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Separation of Oxidative from Phosphorylative Activity by Proteolysis of Glyceraldehyde-3-Phosphate Dehydrogenase*

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Controlled digestion of glyceraldehyde-3-phosphate dehydrogenase with chymotrypsin resulted in marked changes in molecular size and enzymic properties of the protein. After digestion, the enzyme no longer catalyzed the over-all reaction of oxidative phosphorylation of glyceraldehyde-3-phosphate but retained the ability to oxidize glyceraldehyde in the absence of phosphate at an undiminished or even accelerated rate. Glyceraldehyde-3-phosphate was oxidized, in the presence of monothioglycerol as acyl acceptor, at a somewhat slower rate, whereas with arsenate as acceptor over 95% of the activity was lost after digestion. With enzyme acting stoichiometrically as acyl acceptor, the rate of oxidation of glyceraldehyde-3-phosphate by digested enzyme was unimpaired or accelerated. Threose-2,4-diphosphate, a potent inhibitor of glyceraldehyde or glyceraldehyde-3-phosphate oxidation by native enzyme, had little or no effect on digested enzyme. Acylation of digested enzyme by oxidation of aldehyde not only was more rapid but was more extensive as compared with native enzyme. This phenomenon was shown to be due to a pronounced inhibition of aldehyde oxidation by reduced diphosphopyridine nucleotide in the case of native enzyme. These findings are discussed in reference to the mode of action of glyceraldehyde-3-phosphate dehydrogenase and to problems of oxidative phosphorylation linked to electron transport.

The enzyme glyceraldehyde-3-phosphate dehydrogenase catalyzes the oxidative phosphorylation of glyceraldehyde-3-P to 1,3-diphosphoglycerate. Studies on the mechanism of action of this enzyme in several laboratories (*cf.* Racker, 1955) revealed that the reaction proceeds in two steps:

$$E-DPN + \text{glyceraldehyde-3-P} \rightleftharpoons \text{3-P-glyceryl}-E + DPNH + H^+ \quad (1)$$

$$\text{3-P-glyceryl}-E + P_i \rightleftharpoons \text{1,3-di-P-glycerate} + E \quad (2)$$

The enzyme-substrate intermediate formed in the first step has been isolated in crystalline form and has been shown to undergo either reduction in the presence of DPNH or cleavage in the presence of arsenate (Krimsky and Racker, 1955).

It is the purpose of this paper to show that the oxidative activity of the enzyme can be separated from the phosphorolytic activity by controlled proteolytic digestion. The properties of the resulting altered enzyme will be described.

Materials and Methods

Preparation of Glyceraldehyde-3-P Dehydrogenase.—Glyceraldehyde-3-P dehydrogenase was prepared from rabbit muscle (Cori *et al.*, 1948), with the modification that 0.005 M EDTA was present in the extracting fluid and was used in this concentration throughout subsequent procedures. The enzyme was recrystallized three times and then further purified by washing the crystals obtained from one rabbit (about 2 g) three times with 800 ml of 3 M $(NH_4)_2SO_4$ solution, *p*H 7.9. The washed crystals were dissolved in 0.005 M EDTA, *p*H 7.8, to give a protein concentration of about 7%, and stored at 2°. The enzyme was stable in this form for several months.

