The Mechanism of Inactivation of Glyceraldehyde 3-Phosphate Dehydrogenase by Tetrathionate, o-Iodosobenzoate, and Iodine Monochloride*

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SUMMARY

The reversible and irreversible inactivation of pig muscle glyceraldehyde 3-phosphate dehydrogenase (n-glyceraldehyde-3-phosphate:nicotinamide adenine dinucleotide oxidoreductase (phosphorylating), EC 1.2.1.12) with tetrathionate, o-iodosobenzoate, and iodine monochloride has been studied. This investigation has revealed that tetrathionate reacts stoichiometrically with the catalytically active sulfhydryl group of the enzyme to form a sulfenyl thiosulfate derivative under reversible conditions. The sulfenyl thiosulfate derivative of the enzyme is stable at 0$, but decomposes at higher temperatures. During this decomposition the enzyme is irreversibly inactivated. The experimental evidence presented strongly indicates that the catalytically active sulfhydryl group is converted to a stabilized sulfenic acid during its stoichiometric reaction with o-iodosobenzoate at 0°. The reaction of iodine monochloride is complex. However, at 0° it reacts with the catalytically active sulfhydryl group of the enzyme. The product of this reaction is in part a sulfenyl iodide derivative and in part what again appears to be a sulfenic acid derivative of this essential sulfhydryl group. At 26° iodine monochloride irreversibly inactivates the enzyme. During this irreversible inactivation, iodine is not substituted into tyrosine or histidine.

In addition to the pyridine nucleotide-linked oxidative phosphorylation of aldehydes to acyl phosphates, glyceraldehyde 3-phosphate dehydrogenase, under appropriate conditions, catalyzes the hydrolysis of p-nitrophenyl acetate and the transfer of the acyl group of acyl phosphate substrates to a number of acceptors (1–4). It has been shown that all of these activities proceed with the formation of a thiol ester intermediate (4, 5). It is therefore not surprising that the catalytic activity of glyceraldehyde 3-phosphate dehydrogenase is extremely sensitive to sulfhydryl reagents. The enzyme is irreversibly inactivated by a number of alkylating reagents which react with the catalytically active cysteine residue (6, 7). It is also inactivated by a number of reagents which oxidize simple thiols to their corresponding disulfides. Among these reagents are o-iodosobenzoate (8), tetrathionate (9, 10), iodine (11), and certain heavy metal ions (12). Under mild conditions of incubation, the inactivation by the latter reagents can be completely overcome by the addition of excess thiol.

Harris and Perham (13) and Davidson et al. (14) have established that the glyceraldehyde 3-phosphate dehydrogenases isolated from pig and lobster muscle are each composed of four identical polypeptide chains and that each of these chains contains a catalytically active cysteine residue. Comparative studies on the primary structures of the glyceraldehyde 3-phosphate dehydrogenases isolated from 14 species which include Escherichia coli, yeast, three arthropods, two fish, three birds, and four mammals have shown that the amino acid sequence around the catalytically cysteine residue in all of these enzymes is invariant over a range of 12 amino acids (15, 16). This invariant sequence is: Ser–Asp–Ala–Ser–Cys–Thr–Thr–Asn–Cys–Leu–Ala–Pro. The catalytically active cysteine residue in this sequence is marked with an asterisk. Since a unique feature of this invariant sequence is the presence of a 2nd cysteine residue four amino acids removed on the carboxyl side of the catalytically active cysteine residue, it is tempting to suggest that the reversible inactivation of glyceraldehyde 3-phosphate dehydrogenase by oxidative reagents is due to the formation of an intra chain disulfide bond between the catalytically active cysteine residue and this second invariant cysteine 4 residues removed from it. The fact that the glyceraldehyde 3-phosphate dehydrogenases isolated from yeast (11)...
and E. coli (17) are reversibly inactivated by iodine, o-iodosobenzoate, and tetrathionate lends support to this argument. Both of these enzymes appear to contain only 2 cysteines per monomer, both of which reside in the invariant sequence shown above (15, 16). Harris and Perham (18) have reported that the inactivation of pig muscle glyceraldehyde 3-phosphate dehydrogenase by 1 eq per eq of enzyme monomer is accompanied by the simultaneous disappearance of the two sulfhydryl groups that occur in the invariant active center sequence. From their experiments they concluded that inactivation of the enzyme by iodosobenzoate is due to the formation of an intrachain disulfide bridge between these 2 cysteine residues. Since iodosobenzoate inhibition can be reversed by thiols, they suggested that such a reversible oxidation of these 2 cysteine residues may have a physiological role in controlling the concentration in vivo of active enzyme.

