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THE CONVERSION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE TO AN ACYLPHOSPHATASE BY TRINITROGLYCERIN AND INACTIVATION OF THIS ACTIVITY BY AZIDE AND ASCORBATE

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Summary

Trinitroglycerin oxidizes the essential sulfhydryl group, Cys-149, of pig muscle glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate : NAD^+ oxidoreductase(phosphorylating) EC 1.2.1.12) to a sulfenic acid, not to a disulfide. This conclusion is based on the observation that the inactivation of the dehydrogenase activity of the enzyme by the organic nitrate induces the acylphosphatase activity which is catalyzed by the sulfenic acid form of the enzyme. Inorganic nitrite is released during this process which is stoichiometric with the degree of inactivation of the dehydrogenase. The acylphosphatase activity induced by trinitroglycerin, unlike the dehydrogenase activity, is sensitive to CN^-. Treatment of the enzyme oxidized with trinitroglycerin with $^{14}CN^-$ leads to the incorporation of protein-bound $^{14}CN^-$, which is stoichiometric with the degree of inactivation of the dehydrogenase.

Treatment of the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase at pH 5.3 with a 10-fold molar excess of azide over the concentration of enzyme subunit completely inactivates the acylphosphatase reaction catalyzed by the oxidized enzyme. Concomitantly, the dehydrogenase activity catalyzed by the sulfhydryl form of the enzyme reappears which indicates that excess azide reduces the sulfenic acid which is required for the acylphosphatase. Treatment of the oxidized enzyme with a stoichiometric amount of azide at pH 5.3 stimulates the acylphosphatase activity and does not lead to the reappearance of dehydrogenase activity. When the sulfenic acid form of the enzyme is incubated with 20 mM L-ascorbate at pH 5.3, the acylphosphatase activity is completely inactivated and the dehydrogenase activity catalyzed by

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the reduced form of the enzyme is recovered. Thus, L-ascorbate also reduces the protein sulfenic acid which is required for the acylphosphatase activity.

When glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase(phosphorylating), EC 1.2.1.12) is inactivated with *o*-iodosobenzoate, iodine, or H_2O_2 under non-denaturing conditions, the catalytically active sulfhydryl group of the dehydrogenase (Cys-149) is oxidized to a sulfenic acid [1—4]. The oxidation of Cys-149 to a sulfenic acid converts the dehydrogenase to an acylphosphatase [2,3]. The sulfenic acid derivative of Cys-149 is required for the acylphosphatase activity, since the inactivation of the dehydrogenase activity of glyceraldehyde-3-phosphate dehydrogenase with iodoacetate and tetrathionate, which produce the carboxymethyl and sulfenyl thiosulfate derivatives of Cys-149, respectively, is not accompanied by the appearance of acylphosphatase activity [3]. In addition, the acylphosphatase activity catalyzed by the sulfenic acid form of the enzyme is inactivated by a number of nucleophiles which include thiols, thiourea, thiosulfate, dimedone, cyanide, substituted hydrazines, and semicarbazide [3,5—7]. The inactivation of the acyl phosphatase activity by thiols present in excess is accompanied by the full reactivation of the dehydrogenase reaction catalyzed by the sulfhydryl form of glyceraldehyde-3-phosphate dehydrogenase. The inactivation of the acylphosphatase activity by thiourea and thiosulfate is accompanied by partial reaction of the dehydrogenase activity which is dependent on the concentration of these reagents. The reaction described by Eqn 1, where X^- represents a nucleophile, represents the mechanism by which the nucleophiles inactivate the acylphosphatase reaction catalyzed by the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase. The reaction sequence described by Eqn 1 and Eqn 2 represents the mechanism by which thiols, thiosulfate, and thiourea reduce the sulfenic acid derivative of Cys-149 to a sulfhydryl group.

$$ESOH + X^- \rightleftarrows ESX + OH^- \quad (1)$$

$$ESX + X^- + H^+ \rightleftarrows ESH + X - X \quad (2)$$

It has been shown that [^{14}C]dimedone forms a covalent derivative of Cys-149 when it inactivates the acylphosphatase activity catalyzed by the sulfenic acid form of the enzyme [6]. This supports the reaction scheme described by Eqn 1.

Jakschik and Needleman have reported that trinitroglycerin inactivates glyceraldehyde-3-phosphate dehydrogenase [8]. Since the inactivation by the organic nitrate could be reversed by dithiothreitol and since glutathione is oxidized to its disulfide by trinitroglycerin [9], they concluded that the inactivation of glyceraldehyde-3-phosphate dehydrogenase was due to the oxidation of essential sulfhydryl groups to disulfides. We report here that trinitroglycerin inactivates glyceraldehyde-3-phosphate dehydrogenase by oxidizing Cys-149 to a sulfenic acid. We also report that azide and L-ascorbate inactivate the acylphosphatase reaction catalyzed by the oxidized form of the enzyme by reducing the sulfenic acid derivative of Cys-149 to a sulfhydryl group.