Removal of DPN from Glyceraldehyde-3-P Dehydrogenase.—In order to permit digestion by proteolytic enzymes (Racker and Krimsky, 1958), enzyme-bound DPN was removed with charcoal. The charcoal (Norit A, neutral, Fisher Scientific Co.) was prepared by washing 110 g with 2 liters of 1 N HCl twice by decantation. It was then washed on a Buchner funnel with water until the filtrate was essentially free of acid, then with 0.005 M EDTA, *p*H 7.8 (about 16 liters), until the *p*H of the filtrate was 7.8, and finally with 8 liters of H_2O, and then dried. This exhaustive washing of the charcoal was necessary for the preparation of relatively stable digested glyceraldehyde-3-P dehydrogenase. The charcoal was suspended in 0.005 M EDTA, *p*H 7.2, poured into a column of 1-cm diameter, and allowed to settle. A height of 1.5 cm of wet packed charcoal was used for each 100 mg of enzyme, applied as a 7% solution. The enzyme solution was followed by a volume of 0.005 M EDTA, *p*H 7.2, somewhat in excess of the hold-up volume of the column. Air pressure was used to give a flow rate of about 0.4 ml per minute. About 80% of the protein applied to the column was recovered in the effluent. Use of a larger ratio of charcoal to protein gave rise to greater loss of protein. The 280/260 mμ absorbancy ratio of

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the effluent protein solution was about 1.95 and the protein concentration about 4%. The charcoal-treated enzyme solution was stable for several weeks at 2°; however, it was usually used within 2 days after charcoal treatment. It should be noted that the $(NH_4)_2SO_4$ concentration was about 1.0 M.

Proteolytic Digestion of Glyceraldehyde-3-P Dehydrogenase.—The incubation mixture for digestion contained, per ml: 40 μmoles triethanolamine-HCl buffer, *p*H 7.9; 5 μmoles EDTA, *p*H 7.9; 26 mg charcoal-treated glyceraldehyde-3-P dehydrogenase; 180 μg chymotrypsin. After 25 minutes at 30°, 0.02 ml of DPN, 0.04 M, *p*H 6.8, and 0.02 ml of diisopropyl phosphofluoridate, 0.014 M, were added for each ml of incubation mixture, and the mixture was stored in an ice bath.[1] As controls for these preparations of "digested enzyme," "native enzyme" was prepared in the same way except that chymotrypsin was omitted. It should be noted that these standard preparations used throughout this work contain 4.3 moles of DPN, added at the end of the digestion period, per mole of native or digested enzyme.

Assays of Enzyme Activity.—Glyceraldehyde-3-P dehydrogenase activity with glyceraldehyde-3-P as substrate and arsenate or thioglycerol as acceptor was measured in a Beckman Model DU spectrophotometer, in a final volume of 1 ml and with a light path of 10 mm, as the rate of increase in absorbancy at 340 mμ of a solution of triethanolamine-HCl buffer, 0.04 M, *p*H 8.6; EDTA, 0.005 M, *p*H 7.9; DPN, 0.006 M, *p*H 6.8; *d*-glyceraldehyde-3-P, 0.0009 M, *p*H 6.8; sodium arsenate, 0.0045 M, or thioglycerol, 0.55 M. When arsenate was used as acceptor, thioglycerol, 0.006 M, was included in the assay mixture; at this low concentration, thioglycerol did not act as acyl acceptor.

With arsenate as acceptor, the increase in absorbancy between 15 and 45 seconds after addition of enzyme to the reaction mixture was proportional to enzyme concentration when the rate of increase in optical density was between 0.050 and 0.300 per minute.

With thioglycerol as acceptor, the reaction mixture was allowed to stand in the spectrophotometer for about 10 minutes in order to attain optical stability before the addition of enzymes. The increase in optical density between 4 and 8 minutes after addition of enzyme was proportional to enzyme concentration in the range 0.005 to 0.05 per minute.

Glyceraldehyde-3-P dehydrogenase activity in the "back" reaction, *i.e.*, oxidation of DPNH coupled with reduction of 1,3-di-P-glycerate, was assayed as described previously (Wu and Racker, 1959).

Arsenolysis of 1,3-di-P-glycerate catalyzed by glyceraldehyde-3-P dehydrogenase was assayed as the amount of P_i formed in a mixture of Tris, 0.04 M, *p*H 8.6; EDTA, 0.005 M; DPN, 0.004 M; $MgCl_2$, 0.01 M; ATP, 0.02 M; 3-P-glycerate, 0.01 M; thioglycerol, 0.006 M; sodium arsenate, 0.004 M; P-glycerate kinase, 100 μg; and varying amounts of the dehydrogenase. The mixtures (final *p*H of 8.6) were incubated for 10 minutes at 32°, deproteinized with trichloroacetic acid, and centrifuged, and P_i was determined on an aliquot of the supernatant liquid. P_i formation was proportional to dehydrogenase concentration in the range 0.5 to 1.5 μmoles of P_i formed per ml of incubation mixture.

