Acyl Derivatives of Glyceraldehyde-3-Phosphate Dehydrogenase

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Studies of the mechanism of enzyme action have been hampered by difficulties encountered in the isolation of compounds or complexes that may result from interaction of enzymes with their substrates. The availability of glyceraldehyde-3-phosphate dehydrogenase in large quantities has made it possible to use this enzyme not only as a catalyst but, in micromolar amounts, as a reactant. From these studies, which are briefly reviewed here, it has become clear that under certain conditions the enzyme-substrate compound can be stabilized and isolated. The isolation and properties of the crystalline enzyme-substrate intermediate are the subject of this article (*1*).

Mechanism of Action of Glyceraldehyde-3-Phosphate Dehydrogenase

The mechanism of the reversible oxidation and phosphorylation of aldehydes to acyl phosphates by glyceraldehyde-3-phosphate dehydrogenase has been extensively investigated in recent years. An early theory (*2, 3*) postulated a nonenzymatic addition of phosphate to glyceraldehyde-3-phosphate followed by enzymatic oxidation of the hypothetical diphosphoglyceraldehyde to 1,3-diphosphoglyceric acid. In spite of various attempts (*4*), no evidence for the operation of this mechanism during the enzymatic oxidation of glyceraldehyde-3-phosphate has been obtained.

In analogy with the glyoxalase reaction, an alternative theory has been presented (*5, 6*) involving the intermediate formation of a thiol ester. In the proposed sequence of events (Fig. 1), a DPN-enzyme compound (I) interacts with the aldehyde substrate to yield reduced DPN and an acyl enzyme (II) wherein the acyl moiety of the oxidized substrate is attached to a sulfur atom of the protein; this thiol ester is split by inorganic phosphate to yield acyl phosphate and regenerated SH-enzyme (III). Evidence consistent with the essential features of this formulation of the mechanism of enzyme action has been brought forward from a number of laboratories (*6, 7*).

The enzyme as isolated from muscle (*8*) contains firmly bound DPN (*9*). In "reduced" (*10*) enzymes, 3 moles of DPN are bound to each mole of enzyme. (This can be shown either by reducing the DPN on the enzyme with glyceraldehyde-3-phosphate or by precipitating the enzyme with trichloracetic acid and measuring the amount of released DPN in the supernatant fluid.)

The enzyme as isolated from rabbit muscle under conditions that prevent oxidation of sulfhydryl groups (*11*) has an absorption with a broad maximum around 360 mμ. This absorption depends on the presence of bound DPN and SH groups. It disappears when the DPN is removed (by treatment with charcoal) and is restored by the addition of 3 equivalents of DPN. It also disappears when the SH groups are blocked or oxidized, or after addition of the substrates, acetyl phosphate or 1,3-diphosphoglycerate, thus indicating the participation of the DPN-enzyme complex in the enzyme catalyzed reaction (*6*). Similar data have been obtained with yeast enzyme in the presence of DPN.

That the DPN-enzyme complex is directly involved in the activity of the enzyme is also indicated by the fact that disappearance of the absorption at 360 mμ on addition of iodoacetate is closely paralleled by loss of enzymatic activity. After the addition of 3 equivalents of iodoacetate, there is no further reduction in the absorption at 360 mμ. The same amount is needed for complete inactivation of the enzyme (*12*). Inhibition of glyceraldehyde-3-phosphate dehydrogenase activity by irreversible SH blocking reagents, such as iodoacetate and *N*-ethyl maleimide, can be prevented for a time by substrates or by substrate analogs (*13*).

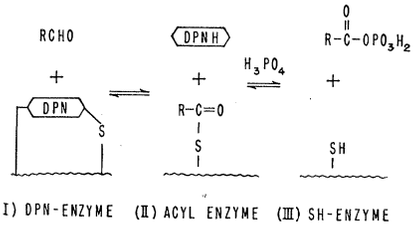

Fig. 1. Mechanism of aldehyde oxidation by glyceraldehyde-3-phosphate dehydrogenase.