On the other hand, observations from several laboratories suggest that the formation of an intrachain disulfide bond does not occur during the reversible oxidation of glyceraldehyde 3-phosphate dehydrogenase by o-iodosobenzoate, tetrathionate, and iodine. With the use of 35S-labeled tetrathionate, Pihl and Lange have shown that radioactive thiosulfate remains protein-bound during the reversible inactivation of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. Since the amount of 35S bound during the inactivation was equivalent to the disappearance of sulfhydryl groups titrable with hydrosulfite, this specific activity increased to 60,000 units per mg of enzyme, which is inactive. Jakoby has shown that arsenite does not inhibit the dehydrogenase activity of glyceraldehyde 3-phosphate dehydrogenase (19), and Olson and Park have shown that the esterase activity is unaffected by arsenite (20). If the 2 cysteine residues at the active center were to form a disulfide bond upon oxidation, one would expect that they would readily join in complex with arsenite, a reagent which is known to react with vicinal diols (21, 22).

Chemical product of the catalytically active cysteine residue of pig muscle glyceraldehyde 3-phosphate dehydrogenase when it is oxidized with tetrathionate, o-iodosobenzoate, and iodine.

**EXPERIMENTAL PROCEDURE**

**Materials**—Pig muscle glyceraldehyde 3-phosphate dehydrogenase was prepared according to the procedure of Elodi and Sorenyi (23) with the following modification. After the first crystallization the enzyme was dialyzed against 0.05 M NaPO4, pH 7.5, which contained 10-3 M EDTA and 10-3 M ß-mercaptoethanol. The protein which precipitated during dialysis was discarded. Batches, 4 g each, of the dialyzed enzyme in 100 ml methanol were then subjected to gel filtration on a column, 5 x 100 cm, of Sephadex G-100 equilibrated with 0.05 M NaPO4, pH 7.5, which contained 10-3 M EDTA and 10-3 M ß-mercaptoethanol. This step removed significant amounts of a brown protein of lower molecular weight from the enzyme. The protein was then recrystallized and was stored as a crystalline suspension in 0.75 saturated ammonium sulfate which contained 10-3 M EDTA. Protein concentrations were determined spectrophotometrically with the use of the absorption coefficient of E280 cm = 9.0 at 280 mg.

The dihydrate of sodium tetrathionate was prepared in the same manner from 35S-thiosulfate (inner sulfur atom labeled). The 35S-thiosulfate was purchased from Trace-System.

Ellman's reagent (5,5'-dithio bis(2-nitrobenzoic acid)) was purchased from Nutritional Biochemicals. Cleland's reagent (dithiothreitol) was purchased from Calbiochem. Solutions of o-iodosobenzoic acid (0.1 M each) were made in 0.1 M NaOH from the recrystallized reagent that was purchased from Mann.

35S o-Iodosobenzoic acid was prepared from 35S-thiosulfate as described by Meyer (24). 35S-o-Iodosobenzoic acid was prepared by exchange as described by Tubis, Eford, and Rawliss (25).

Solutions of iodine monochloride were prepared according to McFarlane (26). 124ICl was prepared by the addition of small amounts of carrier-free 129-potassium iodide to solutions of ICI. The radioactive KI was purchased from Iso/serv Corporation.

**Methods**—The enzyme was assayed as described previously (11) with the exception that the assay mixture contained 0.15 ß-mole of d-glyceraldehyde 3-phosphate (added as the DL-mixture) instead of 0.75 ß-mole of d-glyceraldehyde 3-phosphate added as an equimolar mixture of triosephosphate esters.

Immediately before all experiments the enzyme was activated in the following manner. Solutions of the enzyme (2.0% each) in 0.05 M NaPO4, pH 7.5, prepared from a centrifuged pellet of the stock enzyme were incubated with 0.01 M dithiothreitol for 30 min at room temperature. Excess thiol and ammonium sulfate were removed in the cold by passing the enzyme through a column, 1.5 x 33 cm, of Sephadex G-25 (coarse) which was equilibrated with the buffer to be used in the ensuing experiments. Fractions of the effluent (2.0 ml each) were collected. Fractions which contained more than 1.5 mg per ml of enzyme were combined. Before activation the enzyme had a specific activity of 40,000 units per mg per min expressed in the units defined previously. After incubation with dithiothreitol at room temperature, this specific activity increased to 60,000 units per mg per min. After removal of the thiol by gel filtration, the specific activity varied from 45,000 to 60,000 units per mg per min.

Only preparations with specific activities greater than 55,000 units per mg per min were used in the experiments described below.

Radioactivity was monitored with the use of a Nuclear-Chicago Uniflex scintillation counter. The scintillation fluid used was that described by Bray (27).

**RESULTS**

**Experiments with Tetrathionate**

**Stoichiometry of Inactivation**—Pihl and Lange have reported that a 3-fold m excess of tetrathionate completely inactivated rabbit muscle glyceraldehyde 3-phosphate dehydrogenase with the concomitant loss of three sulfhydryl groups which can be titrated with hydroxymercureibenzoate (9). Fig. 1 shows that pig muscle glyceraldehyde 3-phosphate dehydrogenase, when maximally activated as described under "Methods," is stoichiometrically inactivated by the addition of a 4-fold m excess of tetrathionate. This inactivation is complete within 2 min at 0 and can be reversed completely by the addition of excess ß-mercaptoethanol or dithiothreitol, provided that the inactivated
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enzyme is incubated at 0° and that the thiol is added within 1 hour after the introduction of tetrathionate.