Materials and Methods

Materials

Chemicals were purchased from the following sources: NAD^+ from P-L Biochemicals; *o*-iodosobenzoic acid from Pierce Chemicals; DL-glyceraldehyde-3-phosphate diethylacetal (barium salt) from Calbiochem; $K^{14}CN$ from New England Nuclear; and the sodium salt of L-ascorbate from Calbiochem. Trinitroglycerin was generously supplied as a 10% mixture with lactose by Dr Robert Hosley of Eli Lilly and Co. [^{15}N]potassium azide which contained 99.4 atom % excess ^{15}N in each nitrogen atom was purchased from the Wilmad Glass Co. Glyceraldehyde-3-phosphate dehydrogenase was prepared from swine muscle by the method of Elodi and Szörényi [10] with a modification described previously [1].

Inactivation of the dehydrogenase activity with trinitroglycerin

Inactivation of the enzyme with trinitroglycerin was carried out by incubating 14 mg (0.4 μmol of subunit) of the enzyme with 26.4 μmol of trinitroglycerin in a final volume of 2.0 ml of 50 mM sodium pyrophosphate, pH 8.5, which contained 75 mM lactose, at 35° C for 10 min. The incubation mixtures were then either assayed directly for the acylphosphatase and dehydrogenase activity or filtered through a Sephadex G-25 column (1.2 × 25 cm) to remove compounds of low molecular weight. In most experiments the gel-filtration step was not included since excess reagents and products did not alter the enzyme assays. As a control the enzyme was also treated with 1.6 μmol of *o*-iodosobenzoate under the conditions specified above.

To reactivate the dehydrogenase activity with dithiotreitol, 3.5 mg of the trinitroglycerin—treated enzyme was incubated with 10 μmol of the thiol at 35° C for 25 min and then assayed for both dehydrogenase and acylphosphatase activity.

Inactivation of the acylphosphatase activity with KCN and labelling of the oxidized enzyme with $K^{14}CN$

After the enzyme was incubated with the oxidants as described above, compounds of low molecular weight were separated from the enzyme by filtration through a Sephadex G-25 column (1.2 × 25 cm). KCN, 0.3 μmol, was added to a sample of the enzyme effluent which contained 200 μg of the enzyme and was incubated for 5 min at 23° C at which time it was assayed for the dehydrogenase and acylphosphatase activity.

The enzyme was labelled with $^{14}CN^-$ by incubating 28 mg of the enzyme with 8 μmol of $K^{14}CN$ (4.41×10^5 cpm/μmol) at 35° C for 10 min in the presence of either 26.4 μmol of trinitroglycerin or 0.6 μmol of *o*-iodosobenzoate in 50 mM sodium pyrophosphate, pH 8.5, which contained 75 mM lactose. The final volume was 2.0 ml. After the incubation the reaction mixtures were filtered through a Sephadex G-25 column (1.2 × 25 cm) with 20 mM veronal buffer, pH 7.5. Fractions containing protein were pooled and radioactivity was determined with the use of a Beckman LS-100 liquid scintillation counter.

Oxidation of the enzyme with o-iodosobenzoate

Crystals of the dehydrogenase were collected by centrifugation and were dissolved in 20 mM Veronal, pH 7.6, to give a final protein concentration of 20—25 mg per ml. A neutralized solution of *o*-iodosobenzoate was added to the dissolved enzyme in the presence of 10 mM NAD^+ to a final concentration of 20 mM. After incubating for 1 h at 0°C the oxidized enzyme was removed from excess reagents by gel filtration on a 2.0 × 30 cm column of Sephadex G-25 (medium) which was equilibrated and eluted with the buffer in which the subsequent experiments were carried out.

The determination of $^{30}N_2$ after the addition of ^{15}N-labelled azide to the sulfenic acid form of the glyceraldehyde-3-phosphate dehydrogenase

The various reactions of $K^{15}N_3$ with the oxidized or reduced enzyme were carried out in a dual compartment, tip-in reaction vessel which was attached to a glass manifold which could be alternately evacuated with an oil pump and then flushed with argon. Initially, the reaction vessel contained 1 or 10 μmol of $K^{15}N_3$ in 1.0 ml of 0.1 M sodium acetate, pH 5.0, in one compartment and 36 mg of oxidized or reduced enzyme (1 μmol expressed as subunit concentration) in 10 ml of the same buffer in the other compartment. The reaction vessel was immersed in an ice bath and then carefully evacuated to avoid foaming to a pressure of 0.5 mm of Hg and then flushed with argon. This procedure was repeated 5 times. After the final flush with argon, the reaction vessel was evacuated to 0.5 mm of Hg and the $K^{15}N_3$ and the enzyme were mixed and the reaction vessel was warmed to room temperature in a water bath and incubated for 1 h. Then the reaction mixture was frozen in liquid nitrogen and the sealed reaction vessel was attached to the mass spectrometer for the detection of $^{28}N_2$, $^{30}N_2$, and $^{32}O_2$. A modified version of the mass spectrometer originally described by Nier was used for the gas analyses [11].