P-Glyceryl thioglycerol formation from 1,3-di-P-glycerate and thioglycerol catalyzed by glyceraldehyde-3-P dehydrogenase was measured in the same way as arsenolysis of 1,3-di-P-glycerate except that thioglycerol, 0.55 M, was substituted for arsenate, and that, after deproteinization, thiol ester was assayed.

Analytical Methods.—P-Glyceryl thiol esters, P-glyceryl, and glyceryl groups in acyl enzymes were measured by the hydroxamic acid method (Lipmann and Tuttle, 1945), with the higher $FeCl_3$ concentration used by Wolff and Black (1959). The molar absorbancy index of P-glycerohydroxamic acid was 920. It was determined by complete conversion of a known amount of 3-P-glycerate to the hydroxamic acid in the presence of excess ATP, P-enolpyruvate, pyruvate kinase, and P-glycerate kinase (Krimsky, 1959). The amount of P-glycerate used was measured enzymatically (Racker, 1957) with purified glycolytic enzymes. The molar absorbancy index of glycerohydroxamic acid was taken as 990 (Black and Wright, 1956). P_i and total P were assayed colorimetrically (Lohmann and Jendrassik, 1926; Lohmann, 1928). Sulfhydryl content was assayed with nitroprusside (Stadtman, 1957) or *N*-ethyl maleimide (Gregory, 1955). *N*-ethyl maleimide, purified by sublimation, was used as standard. Glyceraldehyde-3-P dehydrogenase concentration was measured spectrophotometrically (Fox and Dandliker, 1956); the molecular weight was taken as 140,000 (Dandliker and Fox, 1955). Protein concentration of fractions from sucrose gradients was measured colorimetrically (Lowry *et al.*, 1951).

Reagents.—Chymotrypsin (α-chymotrypsin, three times crystallized, salt-free) was purchased from Worthington Biochemical Corp. Crystalline preparations of P-glycerate kinase, triose-P isomerase, α-glycero-P dehydrogenase, enolase, P-glycerate mutase, pyruvate kinase, and lactate dehydrogenase were purchased from Boehringer and Sons. Glyceraldehyde-3-P solutions were prepared from the barium salt of *dl*-glyceraldehyde-3-P diacetal purchased from Schwarz Laboratories, Inc. (Racker *et al.*, 1959). D-Threose-2,4-di-P was a gift from Dr. C. Ballou. Phosphocellulose was purchased from Serva Entwicklungslabor, Heidelberg. P-Enolpyruvate and P-glycerate were products of Boehringer and Sons. α-Monothioglycerol (Thiovanol) was a product of Evans Chemetics, Inc., Waterloo, N. Y. Water from a special glass still (Ballentine, 1954) was used in preparing all solutions.

Properties of Digested Enzyme

Rate of Oxidation.—Controlled digestion of glyceraldehyde-3-P dehydrogenase with chymotrypsin resulted in 95–98% loss of activity in the over-all reaction, glyceraldehyde-3-P $\rightleftharpoons$ 1,3-di-P-glycerate, as measured in either direction. The arsenolysis of 1,3-di-P-glycerate was decreased to the same low level.

In order to study the oxidative step catalyzed by glyceraldehyde-3-P dehydrogenase independently of the phosphorolytic step, three assay procedures were used: (*a*) oxidation of glyceraldehyde in the absence of P_i or arsenate; (*b*) oxidation of glyceraldehyde-3-P with thioglycerol as acyl acceptor; (*c*) oxidation of glyceraldehyde-3-P with stoichiometric amounts of enzyme as acyl acceptor.