Heat Lability of Tetrathionate-inactivated Enzyme—It has been shown that the glyceraldehyde 3-phosphate dehydrogenases isolated from a variety of species, when inactivated with tetrathionate and incubated at 37°, show a time-dependent decrease in activity that can be recovered by the addition of thiol (10). The rate of this irreversible inactivation depends upon pH, protein concentration, and the nature and concentration of buffer, and it varies with glyceraldehyde 3-phosphate dehydrogenase isolated from different species. Fig. 2 shows the rate of irreversible inactivation of the tetrathionate-treated pig muscle glyceraldehyde 3-phosphate dehydrogenase in 0.05 M sodium phosphate buffer, pH 7.5, and in 0.05 M sodium pyrophosphate buffer, pH 8.5. Since controls which contain native enzyme are resistant to heat inactivation, this heat lability is characteristic of the tetrathionate derivative of the enzyme.

Experiments with 35S4O6=—Pihl and Lange have suggested that a sulfenyl thiosulfate derivative of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase is formed when the enzyme is inactivated with tetrathionate, according to the equation (9):

$$ ESH + S_4O_6^- \rightarrow E S-S-S-^- + S_2O_3^- + H^+ $$

The experimental evidence which led Pihl and Lange to reach this conclusion is the following. When the rabbit muscle enzyme was inactivated with 35S4O6= and the reaction mixture was subjected to paper electrophoresis, roughly half of the radioactivity remained protein-bound. Moreover, the gram atoms of 35S bound to the protein were equivalent to the number of hydroxymercurobenzoate-titratable groups which disappeared during the inactivation (9).

Similar experiments have been carried out with pig muscle glyceraldehyde 3-phosphate dehydrogenase, with the exception that gel filtration rather than paper electrophoresis was used to separate the protein-bound radioactivity from the non-protein bound radioactivity. Fig. 3 shows the profile of radioactivity obtained in such an experiment when 0.193 μmole of enzyme was inactivated with 0.094 μmole of 35S4O6=. The enzyme in this reaction mixture was inactivated by 59%. The ratio of the...
radioactivity in the protein bound peak to that in the non-protein-bound peak is 0.81.

When the enzyme, which was completely inactivated by the addition of 1.19 moles of \( \text{Sb}^4\text{O}_7 \) per mole of subunit at \( 0^\circ \), was incubated at \( 37^\circ \) the profile of radioactivity in gel filtration experiments changed with time as shown in Table I. As the enzyme was irreversibly inactivated the amount of \( ^{35}\text{S} \) which remained bound to the enzyme decreased. This decrease in protein-bound radioactivity was roughly equivalent to the degree of irreversible inactivation. These experiments strongly suggest that a sulfenyldisulfate derivative of the catalytically active sulphydryl group is formed during the reversible inactivation at low temperature and that this derivative reacts with a second sulphydryl group in the enzyme to form a disulfide bond and liberate free thiosulfate as shown in Equations 2 and 3.

To show that the stability of the sulfenyldisulfate derivative is a characteristic of the folded structure of the enzyme, the following experiment was carried out. The enzyme was inactivated by the addition of 1 mole of \( \text{Sb}^4\text{O}_7 \) per mole of subunit at \( 0^\circ \) in 1 ml of 0.05 M sodium phosphate, pH 7.5. Then solid urea was added to a final concentration of 8 M. This reaction mixture was then subjected to gel filtration on a column, 1.5 \( \times \) 33 cm, of Sephadex G-25 that was equilibrated and eluted with 0.05 M sodium phosphate, pH 7.5, which contained 8 M urea. The radioactivity and optical density at 280 nm of the collected fractions of the column effluent were determined. None of the radioactivity in the column effluent was associated with the protein. Therefore, dissociation of the enzyme with urea leads to the destruction of the sulfenyldisulfate derivative of the enzyme. The reactions which probably occurred during this experiment are also illustrated by Equations 2 and 3.

\[
\begin{align*}
\text{SH} + \text{Sb}^4\text{O}_7^- & \rightarrow \text{S-SbO}_7^- + \text{H}^+ \\
\text{SH} & \text{SH} \\
\text{E} + \text{Sb}^4\text{O}_7^- & \rightarrow \text{E} + \text{S-SbO}_7^- + \text{H}^+ \\
\text{SH} & \text{SH} \\
\text{S-SbO}_7^- \text{SH} & \xrightarrow{\text{heat, urea}} \text{E} + \text{S-SbO}_7^- + \text{H}^+ \\
\text{SH} & \text{SH}
\end{align*}
\]