Enzyme assays

The dehydrogenase activity was assayed as previously described [1]. The acylphosphatase activity was assayed by the equilibrium displacement method of Ehring and Colowick [2]. In a final volume of 3.0 ml, the assay mixtures contained 20 mM Veronal, pH 7.5, 50 mM potassium phosphate, pH 7.5, 0.5 mM NAD^+, and 0.5 mM D-glyceraldehyde 3-phosphate.

Results

The conversion of glyceraldehyde-3-phosphate dehydrogenase to an acyl phosphatase with trinitroglycerin

Table I shows that incubation of 0.2 mM glyceraldehyde-3-phosphate dehydrogenase (expressed as subunit concentration) with 13.2 mM trinitroglycerin for 10 min at 35°C inactivates the dehydrogenase by about 50%. This inactivation was reversed by dithiothreitol. About 90% of the original dehydrogenase activity reappeared when the trinitroglycerin-treated enzyme was incubated with dithiothreitol. The failure to recover the full original activity is evidently due to the irreversible heat denaturation of the oxidized enzyme [3]. Rafter [12] and Ehring and Colowick [2] have shown that the inactivation of

TABLE I

THE CONVERSION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE TO AN ACYLPHOSPHATASE WITH TRINITROGLYCERIN

The reaction mixtures contained 14 mg of pig muscle glyceraldehyde-3-phosphate dehydrogenase, 26.4 μmol of trinitroglycerin or 1.6 μmol of *o*-iodosobenzoate, and 100 μmol of sodium pyrophosphate (pH 8.5) which contained 75 mM lactose in a final volume of 2.0 ml. The mixtures were incubated for 10 min at 35°C and assayed for the dehydrogenase and acylphosphatase activity. 0.5 ml of the trinitroglycerin-treated enzyme (3.5 mg) was incubated at 35°C for 25 min in the presence of 10 μmol of dithiothreitol. Both the dehydrogenase and the acylphosphatase activity are expressed in μmol of NADH formed. Under the conditions of the assay of the acylphosphatase, formation of 1 μmol of NADH corresponds to 1 μmol of 1,3-diphosphoglycerate hydrolyzed.

Treatment	Dehydrogenase activity		Acylphosphatase activity	
	μmol/mg/min	% Inactivation	μmol/mg/min	%
Native	31.4	0	0	0
o-Iodosobenzoate	0	100	0.73	100
Trinitroglycerin	16.9	46	0.29	40
Trinitroglycerin + dithiothreitol	28.0	11	0	0

glyceraldehyde-3-phosphate dehydrogenase with *o*-iodosobenzoate could be reversed by simple thiols. Table I also shows that the treatment of the enzyme with trinitroglycerin induces the acylphosphatase activity, which disappears upon addition of the dithiothreitol. For comparison, Table I also includes results obtained when the enzyme was treated with 0.8 mM *o*-iodosobenzoate for 10 min at 35°C. The dehydrogenase activity was completely inactivated by *o*-iodosobenzoate under these conditions. The acylphosphatase activity induced by trinitroglycerin is about 40% of that induced by *o*-iodosobenzoate. This value is in good agreement with the degree of inactivation of the dehydrogenase activity by trinitroglycerin.

It was found that 0.174 μmol of inorganic nitrite ion [13] was released during the 10 min incubation of the enzyme with trinitroglycerin. This corresponds to the release of 0.44 μmol of inorganic nitrite per μmol of enzyme subunit when the dehydrogenase is inactivated by 46% by trinitroglycerin.

The inactivation of the acylphosphatase activity by cyanide and the binding of [^{14}C] cyanide to the oxidized enzyme

Table II shows that a low concentration of CN^- inactivates the acylphosphatase activity induced by both trinitroglycerin and *o*-iodosobenzoate. However, CN^- neither affects the dehydrogenase activity of the native enzyme nor the residual dehydrogenase activity of the enzyme treated with trinitroglycerin.

Table III demonstrates that the inactivation of the acylphosphatase activity by cyanide was accompanied by the incorporation of $^{14}CN^-$ into the protein. The native enzyme did not incorporate $^{14}CN^-$ when treated in the absence of oxidants. The amount of radioactivity incorporated corresponds with the degree of inactivation of the dehydrogenase activity by trinitroglycerin and *o*-iodosobenzoate. Under conditions in which the dehydrogenase activity is inactivated by 46% by trinitroglycerin, 0.36 μmol of $^{14}CN^-$ was incorporated per

TABLE II

THE EFFECT OF CYANIDE ON THE DEHYDROGENASE AND THE ACYLPHOSPHATASE ACTIVITIES

The enzyme was treated with the oxidants as described in the legend to Table I and filtered through a Sephadex G-25 column (1.2 × 25 cm). The acylphosphatase activity was then determined with the enzyme (200 μg), which was incubated with 0.3 μmol of cyanide for 5 min at 23°C. The native enzyme was treated in an identical manner in the absence of the oxidants.

