (Watson and Banaszak, 1964; Wassarman and Watson, 1968) and of other dehydrogenases (Zeppezauer *et al.*, 1967; M. G. Rossmann, personal communication) suggest that the addition of NAD^+ to the apoenzyme changes the molecular symmetry of the enzyme, indicative of a conformational change.

Titrations of native and carboxymethylated lobster muscle glyceraldehyde 3-phosphate dehydrogenase with 5,5′-dithiobis(2-nitrobenzoic acid) demonstrate considerable differences in the rates of reactivity of their SH groups. Only 4 of the 16 available SH groups of carboxymethylated lobster muscle glyceraldehyde 3-phosphate dehydrogenase can be titrated with 5,5′-dithiobis(2-nitrobenzoic acid); 12 SH groups are com-

pletely unreactive toward this reagent. Titration of native lobster muscle glyceraldehyde 3-phosphate dehydrogenase with 5,5′-dithiobis(2-nitrobenzoic acid), on the other hand, accounts for all 20 SH groups/mole of enzyme. X-Ray diffraction studies have shown that lobster muscle glyceraldehyde 3-phosphate dehydrogenase does not undergo a measurable conformational change concominant with the carboxymethylation of cysteine residue 148 (Wassarman and Watson, 1968). It must be assumed, therefore, that the inability of 12 SH groups/mole of carboxymethylated lobster muscle glyceraldehyde 3-phosphate dehydrogenase to react with 5,5′-dithiobis(2-nitrobenzoic acid) is not due to a structural change. In fact, these results suggest that only 8 SH groups are actually accessible to 5,5′-dithiobis(2-nitrobenzoic acid) in the native enzyme, 4 of these being "active site" cysteine residue 148. Reaction of the "active site" with 5,5′-dithiobis(2-nitrobenzoic acid) apparently triggers a conformational change which unmasks the remaining SH groups. However, we suggest it is not this reaction directly, but rather the subsequent formation of an intramolecular disulfide bond between cysteine residues 148 and 152, which is responsible for the conformational change. The disulfide bridge could form in the following manner



The lack of stoichiometry between moles of SH groups titrated and moles of 5,5′-dithiobis(2-nitrobenzoic acid) present at low molar ratios of 5,5′-dithiobis(2-nitrobenzoic acid) to lobster muscle glyceraldehyde 3-phosphate dehydrogenase could result from this reaction scheme. The spectrophotometric assay recommended by Ellman (1959) depends upon the quantitative release of only 1 mole of the colored anion of thionitrobenzoate/mole of 5,5′-dithiobis(2-nitrobenzoic acid) reacted (or per mole of SH group reacted). The sequence of reactions presented above would account for the lack of stoichiometry since 2 moles of thionitrobenzoate ion would be relased per mole of 5,5′-dithiobis(2-nitrobenzoic acid) reacted.

Mild oxidation of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase with iodosobenzoate has been shown to stimulate the phosphatase activity of the enzyme (Rafter and Colowick, 1957) and to inhibit the dehydrogenase (Park *et al.*, 1961) and esterolytic (Olson and Park, 1964) activities of glyceraldehyde 3-phosphate dehydrogenase. Perham (1964) demonstrated that in the presence of stoichiometric amounts of iodosobenzoate an intramolecular disulfide bridge was formed between the two cysteine residues at the "active center" of glyceraldehyde 3-phosphate dehydrogenase, which accounted for the observed effects on catalysis. In the case of lobster muscle glyceraldehyde 3-phosphate dehydrogenase the disulfide bridge is formed between cysteine residues 148 and 152 (Davidson *et al.*, 1967).

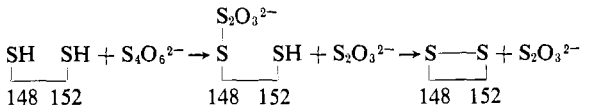
Titrations of oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase indicate that all of the free SH groups per mole of enzyme (11.0 ± 0.5) are accessible to 5,5′-dithiobis(2-nitrobenzoic acid), in sharp contrast to the results obtained with carboxymethylated enzyme (4.5 ± 0.5 SH groups). The differential reactivity of the SH groups of carboxymethylated and oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase suggests that the formation of an intramolecular disulfide bond between cysteine residues 148 and 152 causes the enzyme to undergo a conformational change. This change could explain our observation that the crystallizing properties of oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase resemble those of the apoenzyme, rather than those of the holoenzyme. In this regard it should be noted that Astrachan *et al.* (1957) found that oxidation of the SH groups of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase increased the availability of its bound NAD^+ to other enzymes (*e.g.*, pyrophosphatase and deaminase), suggestive of a structural rearrangement at the "active center."

Intramolecular disulfide formation, *via* the scheme proposed for 5,5′-dithiobis(2-nitrobenzoic acid) reacted lobster muscle glyceraldehyde 3-phosphate dehydrogenase, may account for the effect of sodium tetrathionate on the dehydrogenase activity of the enzyme. Allison and Kaplan (1964a) showed that tetrathionate was a stoichiometric inhibitor of lobster muscle glyceraldehyde 3-phosphate dehydrogenase and indicated that modification of the "active site" cysteine residues led to inactivation of the enzyme in two steps

$$\text{active state (I)} \underset{\text{RSH}}{\overset{S_4O_6^{2-}}{\rightleftharpoons}} \text{reversibly inactive state (II)} \rightarrow \text{irreversibly inactive state (III)}$$

Gel diffusion and complement fixation experiments, using an antibody directed against the fully active holoenzyme, indicated that the reversibly inactivated enzyme (state II) was more antigenic than the irre-

versibly inactivated enzyme (state III). It was concluded that the changes in antigenic activity, following treatment with tetrathionate, were caused by changes in the tertiary structure of lobster muscle glyceraldehyde 3-phosphate dehydrogenase. These structural changes were reflected in the increased susceptibility of the irreversibly inactivated enzyme to proteolysis by trypsin (relative to native lobster muscle glyceraldehyde 3-phosphate dehydrogenase). The two inhibitory states described by Allison and Kaplan (1964) may be analogous with those proposed for 5,5′-dithiobis(2-nitrobenzoic acid) reacted lobster muscle glyceraldehyde 3-phosphate dehydrogenase



Finally, 5,5′-dithiobis(2-nitrobenzoic acid) titrations of native, carboxymethylated, and oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase, carried out in the presence of low concentrations of urea, support the thesis that a conformational change occurs upon formation of an intramolecular disulfide bridge at the "active center" of the enzyme. Urea, at concentrations as low as 0.1–0.5 M, increases significantly the rate of reaction of the SH groups of native and oxidized lobster muscle glyceraldehyde 3-phosphate dehydrogenase with 5,5′-dithiobis(2-nitrobenzoic acid probably due to further unfolding of the polypeptide chains of the enzyme. Retention of both the "initial burst" (4.5 ± 0.5 SH groups) of native lobster muscle glyceraldehyde 3-phosphate dehydrogenase and the normal 5,5′-dithiobis(2-nitrobenzoic acid) titration curve of carboxymethylated lobster muscle glyceraldehyde 3-phosphate dehydrogenase (4.5 ± 0.5 SH groups) suggests that the urea affects the accessibility of the SH groups only after intramolecular disulfide formation, *i.e.*, only after reaction of cysteine residue 148 with 5,5′-dithiobis(2-nitrobenzoic acid), and does not affect the native conformation prior to this reaction.

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