Equations with o-Iodosobenzoate

Rafter was the first to observe that glyceraldehyde 3-phosphate dehydrogenase is inactivated by o-iodosobenzoate and that the inactivation could be reversed by thiols (28). Olson and Park have shown that the addition of 4 moles of o-iodosobenzoate to 1 mole of NAD-free rabbit muscle glyceraldehyde 3-phosphate dehydrogenase completely destroys both the ability to hydrolyze p-nitrophenyl acetate and the dehydrogenase activity. This inactivation occurred with the concomitant disappearance of 2.9 sulphydryl groups which could be alkylated with \( ^{14}\text{C} \)-iodoacetate acid in the absence of urea. When the iodosobenzoate-inactivated enzyme was alkylated with \( ^{14}\text{C} \)-iodoacetate in the presence of 8 M urea, the loss of 6.3 sulphydryl groups/140,000 g of enzyme which could be alkylated was observed (20).

Stoichiometry of Inactivation—Inactivation of pig muscle glyceraldehyde 3-phosphate dehydrogenase occurs slowly at \( 0^\circ \) but is complete in 10 min at \( 25^\circ \). Fig. 4 shows that the inactivation of the enzyme is linear with the concentration of o-iodosobenzoate added until the ratio of the concentration of the inactivator to the concentration of subunit is 0.7, at which point

\[
\begin{align*}
\begin{array}{cccc}
\text{Moles O-Iodosobenzoate/Mole Subunit} & 0.4 & 0.6 & 0.8 & 1.0 & 1.2 & 1.4 \\
\% Activity & 100 & 20 & 20 & 20 & 20 & 20 \\
\end{array}
\end{align*}
\]

more than a stoichiometric amount of o-iodosobenzoate is required for inactivation. The addition of excess dithiothreitol after the 10-min incubation at \( 25^\circ \) completely overcomes the o-iodosobenzoate inactivation.

Heat Lability of o-Iodosobenzoate-inactivated Enzyme—When the pig muscle glyceraldehyde 3-phosphate dehydrogenase
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Figs. 5 and 6. 

Fig. 5. The effect of o-iodosobenzoate inactivation on the heat stability of pig muscle glyceraldehyde 3-phosphate dehydrogenase. Enzyme, 0.015 amole, which had been completely inactivated during a 10-min preliminary incubation in the presence of 0.025 amole of o-iodosobenzoate at 20°, was incubated at 37° in 1.0 ml of 0.1 m sodium phosphate, pH 7.5 ( ), and in 0.05 m sodium pyrophosphate, pH 8.5 ( ). Samples of the reaction mixtures were diluted at the times indicated in 0.01 m dithiothreitol and were incubated and assayed as described in the legend to Fig. 1. A control reaction mixture ( ) which contained 0.015 amole of untreated enzyme was incubated at 37° in 1.0 ml of 0.05 m sodium pyrophosphate, pH 8.5, was diluted and assayed at the times indicated.

which had been reversibly inactivated after a 10-min incubation at 26° was heated at 37°, the amount of activity which could be regained by the addition of excess Cielegand’s reagent decreased with time, as shown in Fig. 5. The rate of this inactivation is greater in 0.05 m sodium pyrophosphate buffer, pH 8.5, than it is in 0.1 m sodium phosphate buffer, pH 7.5. This irreversible inactivation is characteristic of the iodosobenzoate-inactivated enzyme, since the native enzyme is only slightly inactivated after 2 hours at 37°.

Experiments with 131I-o-Iodosobenzoate—In order to see whether a stable o-iodosobenzoate-enzyme complex was the reaction product which caused the reversible inactivation of the enzyme by o-iodosobenzoate, 131I-labeled o-iodosobenzoate was prepared. Pig muscle glyceraldehyde 3-phosphate dehydrogenase, 10.5 mg, was incubated with 0.30 amole of 131I-o-iodosobenzoate for 10 min at 26°, at which time 90% of the enzyme activity disappeared. At the end of this incubation the reaction mixture was subjected to gel filtration on a column of Sephadex G-25. The results of this gel filtration experiment are shown in Fig. 6. Since none of the radioactive was protein-bound in the column effluent and since the protein was still inactive after gel filtration, an enzyme-inhibitor complex was not responsible for the inactivation. After gel filtration 88% of the enzyme activity could be recovered by the addition of excess dithiothreitol. The radioactive material in the small molecule peak was qualitatively identified as o-iodosobenzoate by the determination of the ultraviolet absorption spectrum of this material.