(*a*) *Oxidation of Glyceraldehyde.*—As shown in Figure 1, oxidation of glyceraldehyde in the absence of phosphate proceeded at a somewhat faster rate after digestion. Addition of phosphate, which markedly stimulated the native enzyme, had little or no effect on the digested enzyme.

D-Threose-2,4-di-P, a potent and specific inhibitor of glyceraldehyde-3-P dehydrogenase (Racker *et al.*, 1959; Fluharty and Ballou, 1959), inhibited glyceraldehyde oxidation by native enzyme but had little effect

[1] We are indebted to Dr. G. E. Boxer, Merck Institute for Therapeutic Research, Rahway, N. J., for giving us diisopropyl phosphofluoridate.

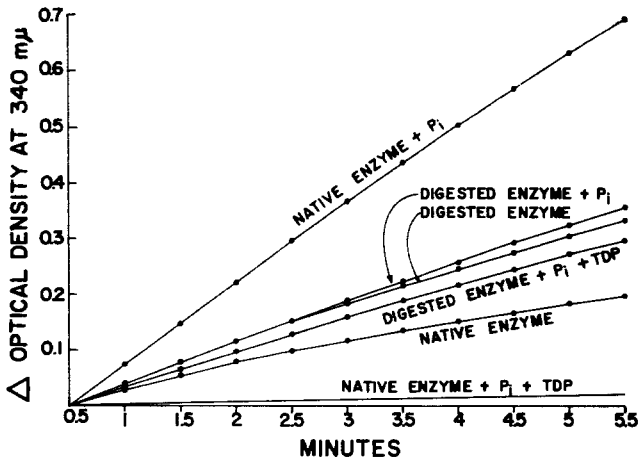

FIG. 1.—Rate of glyceraldehyde oxidation. The measurements were made in a Beckman model DU spectrophotometer in solutions of 0.04 M Tris-HCl buffer, *p*H 7.9; 0.005 M EDTA, *p*H 7.9; 0.0012 M DPN; 0.011 M *dl*-glyceraldehyde; 0.006 M thioglycerol; 1.25 mg per ml native or digested glyceraldehyde-3-P dehydrogenase; also, where indicated, 0.001 M potassium phosphate, *p*H 7.9; 0.000053 M threose di-P. Temperature, 16°.

on the oxidation by digested enzyme, as shown in Figure 1. Phosphate had no effect on the oxidation rate in the presence of inhibitor with either native or digested enzyme.

(*b*) *Oxidation of Glyceraldehyde-3-P with Thioglycerol as Acceptor.*—The requirement for an acyl acceptor in the catalysis of glyceraldehyde-3-P oxidation by the enzyme can be met by thiols as well as by P_i or arsenate (Racker and Krimsky, 1952; Wolff and Black, 1959). With glutathione and methyl mercaptan the corresponding thiol esters were shown to be formed. Although the oxidation rates were much slower than with P_i, this offered a possibility of measuring catalysis of glyceraldehyde-3-P oxidation independently of the phosphorolytic mechanism.