Treatment	Dehydrogenase activity (μmol/mg/min)		Acylphosphatase activity (μmol/mg/min)	
	$-CN^-$	$+CN^-$ (0.6 μmol)	$-CN^-$	$+CN^-$ (0.3 μmol)
Native	23.7	23.7	0	—
Trinitroglycerin treated	12.0	12.0	0.26	0
o-Iodosobenzoate treated	0	—	0.65	0.03

μmol of subunit and under conditions in which the dehydrogenase activity was completely inactivated by *o*-iodosobenzoate, 0.91 μmol of $^{14}CN^-$ was incorporated per μmol of subunit.

The reduction of the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase by excess azide

When 1 mM NaN_3 is incubated with 3.6 mg (0.1 μequiv. of enzyme subunit) of oxidized enzyme in a final volume of 1.0 ml of sodium acetate, pH 5.3 at room temperature, the acylphosphatase activity in 0.10 ml samples, which were assayed at pH 7.6, decreases with time as shown in Fig. 1. Under these conditions in which azide was present in a ten-fold molar excess over the concentration of the enzyme expressed as subunit, the acylphosphatase was completely inactivated within 30 min at room temperature. Fig. 1 also shows that the dehydrogenase activity returns in samples of the same reaction mixtures which were diluted and assayed in 0.05 M sodium pyrophosphate, pH 8.5 as the acylphosphatase activity disappears. Treatment of the sulfenic acid form

TABLE III

LABELING OF THE NATIVE AND OXIDIZED GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE WITH [^{14}C]KCN

The enzyme, 28 mg, was incubated with 8 μmol of [^{14}C]KCN (4.41×10^5 cpm/μmol) in the presence of either 26.4 μmol of trinitroglycerin or 1.6 μmol of *o*-iodosobenzoate. The enzyme was separated from the rest of incubation mixtures by filtering through a Sephadex G-25 column (1.2 × 25 cm). Fractions containing protein were pooled and the total radioactivity of the pooled fractions was determined.

Treatment	Protein-bound $^{14}CN^-$	
	cpm	$\frac{\mu mol\ of\ ^{14}CN^-}{\mu mol\ of\ subunit}$
Native	0	0
o-Iodosobenzoate	3.24×10^5	0.91
Trinitroglycerin	1.29×10^5	0.36

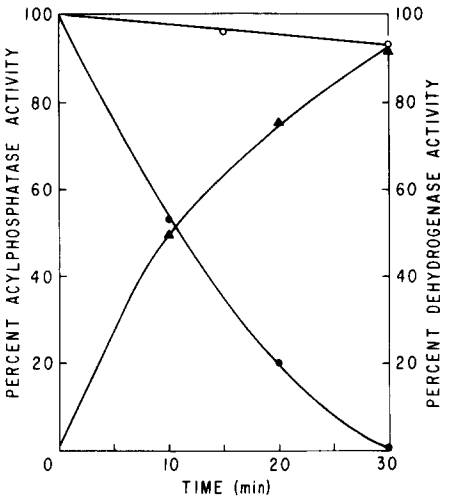


Fig. 1. Reduction of the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase with excess azide. The reaction mixture contained in 1.0 ml of 0.1 M sodium acetate, pH 5.3, 3.6 mg of oxidized enzyme (0.1 μmol expressed as subunit concentration) and 1.0 μmol of NaN_3. At the times indicated 0.10 ml samples of the reaction mixture were withdrawn and assayed for acylphosphatase activity. To assay the dehydrogenase activity, samples of 0.05 ml were diluted at the times indicated in 0.1 M sodium acetate, pH 5.3, to a final enzyme concentration of 0.10 mg per ml. Samples, 0.05 ml, of these dilutions were used to initiate the assay of the dehydrogenase activity within 30 s. Percent reactivation of the dehydrogenase activity was calculated on the basis of the activity of the unoxidized enzyme which had a specific activity of 38 μmol NADH/mg/min. (●), percent acylphosphatase activity of oxidized glyceraldehyde-3-phosphate dehydrogenase treated with NaN_3; (▲) percent dehydrogenase activity reactivated by addition NaN_3 to oxidized glyceraldehyde-3-phosphate dehydrogenase; and (○) percent acylphosphatase activity of an untreated control.

of the enzyme with a ten-fold molar excess of azide under these conditions led to 92% recovery of the dehydrogenase in 30 min.