Reaction of o-Iodosobenzoate-inactivated Enzyme with 14C-Thiourea and 35S-Thiosulfate. In order to gain more information about the nature of the oxidation state of the catalytically active sulffhydril group of the enzyme following reversible inactivation with o-iodosobenzoate, the following experiments were carried out. Pig muscle glyceraldehyde 3-phosphate dehydrogenase was inactivated by o-iodosobenzoate and was separated from excess reagent and o-iodosobenzoate by gel filtration. The inactive enzyme was then treated in separate experiments with 125I-, 35S-O3S2-, and 14C-thiourea. Each of these reaction mixtures was subjected to gel filtration and the radioactivity which was bound to the protein was determined. When the reversibly inactivated enzyme was incubated with 125I-, no radioactivity was incorporated into the enzyme. However, in such experiments with radioactive thiosulfate and thiourea, the radioactive reagents were incorporated into the enzyme. When the reversibly inactivated enzyme was incubated with a 2-fold excess of 35S-O3S2, 0.14 mole of radioactive reagent per mole of subunit remained bound to the protein after gel filtration on Sephadex G-25. This radioactivity was removed from the protein by adding dithiothreitol and subjecting the enzyme to a second gel filtration. This treatment reactivated the enzyme. When such an experiment was carried out on the native enzyme, there was no inactivation of the enzyme by 35S-O3S2 and no radioactivity was bound to the enzyme upon gel filtration. Therefore, the radioactive thiosulfate was not contaminated with tetrathionate.

When the reversibly inactivated enzyme was incubated with a 5- and 50-fold excess of 14C-thiourea at 0° for 10 min, 0.10 g atom of 14C remained protein bound after gel filtration on Sephadex G-25. This radioactivity was freed from the protein by the addition of dithiothreitol and subjecting the enzyme to a second gel filtration. Again, this treatment reactivated the enzyme.

Reactivation of o-Iodosobenzoate-inactivated Enzyme with Arsenite—Gutmann has reported that sodium ethyl thiosulfate under alkaline conditions is reduced by arsenite to form ethyl merc-
The enzyme, 0.58 μ mole, was inactivated with 0.60 μ mole of o-iodosobenzoate in 1 ml of 0.05 M sodium phosphate, pH 7.5, which contained 0.4 M (NH₄)₂SO₄ by a 10-min incubation at 20°. The inactivated enzyme was removed from reaction products and (NH₄)₂SO₄ by gel filtration on a column, 1.5 X 33 cm, of Sephadex G-25 which was equilibrated and eluted with 0.05 M sodium phosphate which contained millimolar EDTA. After gel filtration, the inactivated enzyme, 0.05 μ mole, was incubated with sodium arsenate at the concentrations indicated in 0.05 M sodium phosphate, pH 7.5, at 26°. Samples which contained 5 μg of enzyme were withdrawn from these reaction mixtures and assayed at the times indicated.

<table>
<thead>
<tr>
<th>[Arsenite]: [enzyme]</th>
<th>Time at 26°</th>
<th>Activity¹</th>
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<td>1800</td>
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¹ Percentage activity is expressed as the activity restored by reduction with arsenite relative to that of the maximally activated enzyme prior to inactivation. After gel filtration maximal activity could be attained by reduction with dithiothreitol in this experiment.

captan, sodium sulfite, and arsenate (29). He suggested that an intermediate in the reaction might be ethyl sulfenic acid which is reduced by arsenite to form the mercaptan. If the product of the reaction between the catalytically active cysteinic residue of the enzyme and o-iodosobenzoate was a stabilized sulfenic acid, it might be expected on the basis of Gutmann's results and hypothesis that arsenite might reactivate the enzyme oxidized by o-iodosobenzoate. Pig muscle glyceraldehyde 3-phosphate was inactivated with o-iodosobenzoate, separated from iodobenzoate by gel filtration, and was incubated with increasing concentrations of sodium arsenite at 26°. It was found that the enzyme which was inactivated with o-iodosobenzoate was indeed reactivated by arsenite. The rate of the reactivation by arsenite is relatively slow and depends upon the arsenite concentration, as shown in Table II. However, at an 1800 M excess of arsenite over the concentration of oxidized enzyme subunit, 75% of the enzyme activity could be recovered. Under these conditions arsenite did not reduce Ellman's reagent, 5,5'-dithio bis(2-nitrobenzoic acid), as determined spectrophotometrically at 412 nm (30). At the same concentrations arsenite did not reconvert the inactive tetrahydrothione derivative of the enzyme.

Experiments with Iodine Monochloride

Ehring and Colowick (8) have reported that the dehydrogenase activity of rabbit muscle glyceraldehyde 3-phosphate is lost upon the addition of 1.5 moles of iodine per mole of essential sulfhydryl group. Concurrent with the inactivation of dehydrogenase activity, the acyl phosphatase activity which is characteristic of the oxidized form of the enzyme appeared (11, 31). Upon the addition of another 1.5 moles of iodine per mole of essential sulfhydryl group, they reported that the acyl phosphatase activity disappeared and that the dehydrogenase activity could not be recovered by the addition of β-mercaptoethanol (8). The following experiments were carried out to find the nature of the iodine derivative of the enzyme which was characteristic of the reversibly inactivated enzyme.