Various thiols were tested, including ethyl mercaptan, glutathione, and cysteine. The most suitable were thioglycolate (0.05 M) and α-monothioglycerol (0.5 M), since in their presence the rate of reduction of DPN measured at 340 mμ was proportional to the amount of enzyme over a fairly broad range. Although the rate with thioglycerol was only 10% that with thioglycolate and only 0.6% that with arsenate, thioglycerol was the preferred acceptor because, after digestion of the enzyme, it yielded results that could be more readily evaluated. The rate of glyceraldehyde-3-P oxidation with either thiol as acceptor was decreased by digestion; the percentage decrease, relative to native enzyme, was much greater with thioglycolate than with thioglycerol. It appears that the site that allows for the more efficient utilization of the charged thioglycolate is affected by digestion similarly to the site that reacts with phosphate. The thiol esters formed in the oxidation of glyceraldehyde-3-P were isolated in the case of both thioglycolate and thioglycerol. The analysis of the reaction product obtained after alkaline hydrolysis of the thioglycolate ester gave good stoichiometry (*e.g.*, disappearance of thiol ester, 4.7 μmoles/ml; appearance of glycerate-3-P, 4.4 μmoles/ml; appearance of SH, 4.7 μmoles/ml). The thioglycerol ester, when measured immediately after its formation, reacted quantitatively with NH_2OH at *p*H 6.5 within 10 minutes. However, after isolation of the alcohol-insoluble calcium salt, incubation at alkaline *p*H was required for the rapid formation of the hydroxamate. Alkaline hydrolysis of the isolated ester yielded 4.2 μmoles of glycerate-3-P after hydrolysis of 4.3 μmoles of ester, but the analysis for sulfhydryl groups revealed 4.4 μmoles before and 4.2 μmoles after hydrolysis. Apparently an S to O shift of the P-glyceryl group had taken place during isolation.

After digestion of glyceraldehyde-3-P dehydrogenase with chymotrypsin under the standardized conditions, in nine consecutive experiments between 42% and 63% (average 56%) of the oxidative activity had been retained with thioglycerol, but only 1.2% to 4.7% (average 2.6%) with arsenate as acyl acceptor. In view of the rather sluggish transfer of the acyl group to the thiol, it was desirable to compare the forward and back reactions with the same thiol acceptor. As can be seen from Table I, the rates of the forward and back reactions were about the same with native enzyme, whereas with digested enzyme the forward reaction was about five times as fast as the back reaction.

TABLE I

RATE OF PHOSPHOGLYCERYL THIOGLYCEROL FORMATION IN FORWARD AND REVERSE DIRECTIONS

Measurements were carried out as described under Assays of Enzyme Activity.

Enzyme	Substrate	μmoles Phosphoglyceryl Thioglycerol/min./mg
Native	Glyceraldehyde-3-P	0.43
	1,3-di-P-glycerate	0.38
Digested	Glyceraldehyde-3-P	0.24
	1,3-di-P-glycerate	0.05

In the oxidation of glyceraldehyde-3-P with thioglycerol as acceptor, native enzyme was 50% inhibited at 2×10^{-7} M threose-2,4-di-P and was maximally inhibited (> 95%) at 1×10^{-6} M. Digested enzyme preparations showed varying inhibition (0 to 40%) by threose-2,4-di-P. The inhibitable portion of digested enzyme activity was also 50% inhibited at 2×10^{-7} M threose-2,4-di-P, and inhibition was maximal at 1×10^{-6} M. The uninhibitable portion of digested enzyme was not affected even at 1×10^{-5} M threose-2,4-di-P.

The experiments described above, although strongly suggesting a greater loss of phosphorylative than of oxidative activity, have the defect that the measurements include a second step (hydrolysis or transfer) which complicates the evaluation of the rate changes. Direct measurement of the rate of formation of acyl enzyme was therefore attempted.

(*c*) *Oxidation of Substrate with Enzyme as Acyl Acceptor.*—P-Glyceryl enzyme can be isolated in an inactive form from a reaction mixture of substrate and enzyme by precipitation with trichloroacetic acid (Krimsky and Racker, 1955). It was found that native and digested enzyme were maximally acylated by glyceraldehyde-3-P within 30 seconds at 0°, making rate measurements by this method unfeasible.

With the collaboration of Dr. B. Chance, initial rate measurements of DPN reduction were carried out spectrophotometrically at relatively low concentrations of glyceraldehyde-3-P with enzyme as acyl acceptor. Although the rates were accordingly slow, it was found that DPNH formation was more rapid and more extensive with digested than with native enzyme.