Approximately 2 equivalents of glutathione (as measured by glutathione reductase) are released from glyceraldehyde-3-phosphate dehydrogenase by tryptic digestion. (The method used precludes complete recovery of glutathione.) Treatment of the enzyme with 3 equivalents of iodoacetate preceding digestion prevents the appearance of free glutathione. This parallels the afore-mentioned inhibition of over-all enzyme activity. Similarly, formation of thiol ester (acyl enzyme) by the interaction of acetyl phosphate and the enzyme is suppressed by 2 to 3 equivalents of iodoacetate (*14*).

Isolation and Properties of Acyl Enzyme

It was recently found that the formation of acyl enzyme (II) from the acyl phosphate (III) occurs with enzyme free of DPN and that the resulting acyl enzyme had considerable stability as compared with similar preparations obtained in the presence of DPN (*15*). Kinetic and spectrophotometric evidence for the formation of an acyl enzyme was advanced from several laboratories (*6, 7*), but the isolation of the enzyme-substrate compound had not been reported previously. Efforts were therefore directed toward the isolation of this postulated enzyme derivative. The observations on the stabilization of acyl enzyme by removal of DPN made it possible to isolate the acyl derivative of the enzyme.

Acetyl and 3-phosphoglyceryl enzyme have been prepared from glyceraldehyde-3-phosphate dehydrogenase isolated from both rabbit muscle (*8*) and baker's yeast (*16*) in the presence of ethylene diamine tetraacetate. The muscle enzyme was treated once or twice with 40 mg of charcoal per milliliter of a 3 to 6 percent solution to remove bound DPN; recrystallized glyceraldehyde-3-phosphate dehydrogenase from yeast is sufficiently free of DPN.

Either acetyl phosphate or a mixture of 3-phosphoglycerate, ATP, Mg^{++}, and phosphoglycerate kinase were incubated with the enzyme for a few minutes at *p*H 7; the protein was precipitated by addition of a cold saturated solution of ammonium sulfate at *p*H 6, the mixture was centrifuged, and the sediment was washed several times with saturated ammonium sulfate solution. Finally, the sediment was dissolved in a small amount of cold water. The protein crystallized

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during the washing procedure; also, it was readily recrystallized from solution by the addition of ammonium sulfate.

Like thiol esters, the acyl enzymes reacted with hydroxylamine at *p*H 6.5 to form hydroxamic acids. The acyl-enzyme bond of acetyl enzyme was, like the thiol ester of acetyl glutathione, stable to heating at 100° C for 5 min at *p*H 4.5. The thiol ester of phosphoglyceryl enzyme was found to be destroyed by this treatment. The thiol ester bond of both acetyl and phosphoglyceryl enzyme was stable in cold 5-percent trichloracetic acid. Formation of the acyl enzymes was prevented by treatment of the enzymes with approximately 3 equivalents of either iodoacetate (in the presence of DPN) or *N*-ethyl maleimide (in the absence of DPN) before mixing with the substrates. The acyl enzymes were sufficiently stable in the cold at neutral *p*H to permit study of their enzymatic properties; they lost approximately 30 percent of their hydroxamic acid-forming capability in the course of 4 hr at 0° C. Under the aforedescribed conditions for the preparation of the acyl enzymes, 0.8 to 1.5 moles of acyl groups (measured as hydroxamic acid) were found to be present per mole of enzyme.

The acyl enzymes oxidized DPNH in amounts approximately equivalent to the amounts of hydroxamic acid they could form, as is shown in Table 1. (The absorption coefficient of phosphoglyceryl hydroxamic acid-Fe complex is here assumed to be that of acetyl hydroxamic-Fe complex.) In the case of acetyl enzyme, an additional amount of DPNH was oxidized on adding alcohol dehydrogenase to the mixture. It may be assumed, then, that the acetyl group of acetyl enzyme was reduced to acetaldehyde, which in turn was reduced to ethanol. Similarly, in the case of phosphoglyceryl enzyme, addition of triose phosphate isomerase and α-glycerophosphate dehydrogenase caused further oxidation of DPNH; here, glyceraldehyde-3-phosphate formed by reduction of the phosphoglyceryl group was isomerized to dihydroxyacetone phosphate, which was then reduced to α-glycerophosphate. The oxidation of DPNH by phosphoglyceryl enzyme from yeast and muscle and by acetyl enzyme from muscle is rapid; oxidation of DPNH by acetyl enzyme from yeast is slow. These rates are in good agreement with the rates of oxidation of DPNH by the two acyl phosphates in the presence of catalytic amounts of the enzymes.