Stoichiometry of Inactivation—When ICI was added to the enzyme in 0.05 M sodium phosphate buffer, pH 7.5, the dehydrogenase activity disappeared linearly with increasing ICI added. However, 2 moles of ICI per mole of subunit were required for this inactivation, as shown in Fig. 7. The inactivation occurred within 2 min at 0°. When the dehydrogenase activity was completely inactivated by the addition of 2 moles of ICI per mole of subunit, only 60% of the enzyme activity could be recovered by the prompt addition of excess dithiothreitol as shown in the upper curve in Fig. 7. Since the inactivation requires 2 moles of ICI per mole of subunit, it appears that competing or consecutive reactions, or both, occur during the inactivation of dehydrogenase activity. Upon the addition of more than 2.0 moles of ICI per mole of subunit at 0°, the amount of dehydrogenase activity which could be recovered by the addition of dithiothreitol did not decrease linearly, but rather leveled off as shown in Figs. 7 and 9. On the other hand, when the enzyme was inactivated with ICI at 26° the enzyme was irreversibly inactivated. This irreversible inactivation at 26° requires 2 moles of ICI per mole of enzyme subunit, as shown in Fig. 9.

Heat Stability of Enzyme Inactivated at 0° with ICI—When the enzyme which had been completely inactivated by the addition of 2 moles of ICI per mole of subunit was incubated at 26°, the amount of enzyme activity that could be recovered by the addition of dithiothreitol decreased with time, as shown in Fig. 8. In this experiment only 68% of the enzyme activity could be recovered by the prompt addition of excess dithiothreitol to the enzyme after the addition of 2.0 moles of ICI per mole of

![Fig. 7. The stoichiometry of ICI inactivation at 0°. The enzyme, 0.10 μ mole, in 2.4 ml of 0.05 M sodium phosphate, pH 7.5, which contained 0.25 M NaCl was inactivated with increasing concentrations of ICI at 0°. After 2 min, samples were diluted in 0.05 M sodium phosphate, pH 7.5, with and without 0.01 M dithiothreitol. The dilutions were incubated and assayed as described in the legend to Fig. 1. ○, samples assayed in the absence of dithiothreitol; □, samples assayed in the presence of dithiothreitol.](http://www.jbc.org/content/jbc/169/3/185/F7.large.jpg)
subunit at 0°. When the enzyme which had been inactivated with ICl was maintained at 0°, the amount of enzyme activity that could be recovered by the addition of dithiothreitol remained constant even after 30 min. However, when an identical reaction mixture was warmed to 26°, part of the enzyme was irreversibly inactivated, as shown in Fig. 8. With the exception of the initial 30 to 35% irreversible inactivation which occurred immediately at 0°, the irreversible inactivation of the enzyme inactivated with ICl at 26° is reminiscent of the irreversible inactivation of the enzyme inactivated with either tetrathionate or o-iodosobenzoate at 37°.

**Experiments with 125ICl**—The inactivation experiments described above were carried out with 125I-labeled ICl to determine whether substitution reactions occur during the irreversible inactivation of the dehydrogenase activity. The following experiments were carried out at 0° and 26°.

Increasing concentrations of 125ICl were added to the maximally activated enzyme at 0° and 26° in separate experiments. After a 20-min incubation, samples were diluted in buffer which contained 0.01 M dithiothreitol to a final substrate concentration of 100 μg per ml and the dilutions were incubated for 20 min at 25°C, at which time samples of the dilutions which contained 5 μg of enzyme were assayed for activity. β-Mercaptoethanol was added to the remainder of the initial reaction mixtures to a final concentration of 0.1 M at the end of each of the 20-min incubation periods at 0° and 26°. These reaction mixtures were then dialyzed exhaustively against 10⁻³ M β-mercaptoethanol which contained 10⁻³ M EDTA, pH 7.0. At the end of dialysis samples of each reaction mixture were counted to determine the extent of 125I incorporation into the protein. The results of these experiments are illustrated in Fig. 9. The experiments carried out at 0° show that both the reversible and irreversible inactivation of the dehydrogenase activity are not accompanied by substitution of radioactivity into the enzyme which is stable to the addition of thiol. When 125ICl was added to the enzyme at 0°, complete inactivation of the enzyme was observed when 125ICl was present in a 2:1 molar excess over the enzyme subunit concentration. At this point of complete inactivation in the absence of thiol, 60 to 70% of the original activity could be recovered by incubation with dithiothreitol. No significant incorporation of 125I into the enzyme occurred, which shows that the 30 to 35% irreversible inactivation was due to oxidative reactions and not substitution. Fig. 9 shows that the addition of more than 2.0 moles of 125ICl per mole of subunit at 0° leads to significant substitution of 125I into the enzyme. When 4.5 moles of 125ICl are added per mole of subunit at 0°, approximately 0.7 g atom of 125I is substituted per mole of enzyme subunit. However, the substitution reaction which occurs at these high concentrations of ICl is not accompanied by significant inactivation of the dehydrogenase activity, as shown in Fig. 9. The irreversible inactivation promoted by ICl at 0° does not increase linearly with increasing ICl concentration but levels off.
The reaction mixture was subjected to gel filtration on a column, 1.5 X 33 cm, of Sephadex G-25 (coarse) which was equilibrated and eluted with 0.05 M sodium phosphate, pH 7.0. The optical density of the collected fractions (2 ml) was determined at 280 m\( \mu \) (\( \Delta \)). Samples, 50 \( \mu l \), of these fractions were assayed for radioactivity (\( \bullet \)).