In contrast to the rate with glyceraldehyde-3-P, the rate of acyl enzyme formation with glyceraldehyde was slow enough at 0° to be measured by the determination of acyl groups at short intervals, as shown in

Figure 2. The digested enzyme was acylated more rapidly and to a greater extent. This departure from the apparent equilibrium obtained with native enzyme, which was also observed by Dr. Chance in the spectrophotometric experiments with glyceraldehyde-3-P as substrate, was further investigated. As shown in Table II, experiment 1, with either glyceraldehyde or

TABLE II

ACYL ENZYME FORMATION FROM GLYCERALDEHYDE-3-P AND FROM GLYCERALDEHYDE

Incubation volume 1 ml. In expt. 1, the mixture contained 0.08 M triethanolamine-HCl buffer, *p*H 7.28, and 11 mg of native or digested glyceraldehyde-3-P dehydrogenase; also, where indicated, 0.048 M potassium pyruvate, 100 μg lactate dehydrogenase, 0.00132 M *dl*-glyceraldehyde-3-P, 0.066 M *dl*-glyceraldehyde. Incubation time, 6 minutes; temperature, 0°. In expt. 2, the mixture contained 0.04 M Tris-HCl buffer, *p*H 7.9; 11 mg of native or digested enzyme; 0.018 M glyceraldehyde-3-P; 0.004 M DPN; also, where indicated, 0.0048 M potassium pyruvate and 50 μg lactate dehydrogenase. Incubation time, 2 minutes; temperature, 25°. Determination of acyl groups was made as described in Fig. 2.

Expt.	Enzyme	Lactate Dehydrogenase and Pyruvate	Moles of Acyl Groups/Mole of Glyceraldehyde-3-P Dehydrogenase	
			d-Glyceraldehyde-3-P	*d*-Glyceraldehyde
1	Native	absent	0.99	0.44
		present	2.16	1.50
	Digested	absent	1.86	1.99
		present	2.27	2.32
2	Native	absent	1.3	
		present	3.1	
	Digested	absent	3.2	
		present	3.2	

glyceraldehyde-3-P as substrate the amount of acyl groups that can be formed per mole of enzyme is considerably larger with digested than with native enzyme. Previous observations (Racker *et al.*, 1959) have implicated DPNH as a potent inhibitor of the forward reaction. Acyl enzyme formation with both substrates was therefore measured in the presence of a DPNH-removing system consisting of lactate dehydrogenase and pyruvate. As can be seen, this resulted in a pronounced stimulation of acyl enzyme formation with native enzyme (experiment 1). The difference between native and digested enzyme decreased under these conditions with either substrate (experiment 1) and disappeared completely at higher concentrations of glyceraldehyde-3-P and DPN (experiment 2). It should be mentioned that in the incubation time used (6 minutes) the amount of acyl groups formed with either substrate had reached a constant level.

With native enzyme the high concentration of DPN required to overcome the inhibition of glyceraldehyde-3-P oxidation by DPNH (Racker *et al.*, 1959) contrasts with the relatively high affinity of the enzyme to DPN in the absence of substrate. Moreover, considerable excess of DPNH is required to displace the DPN (Velick, 1958). These observations point to a decrease in the affinity of the enzyme to DPN during catalysis. This was demonstrated directly by the following experiments. P-Glyceryl enzyme, prepared by exposing the enzyme to a system generating a high 1,3-di-P-glycerate concentration (Krimsky, 1959) was passed through a Sephadex column (G-50, 3.5 × 16.5 cm). The emerging protein was analyzed and found to have