Figure 2 illustrates an experiment in which phosphoglyceryl enzyme prepared from muscle glyceraldehyde-3-phosphate dehydrogenase was used to oxidize DPNH. The first point in the curve represents the optical density at 340 mμ calculated from the density of the enzyme solution plus that of DPNH, which was added last. The acyl enzyme caused a rapid oxidation of DPNH during the first minute in contrast to the control, in which acylation of the enzyme was prevented by prior treatment with *N*-ethyl maleimide. The slow oxidation of DPNH that follows may be explained by trace contamination of the glyceraldehyde-3-phosphate dehydrogenase (7.5 mg of enzyme was used) with triose phosphate isomerase and α-glycerophosphate dehydrogenase. Addition of triose phosphate isomerase and α-glycerophosphate dehydrogenase caused a further sharp decrease in DPNH, corresponding approximately to the theoretical amount of triose formed from phosphoglyceryl enzyme.

Arsenolysis and Hydrolysis of Acyl Enzyme

It is known that arsenate can substitute for phosphate in the enzyme catalyzed oxidation of glyceraldehyde-3-phosphate by DPN (*2*). However, in the presence of arsenate, an equilibrium mixture of aldehyde and acyl compound is not obtained; instead the oxidation of the aldehyde to the free acid goes to completion, presumably because of the spontaneous hydrolysis of the hypothetical acyl arsenate. In the presence of arsenate, acetyl

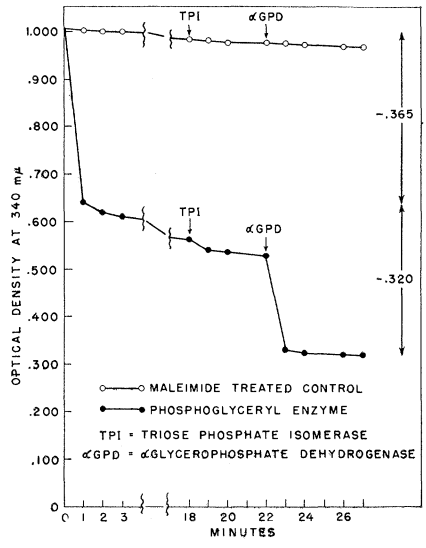

Fig. 2. Oxidation of reduced DPN by phosphoglyceryl enzyme.

phosphate and 1,3-diphosphoglycerate are rapidly hydrolyzed by glyceraldehyde-3-phosphate dehydrogenase. This arsenolysis has been shown to depend on DPN (*6*).

"Oxidized" (*10*) glyceraldehyde-3-phosphate dehydrogenase catalyzes a DPN-dependent hydrolysis of acetyl phosphate; glutathione or potassium cyanide inhibited this reaction without inhibiting arsenolysis (*17*). In the course of the present investigation it was found that preparations of "reduced" (*10*) enzyme likewise exhibit hydrolytic activity which is relatively slow compared with the rate of arsenolysis. Hydrolysis is accelerated by prior treatment of the enzyme with iodoacetate in contrast to arsenolysis, which is inhibited. On the other hand, potassium cyanide and glutathione, as well as semicarbazide, phenyl hydrazine, and hydroxylamine, inhibited hydrolysis of acetyl phosphate by iodoacetate-treated or untreated enzyme without affecting the rate of arsenolysis of acetyl phosphate by untreated enzyme. They also stabilize acyl enzyme in the presence of DPN against hydrolysis but do not prevent the very rapid arsenolysis of the acyl enzyme. A typical experiment is shown in Table 2 demonstrating the effectiveness of the inhibitors in stabilizing the acyl groups. Since these inhibitors are known to react with carbonyl groups, the possibility was considered that an aldehyde group of the enzyme participates in the hydrolytic activity. It was found that heating of the neutral protein solution at 100°C released a small amount of steam-distillable aldehyde, which served as substrate for alcohol dehydrogenase in the presence of DPNH. Since several other highly purified proteins also yielded an aldehyde after heat denaturation, the relation of the released

Table 1. Comparison of hydroxamic acid formed and DPNH oxidized by equal amounts of acyl enzyme.