**Fig. 10.** The incorporation of \(^{125}\)I in the absence of added thiol during the inactivation of pig muscle glyceraldehyde 3-phosphate dehydrogenase with \(^{125}\)ICl. The enzyme, 0.10 \( \mu mole \), was completely inactivated with 0.21 \( \mu mole \) of \(^{125}\)ICl in 2.4 ml of 0.05 M sodium phosphate, pH 7.5, which contained 0.25 M NaCl at 0\(^\circ\). The reaction mixture was subjected to gel filtration on a column, 33 X 1.5 cm, of Sephadex G-25 (coarse) which was equilibrated and eluted with 0.05 M sodium phosphate, pH 7.0. The optical density of the collected fractions (2 ml) was determined at 280 m\( \mu \) (\( \Delta \)). Samples, 50 \( \mu l \), of these fractions were assayed for radioactivity (\( \bullet \)).

After 2.0 moles of ICl are added per mole of enzyme subunit, 60 to 70% of the dehydrogenase activity could be regained by the addition of excess dithiothreitol reagent and removed from the protein either by subsequent gel filtration or dialysis. In this experiment 0.14 mole of \(^{125}\)I per mole of enzyme subunit remained enzyme-bound in the absence of thiol, suggesting that, at least in part, the reversible inactivation of the enzyme is due to the formation of a sulfenyl iodide derivative of the catalytically active sulfhydryl group. Since approximately 60 to 70% of the enzyme activity can be recovered by the addition of thiol, the reversible inactivation is only in part accountable by sulfenyl iodide formation as assessed by the gel filtration experiment described above. Cunningham and Nuenke (32) have shown that the sulfenyl iodide of \( \beta \)-lactoglobulin reacts with thiourea to form a mixed disulfide according to Equation 4.

\[
\text{PSI} + \text{H}_2\text{N} - \text{C} - \text{NH}_2 \to \text{P} - \text{S} - \text{S} - \text{C} - \text{NH}_2 + \text{H}^+ + \text{I}^- \quad (4)
\]

To pig muscle glyceraldehyde 3-phosphate dehydrogenase which had been inactivated by the addition of 2 moles of ICl per mole of subunit were added 1.6 moles of \(^{14}\)C-thiouracil of known specific activity. Within 5 min after the addition of the radioactive thiouracil the reaction mixture was subjected to gel filtration on a column of Sephadex G-25. Fig. 11 shows the profile of radioactivity of this chromatogram; 0.41 g atom of \(^{14}\)C was bound per mole of subunit applied to the column in this experiment. If it is assumed that a hydrolytic equilibrium of the type described by Equation 5 is perturbed during the gel filtration process, in which the enzyme activity can be recovered by the addition of thiol is only in part accountable by the formation of a sulfenyl iodide derivative of the catalytically active sulfhydryl group. Since approximately 60 to 70% of the enzyme activity can be recovered by the addition of thiol, the reversible inactivation is only in part accountable by sulfenyl iodide formation as assessed by the gel filtration experiment described above. Cunningham and Nuenke (32) have shown that the sulfenyl iodide of \( \beta \)-lactoglobulin reacts with thiourea to form a mixed disulfide according to Equation 4.

\[
\text{ESI} + \text{H}_2\text{O} \rightleftharpoons \text{ESOH} + \text{H}^+ + \text{I}^- \quad (5)
\]

of the \(^{125}\)I-enzyme reaction mixture, the amount of radioactive bound to the enzyme following the reaction of the ICl-treated enzyme with \(^{14}\)C-thiouracil may be a more valid indication of the amount of sulfenyl iodide present before gel filtration. However, the amount of enzyme activity that can be recovered by the addition of thiol is only in part accountable by the formation of a sulfenyl iodide derivative of the catalytically active sulfhydryl group. Since approximately 60 to 70% of the enzyme activity can be recovered by the addition of thiol, the reversible inactivation is only in part accountable by sulfenyl iodide formation as assessed by the gel filtration experiment described above. Cunningham and Nuenke (32) have shown that the sulfenyl iodide of \( \beta \)-lactoglobulin reacts with thiourea to form a mixed disulfide according to Equation 4.