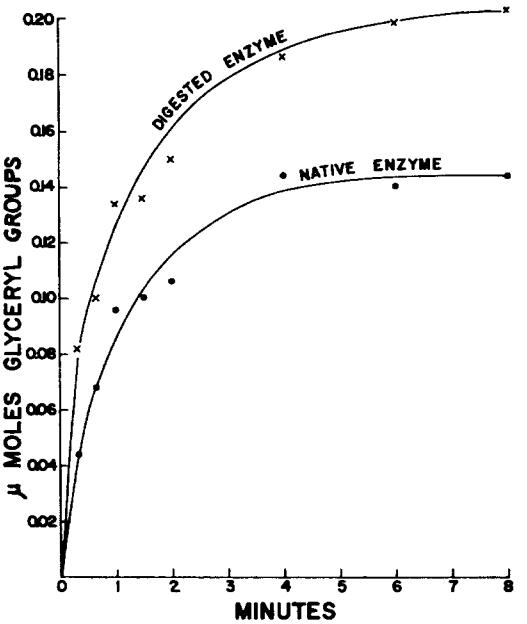

FIG. 2.—Rate of glyceryl enzyme formation. Incubation mixtures, 1 ml volume, were deproteinized at the times indicated. The mixtures contained 0.08 M triethanolamine-HCl buffer, *p*H 7.28; 0.006 M *dl*-glyceraldehyde; 0.048 M potassium pyruvate; 150 μg lactate dehydrogenase; and, as last addition, 11 mg native or digested glyceraldehyde-3-P dehydrogenase (including 4.3 moles of DPN per mole of enzyme). Temperature, 0°. 7 ml of cold 6% trichloroacetic acid was added, the protein was sedimented, washed 3 times with 8.5 ml 5% trichloroacetic acid, and assayed for glyceryl enzyme as described under Analytical Methods.

lost over 75% of its DPN content. Control experiments with untreated enzyme and with enzyme exposed to the 1,3-di-P-glycerate generating system, from which only ATP was omitted, revealed no loss of DPN. A lowered affinity to DPN on acylation of the enzyme is also apparent from the relatively high requirement for DPN in the arsenolysis of 1,3-di-P-glycerate (Racker and Krimsky, 1952). Recent measurements have shown that 1.6×10^{-3} M DPN is required for saturation of the enzyme (10 μg protein per ml) in the arsenolysis reaction.

These experiments show that during catalysis there is a change in the relative affinity of the enzyme to DPN and DPNH. Acyl enzyme binds the latter relatively more firmly. This possible control mechanism in the oxidative step is apparently lost on proteolytic digestion of the enzyme.

Stability.—The standard preparation of digested enzyme lost about 10% of its oxidative activity with thioglycerol as acceptor per 24 hours storage at 2°. At room temperature, digested enzyme solutions became increasingly turbid with time, with accompanying loss of enzymatic activity. This turbidity interfered with optical measurements of concentrated solutions. Prolonged dialysis or dilution of the enzyme resulted in marked inactivation, which could be prevented by 0.8 mM DPN. Under these same conditions, native enzyme was completely stable.

Effect of Glyceraldehyde-3-P and Threose di-P on the Absorbancy of DPN-Enzyme at 405 mμ.—It has been shown (Racker and Krimsky, 1952) that the absorption at 360 mμ due to the interaction between DPN and enzyme is abolished by acylation of the enzyme with its substrate or by alkylation with iodoacetate. Although the absorbancy of the DPN-enzyme at 405 mμ is only about one-half that at 360 mμ, Chance (1954) recommended the longer wavelength to avoid