The reduction of the sulfenic acid form of the enzyme by excess azide is pH dependent as shown in Table IV. Treatment of the oxidized enzymes with a

TABLE IV

REDUCTION OF THE SULFENIC ACID FORM OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE BY EXCESS AZIDE AS A FUNCTION OF pH

The reaction mixtures contained in 1.0 ml of the buffer indicated; 0.96 mg of oxidized enzyme (0.027 μmoles of enzyme expressed as subunit), and 0.3 μmoles of NaN_3. The reaction mixtures were incubated for 30 min at 25° at which time 5 μl samples were withdrawn and assayed for dehydrogenase activity and 0.08 ml samples were withdrawn and assayed for acyl phosphates activity.

Buffer	% Acyl phosphatase activity after 30 min	% Dehydrogenase activity after 30 min
0.1 M Sodium		
acetate (pH 5.3)	0	89
succinate (pH 6.0)	0	92
phosphate		
(pH 6.5)	45	22
(pH 7.0)	96	0
(pH 7.6)	100	0

ten-fold molar excess of azide for 30 min at 25°C does not inactivate the acylphosphatase activity, nor does it lead to a reactivation of the dehydrogenase activity at pH 7.0 and pH 7.6. The acylphosphatase activity is completely abolished when the oxidized enzyme is incubated with a ten-fold molar excess of azide for 30 min at 25°C at pH 5.3 and pH 6.0. Approximately 90% of the dehydrogenase activity catalyzed by the reduced form of the enzyme returns under these conditions. Incubation of the oxidized enzyme with a ten-fold molar excess of azide for 30 min at 25°C at pH 6.5 leads to the partial inactivation (55%) of the acylphosphatase activity and partial reappearance (22%) of the dehydrogenase activity.

A reaction scheme which is consistent with the reduction of the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase by excess azide is described by Eqns 3—5.

$$ESOH + N_3^- \rightarrow ESN_3 + OH^- \quad (3)$$

$$ESN_3 \rightarrow ES\ddot{N}: + N_2 \quad (4)$$

$$ES\ddot{N}: N_3^- + H^+ \rightarrow [ESN_4^-] \rightarrow ESH + 2N_2 \quad (5)$$

Eqn 3 suggests that the reduction is initiated by the reaction of N_3^- with the sulfenic acid at the active site of the acylphosphatase by displacement of hydroxide ion to form a sulfenyl azide. The sulfenyl azide then eliminates N_2 to form a sulfenyl nitrene derivative of the enzyme as described in Eqn 4. The sulfenyl nitrene then reacts with a second molecule of azide to form a reactive intermediate which decomposes to form 2 molecules of N_2 and the sulfhydryl form of the enzyme as described by Eqn 5. The overall stoichiometry of the reaction which is consistent with the 2-electron reduction observed is described by Eqn 6.

$$ESOH + 2N_3^- + H^+ \rightarrow ESH + 3N_2 + OH^- \quad (6)$$

The stimulation of the acylphosphatase activity by the addition of a stoichiometric amount of azide to the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase

If the reduction of the sulfenic acid form of the enzyme by excess azide proceeds by the reactions described by Eqns 3—5, it was expected that the addition of 1 equiv. of N_3^- to 1 equiv. of oxidized enzyme subunit would lead to the inactivation of the acylphosphatase activity which would not be accompanied by the complete recovery of the dehydrogenase reaction catalyzed by the reduced form of the enzyme. Unexpectedly, the addition of 0.1 μmol of NaN_3 to 0.1 μmol of oxidized enzyme subunit in a total volume of 1.0 ml of either 0.10 M sodium acetate or 0.05 M $(NH_4)_2 SO_4$ at pH 5.0 does not inactivate, but rather stimulates the acylphosphatase activity catalyzed by the enzyme when samples were assayed at pH 7.6. In both buffers at pH 5.0 the addition of a stoichiometric amount of azide to the oxidized enzyme stimulated the acylphosphatase activity in a time-dependent manner which was followed by a decrease in activity to its original value. Results similar to those in Fig. 2 were obtained at pH 5.5 in 0.1 M sodium acetate and at pH 6.0 in 0.1 M sodium succinate when the oxidized enzyme was treated with a stoichiometric