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Reaction of the enzyme inactivated with o-iodosobenzoate by reactivation of the enzyme as shown in Table III. Like the filtration, and incubated with arsenite at 26°. The addition of inactivated with ICl, separated from iodide and chloride by gel filtration, and incubated with arsenite at 26°. The addition of arsenite to the enzyme treated in this manner did lead to a slight reactivation of the enzyme as shown in Table III. The reaction of the enzyme inactivated with o-iodosobenzoate by arsenite, this reactivation is slow and requires high concentrations of arsenite. However, significant enzyme activity can be recovered by arsenite reduction which indicates that the part of the inactive derivative of the enzyme which is not accountable by sulfenic acid is not a disulfide, but rather a sulfenic acid derivative of the catalytically active sulfhydryl group.

**DISCUSSION**

The results presented show that a disulfide bond is not the immediate chemical product when pig muscle glyceraldehyde 3-phosphate dehydrogenase is inactivated with tetrathionate, o-iodosobenzoate, or iodine monochloride. Each of these reagents oxidizes the catalytically active sulfhydryl group of the enzyme to the oxidation state of a sulfenic acid. The evidence of Phil and Lange (9) and that presented in this study are quite convincing that tetrathionate reacts with the catalytically active sulfhydryl group to form a sulfenyl thiosulfate derivative of the enzyme which is inactive. The stability of this derivative is due to the structural characteristics of the enzyme which hold the other sulfhydryl groups along a given polypeptide chain in a masked configuration and prevent the immediate formation of a disulfide bond. The lack of reactivity of the sulfhydryl groups other than the catalytically active cysteine residue of the enzyme with alkylating agents in the absence of dissociating reagents indicates that the sulfhydryl groups other than the ones required for catalysis are buried in the three-dimensional structure of the enzyme. The irreversible inactivation of the enzyme treated with tetrathionate which occurs at higher temperatures is due to the reaction of the sulfenyl thiosulfate derivative of the catalytically active sulfhydryl group with other sulfhydryl groups in the molecule to form disulfide bonds. It seems probable that during this irreversible inactivation a disulfide bond is formed between the catalytically active sulfhydryl group and the neighboring cysteine 4 residues removed from it. Since extensive protein precipitation occurs during the irreversible inactivation, it has not been possible to determine whether this is indeed the case. However, when the sulfenyl thiosulfate derivative of lobster muscle glyceraldehyde-3-phosphate dehydrogenase is dissociated in 8 M urea in the presence of ICl, two sulfhydryl groups which can be alkylated disappear. A radioautograph of a peptide map of the tryptic digest of the lobster muscle enzyme which was carbamoylated in this fashion showed that disulfide bond formation occurred exclusively between the 2 cysteine residues which are 4 residues apart in the primary sequence.

The experimental evidence presented indicates quite strongly, but does not firmly establish, that a sulfenic acid derivative of the catalytically active cysteine residue is formed when glyceraldehyde 3-phosphate dehydrogenase is inactivated with o-iodosobenzoate. Although sulfenic acids have been proposed as intermediates in hydrolytic reactions which involve a number of organic sulfur compounds (33, 34), Fries acid (35), Furanquinone sulfenic acid, and two other closely related compounds (36, 37) are the only sulfenic acids that have been isolated successfully. It has been proposed that the stability of these particular sulfenic acids is probably due to hydrogen bonding or to other strong interactions of the oxidized sulfur with the aromatic 9,10-quinone system (37). Aliphatic sulfenic acids lack this stabilization and therefore cannot be isolated. The stability of the sulfenic acid derivative of the catalytically active sulfhydryl group of glyceraldehyde 3-phosphate dehydrogenase can be attributed to the steric and chemical environment of this modified amino side chain which is provided by the unique three-dimensional structure of the dehydrogenase. The results of this study indicate that a disulfide bond is not formed freely and reversibly between the catalytically active sulfhydryl group and the invariant cysteine residue four amino acids removed from it in the primary structure of glyceraldehyde 3-phosphate dehydrogenase. Therefore, it appears that the reversible formation of such a disulfide bridge does not occur under physiological conditions as has been suggested (18).

Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, when isolated in the absence of heavy metal chelating agents and thiols, as described by Cori, Stein, and Cori requires prior incubation with thiols for full activity (38). This is a characteristic of all of the glyceraldehyde 3-phosphate dehydrogenases isolated (17, 38-40). This activation of the enzyme by thiols has been attributed to the conversion of an oxidized state of the enzyme to a reduced state (41). From the results presented in this study it may be concluded that the oxidized state of the enzyme which occurs during isolations in the absence of thiols and chelating agents does not exist in an intramolecular disulfide form. The catalytically active sulfhydryl group of these reversibly inactivated preparations may exist as a mixed disulfide, heavy metal mercaptide, or stabilized sulfenic acid by reactions which can occur during the preparation and fractionation of the homogenates.
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The Mechanism of Inactivation of Glyceraldehyde 3-Phosphate Dehydrogenase by Tetrathionate, o-Iodosobenzoate, and Iodine Monochloride

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