Acyl enzyme	Hydroxamic acid (moles per mole of glyceraldehye-3-phosphate dehydrogenase*)	DPNH oxidized (moles per mole of glyceraldehyde-3-phosphate dehydrogenase*)
Acetyl (muscle)	0.80	0.82
Phosphoglyceryl (muscle)	0.96	1.04
Acetyl (yeast)	0.87	0.46†
Phosphoglyceryl (yeast)	0.78	0.60‡

* The molecular weight of the enzyme is taken as 120,000 (*18*).
† Minimum figure (oxidation incomplete after 3 hr).
‡ DPNH oxidation was measured about 2 hr after hydroxamic acid formation.

Table 2. Stabilization of phosphoglyceryl enzyme (muscle) in the presence of DPN by aldehyde reagents.

Additions	Decomposition of acyl enzyme in 12 min at 25°C (%)
None	53
Potassium cyanide (0.001M)	0
Glutathione (0.001M)	0
Semicarbazide (0.03M)	6
Phenyl hydrazine (0.03M)	22

aldehyde to the hydrolysis of acyl groups remains to be elucidated.

Summary

Acetyl phosphate and 1,3-diphosphoglycerate react with glyceraldehyde-3-phosphate dehydrogenase to form relatively stable enzyme substrate compounds. These compounds appear to be thiol esters, and their properties indicate that they are intermediates in the catalytic activity of the enzyme: they undergo hydrolysis and arsenolysis in the presence of DPN and are reduced by DPNH to form aldehydes. These results are in agreement with the mechanism previously proposed for the oxidation of aldehydes in which a thiol ester formed on the enzyme, with concomitant reduction of DPN, is split in the presence of phosphate to acyl phosphate and regenerated enzyme.

References and Notes

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I cannot refrain from marveling that Sarsi will persist in proving to me, by authorities, that which at any moment I can bring to the test of experiment. We examine witnesses in things which are doubtful, past, and not permanent, but not in those things which are done in our own presence. If discussing a difficult problem were like carrying a weight, since several horses will carry more sacks of corn than one alone will, I would agree that many reasoners avail more than one; but discoursing is like coursing, and not like carrying, and one barb by himself will run farther than a hundred Friesland horses. When Sarsi brings up such a multitude of authors, it does not seem to me that he in the least degree strengthens his own conclusions, but he ennobles the cause of Signor Mario and myself, by showing that we reason better than many men of established reputation. If Sarsi insists that I must believe, on Suidas's credit, that the Babylonians cooked eggs by swiftly whirling them in a sling, I will believe it; but I must say, that the cause of such an effect is very remote from that to which it is attributed, and to find the true cause I shall reason thus. If an effect does not follow with us which followed with others at another time, it is because, in our experiment, something is wanting which was the cause of the former success; and if one thing is wanting to us, that one thing is the true cause. Now we have eggs, and slings, and strong men to whirl them, and yet they will not become cooked; nay, if they were hot at first they more quickly become cold; and since nothing is wanting to us but to be Babylonians, it follows that being Babylonians is the true cause why the eggs become cooked, and not the friction of the air, which is what I wish to prove. Is it possible that in traveling post, Sarsi has never noticed what freshness is occasioned on the face by the continued change of air? And if he has felt it, will he rather trust the relation by others of what was done two thousand years ago at Babylon, than what he can at this moment verify in his own person? I, at least, will not be so wilfully wrong and so ungrateful to nature and to God, that having been gifted with sense and language I should voluntarily set less value on such great endowments than on the fallacies of a fellow-man, and blindly and blunderingly believe whatever I hear, and barter the freedom of my intellect for slavery to one as liable to error as myself.
—GALILEO GALILEI, *Il Saggiatore.* 1623. Translated by Giorgio De Santillana.