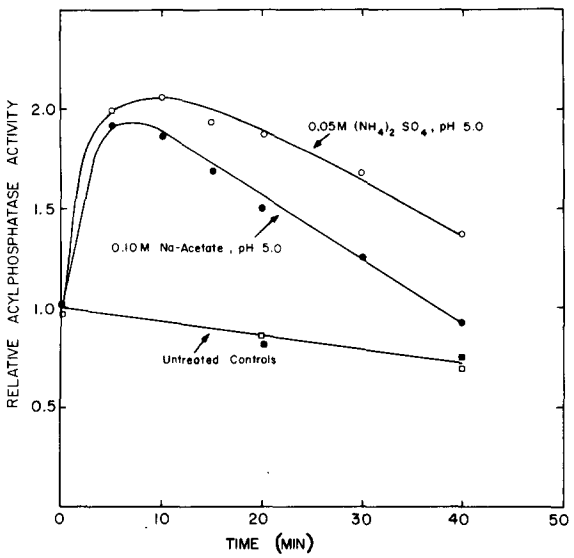


Fig. 2. Stimulation of the acylphosphatase activity by the addition of a stoichiometric amount of azide to the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase. The reaction mixtures contained in 3.0 ml of the buffers indicated 10.8 mg of oxidized glyceraldehyde-3-phosphate (0.3 μmol expressed as enzyme subunit concentration) and 0.3 μmol of NaN_3. At the times indicated 0.10 ml samples were assayed for acylphosphatase activity. (○), the reaction mixture which contained 0.1 mM oxidized glyceraldehyde-3-phosphate dehydrogenase and 0.1 mM NaN_3 in 0.1 M sodium acetate, pH 5.0. (●), the reaction mixture which contained 0.1 mM oxidized enzyme and 0.1 mM NaN_3 in 0.05 M $(NH_4)_2SO_4$, pH 5.0; (□), an untreated control reaction mixture in 0.05 M $(NH_4)_2SO_4$, pH 5.0; and (■) an untreated control reaction mixture in 0.1 M sodium acetate, pH 5.0.

concentration of NaN_3. The maximal stimulation of the acylphosphatase activity observed at pH 5.5 was approximately 35% and the maximal stimulation observed at pH 6.0 was approximately 15%. In all of the experiments in which azide was added to oxidized enzyme in stoichiometric proportions, the acylphosphatase activity was not inactivated nor did any dehydrogenase activity reappear.

The qualitative detection of $^{30}N_2$ after the addition of ^{15}N-labelled azide to the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase

Qualitative evidence is consistent with the stoichiometry suggested by Eqn 3 and Eqn 6 was obtained by the determination by mass spectrometry of the amount of $^{30}N_2$ evolved after the addition of 1 and 10 μmol of $K^{15}N_3$, which was uniformly labelled with a 99.4 atom % excess of ^{15}N, to 1 μmol of oxidized enzyme expressed as subunit concentration.

The results of the gas analyses of reaction mixtures which contained 10 μmol of $K^{15}N_3$ and 36 mg of reduced enzyme, 10 μmol of $K^{15}N_3$ and 36 mg of oxidized enzyme, and 1 μmol of $K^{15}N_3$ and 36 mg of oxidized enzyme are shown in Fig. 3. Qualitatively, these analyses show that no $^{30}N_2$ is formed when a ten-fold molar excess of $K^{15}N_3$ is added to reduced enzyme, $^{30}N_2$ is formed when a ten-fold molar excess is added to oxidized enzyme, and less $^{30}N_2$ is formed when a stoichiometric amount of $^{15}N_3$ is added to oxidized enzyme.

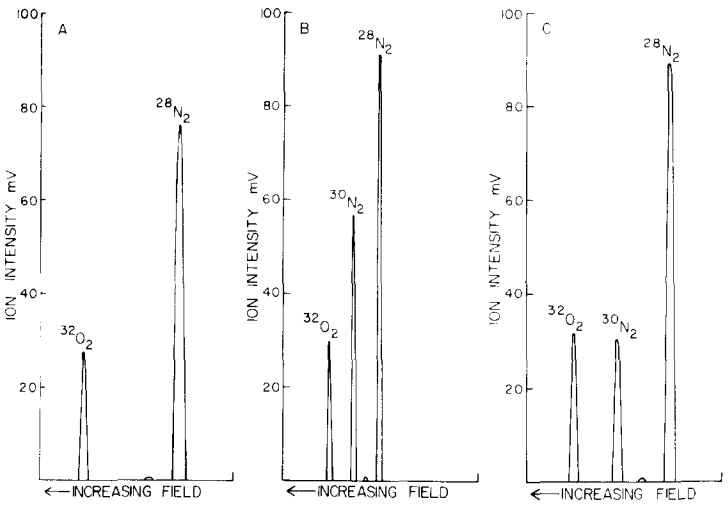


Fig. 3. Qualitative analysis of $^{30}N_2$ after treating oxidized and reduced glyceraldehyde-3-phosphate dehydrogenase with $^{15}N_3$. (A) The mass spectrum after treating 36 mg of reduced enzyme with 10 μmol of $^{15}N_3$ in which each nitrogen contained 99.4 atom % excess of ^{15}N. (B) The mass spectrum after treating 36 mg of oxidized enzyme with 10 μmol of $^{15}N_3$ in which each nitrogen contained a 99.4 atom % excess of ^{15}N. (C) The mass spectrum after treating 36 mg of oxidized enzyme with 1 μmol of ^{15}N. The preparation of the reaction mixtures for gas analysis by mass spectrometry is described in the text.

The reduction of the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase with excess L-ascorbate

Table V shows that the sulfenic acid form of the enzyme is reduced when it is incubated with L-ascorbate at pH 5.3 at 25°C. The acylphosphatase reaction catalyzed by the oxidized enzyme is completely inactivated by 1 mM L-ascorbate within 5 min at this pH. Treatment of the oxidized enzyme with

TABLE V

REDUCTION OF THE SULFENIC ACID FORM OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE WITH L-ASCORBATE AT pH 5.3

The reaction mixtures contained in 1.0 ml of 0.1 M sodium acetate, pH 5.3; 3.96 mg of oxidized glyceraldehyde-3-phosphate dehydrogenase (0.11 μmol of enzyme expressed as subunit) and the concentrations of L-ascorbate indicated. At the times indicated 0.03 ml samples were assayed for acylphosphatase activity. To assay the dehydrogenase activity 0.1 ml samples of the reaction mixture were diluted to 1.0 with 0.1 M sodium acetate, pH 5.3, and within 30 s 10-μl samples of the dilutions were assayed. The dehydrogenase activity is expressed as a percentage of the activity of the unoxidized enzyme which had a specific activity of 30 μmol NADH/mg/min.

[Ascorbate]-(mM)	Incubation time (min)	% Acyl phosphatase activity	% Dehydrogenase activity
0	30	100	0
1	5	0	70
1	10	0	70
1	15	0	70
1	30	0	70
5	30	0	79
10	30	0	83
20	30	0	95

1 mM L-ascorbate leads to the reappearance of 70% of the dehydrogenase activity in 5 min. The reappearance of the dehydrogenase activity did not increase with time when the oxidized enzyme was incubated with 1 mM ascorbate. However, the extent of reduction increased as the ascorbate concentration was increased. Incubation of the oxidized enzyme for 30 min with 20 mM L-ascorbate at pH 5.3 at 25°C led to the reappearance of 95% of the dehydrogenase activity.

The inactivation of the acylphosphatase activity and the reappearance of the dehydrogenase activity when the oxidized enzyme is treated with 20 mM L-ascorbate is pH dependent. Incubation of the oxidized enzyme with 20 mM ascorbate for 30 min at 25°C in 0.1 M sodium phosphate, pH 7.0, inactivates the acylphosphatase activity by 57% and leads to the reappearance of 30% of the dehydrogenase activity. When the oxidized enzyme is incubated with 20 mM L-ascorbate under similar conditions in 0.1 M sodium phosphate, pH 6.5, the acylphosphatase activity in inactivated by 60% and 41% of the dehydrogenase activity is reactivated.

It is interesting to note that treatment of the oxidized enzyme with 1 mM ascorbate at pH 5.3 completely inactivates the acylphosphatase and only 70% of the dehydrogenase activity reappeared. When the oxidized enzyme is treated with 20 mM ascorbate at pH 6.5 and pH 7.0, the reactivation of the dehydrogenase is less than that expected on the basis of inactivation of the acylphosphatase. These observations suggest that a covalent derivative between ascorbate and the oxidized enzyme is present under certain conditions.

Discussion

The results presented indicate that the inactivation of glyceraldehyde-3-phosphate dehydrogenase by trinitroglycerin oxidizes Cys-149 to a sulfenic acid. Since *o*-iodosobenzoate converts the dehydrogenase to an acylphosphatase by the specific oxidation of the catalytically active sulfhydryl group (Cys-149) to a stabilized sulfenic acid [1—3], it can be concluded that trinitro glycerin brings about the same oxidation. However, a relatively high concentration of trinitroglycerin is required to produce only partial oxidation when compared to the oxidation with *o*-iodosobenzoate. The formation of intramolecular disulfide bonds in glyceraldehyde-3-phosphate dehydrogenase results in an extensive conformational change and the enzyme becomes irreversibly inactivated [1,14]. Therefore, the reactivation of the dehydrogenase activity from the trinitroglycerin-treated enzyme with dithiothreitol is not due to the reduction of a disulfide as Jakschik and Needleman concluded [8], but due to the reduction of sulfenic acid to sulfhydryl group.

Needleman and Hunter [9] have shown that the oxidation of glutathione by trinitroglycerin is accompanied by the liberation of inorganic nitrite ion. The oxidation of the essential sulfhydryl group of glyceraldehyde-3-phosphate dehydrogenase by trinitroglycerin is also accompanied by the release of nitrite.

Allison and Connors [3] have reported that the acylphosphatase reaction catalyzed by the sulfenic acid form of the enzyme is inactivated by various nucleophiles such as cyanide and thiosulfate, and proposed the following reaction for this inactivation: $ESOH + X^- \rightarrow ESX + OH^-$, where X^- denotes the

nucleophile. The labelling of the acylphosphatase with $^{14}CN^-$ supports this mechanism.

Since the acylphosphatase activity, catalyzed by the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase, is inactivated by excess azide which is accompanied by the concomitant reappearance of the dehydrogenase reaction catalyzed by the sulfhydryl form of glyceraldehyde-3-phosphate dehydrogenase, excess azide reduces the sulfenic acid to a sulfhydryl group at pH 6.0 and pH 5.3. The reaction scheme described by Eqns 3—5, which suggests that a sulfenyl nitrene is a reactive intermediate, is consistent with the two-electron reduction which occurs during the conversion of the sulfenic acid form of the enzyme to the sulfhydryl form of the enzyme by excess azide. Although sulfenyl nitrenes have not been described in the literature, sulfonyl nitrenes are produced by the thermal and photochemical decomposition of sulfonyl azides [15]. In experiments designed to synthesize sulfenyl azides Kharasch observed that azide reacts with sulfenyl chlorides to produce nitrogen and the corresponding disulfide and has suggested that stoichiometry shown by Eqn 7 for this reaction [16]:

$$2RSCl + 2\,N_3^- \rightarrow RSSR + 2\,Cl^- + 3\,N_2 \qquad (7)$$

If two molecules of azide were to reduce one molecule of the sulfenyl chloride by a reaction sequence similar to that described by Eqns 5—7, one would expect that the thiol produced would react rapidly with a second molecule of sulfenyl chloride to produce the disulfide.

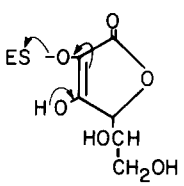
The stimulation of the acylphosphatase activity when the sulfenic acid form of the enzyme is treated with a stoichiometric amount of azide is difficult to explain. Qualitative experiments have shown that $^{30}N_2$ is evolved when the oxidized enzyme is treated with a stoichiometric quantity of $K^{15}N_3$. In addition no dehydrogenase activity is recovered when the sulfenic acid form of the enzyme is treated with an equivalent amount of azide. The posssibility that the stoichiometric addition of azide to the sulfenic acid form of the the enzyme leads to the formation of a sulfenyl nitrene which then reacts with water to form a sulfenyl hydroxylamine derivative of enzyme which would then hydrolyze to produce hydroxylamine and regenerate the sulfenic acid was considered. Such a reaction scheme is illustrated by Eqn 8.

$$\ddot{E}S\ddot{N}: + H_2O \rightarrow ESN(H)-OH \xrightarrow{H_2O} ESOH + NH_2OH \qquad (8)$$

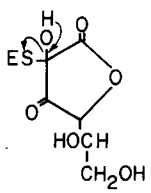
Ionized species of such a transient sulfenyl hydroxylamine would be potent nucleophiles and might increase the efficiency of the hydrolysis of acylphosphates bound at the active site of the enzyme. However, no hydroxylamine was detected in reaction mixtures at various times after a stoichiometric amount of azide was added to the sulfenic acid form of the enzyme by a modification of the method of Csáky [17] which would have detected 0.01 μmol of hydroxylamine if it were formed during the addition of 0.1 μmol of azide to 0.1 μmol of oxidized enzyme.

Both the inactivation of the acylphosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde-3-phosphate dehydrogenase by excess azide and the stimulation of the acylphosphatase activity by the addition of a stoichiometric amount of azide to oxidized glyceraldehyde-3-phosphate dehydrogenase occur more readily at pH values near 5.0 and do not occur at pH 7.0. This might suggest that the pK_a of the protein sulfenic acid is considerably below 7 and N_3^- displaces hydroxide from the undissociated sulfenic in its initial reaction with the oxidized enzyme as suggested by Eqn 3.

Since L-ascorbate also inactivates the acylphosphatase activity catalyzed by the sulfenic acid form of the enzyme which is accompanied by the reappearance of the dehydrogenase activity, it too reduces the protein sulfenic acid to a sulfhydryl group. Ascorbic acid is an ene-diol with a pK_a of 4.2 [18]. To initiate the reduction of the sulfenic acid form of the enzyme, either an enolate tautomer or the C_2 carbanion tautomer of ascorbate could attack the sulfenyl sulfur with the displacement of hydroxide to form the respective intermediates illustrated by Structures I and II. The sulfur atom in both intermediates could



STRUCTURE I



STRUCTURE II

accept electrons as illustrated in Structures I and II to produce dehydroascorbate and the sulfhydryl form of the enzyme. The observation that ascorbate completely inactivates the acylphosphatase reaction catalyzed by the sulfenic acid form of the enzyme which is not accompanied by the full reactivation of the dehydrogenase suggests that a covalent derivative between the oxidized enzyme and ascorbate exists under certain conditions.

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