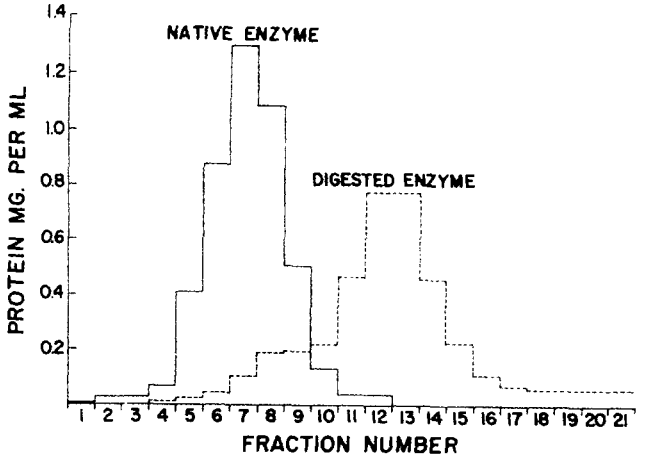

FIG. 3.—Sucrose gradient centrifugation patterns of native and digested enzyme. The method of Martin and Ames (1961) was used, with the modification that the 5 to 20% sucrose gradient contained 0.01 M Tris-HCl buffer, *p*H 7.9; 0.0013 M EDTA; and 0.0008 M DPN. Glyceraldehyde-3-P dehydrogenase (0.15 ml of a solution of 6.25 mg per ml of native or digested enzyme) was layered on the gradient. Centrifugation was for 13 hours at 100,000 × *g* at 0°.

interference due to DPNH absorption. The effect of threose di-P and glyceraldehyde-3-P on the absorption at 405 mμ of native and digested enzyme is shown in Table III. It can be seen that threose di-P caused complete loss of absorbancy at 405 mμ (bleaching) of native enzyme, but only 7% bleaching of digested enzyme. These effects of threose di-P on the absorbancy were parallel to its inhibition of glyceraldehyde-3-P oxidation with thioglycerol as acceptor (native, 97% inhibited; digested, 12% inhibited). On the other hand, bleaching due to the formation of acyl enzyme with glyceraldehyde-3-P as substrate is only partial with native enzyme but is complete with digested enzyme. These values parallel the values obtained for acyl enzyme formation by direct measurement with hydroxylamine (*cf.* Table II, experiment 2), and point again to a decreased affinity to the inhibitory DPNH. Apparently the digested enzyme also combined less readily with the oxidized nucleotide. Whereas addition of 4.3 equivalents of DPN per mole of enzyme (see Methods) gave maximum absorption at 360 mμ with native enzyme (absorbancy index = 3.3×10^3), the digested enzyme gave only 56% of the absorbancy of native enzyme, a value which could be increased to 72% by the addition of about 25 equivalents of DPN.

P-Glyceryl Enzyme Formation from 1,3-di-P-glycerate.—As shown in Table IV, the rate of acylation of digested enzyme by 1,3-di-P-glycerate is much slower than that of native enzyme. Since acylation was completed in 30 seconds with native enzyme, an accurate quantitative evaluation was impossible. It should be stressed, however, that, in spite of the markedly decreased rate, the extent of acylation of digested enzyme is about the same as that of native enzyme. Since about 2% to 5% of the over-all catalytic activity was retained after proteolytic digestion, the possibility was considered that the acylation might be dependent on traces of undigested native enzyme. However, addition of native enzyme in amounts equal to or twice the residual activity of the digest did not significantly affect the rate of acylation (Table V).

TABLE III

BLEACHING OF DPN-ENZYME COMPLEX

The mixtures contained, in 1 ml, 9.55 mg native or digested enzyme; 0.04 M Tris-HCl buffer, *p*H 7.9; 0.006 M thioglycerol; 0.002 M DPN. Optical density readings were taken before and after addition of 0.0048 M *dl*-glyceraldehyde-3-P or 0.0013 M threose di-P. The resulting decreases in optical density were calculated as percentage of decrease produced by 0.002 M potassium iodoacetate.

Enzyme	Decrease in Optical Density at 405 mμ (as % of decrease with excess iodoacetate)	
	Glyceraldehyde-3-P	Threose-2,4-di-P
Native	30	100
Digested	100	7

TABLE IV

PHOSPHOGLYCERYL ENZYME FORMATION WITH 1,3-DI-P-GLYCERATE

Incubation mixtures, 1 ml volume, were deproteinized at the time indicated. The mixtures contained 0.1 M Tris-HCl buffer, *p*H 7.9; 0.005 M EDTA; 0.004 M DPN; 0.01 M 3-P-glycerate; 0.01 M $MgCl_2$; 0.02 M ATP; 100 μg P-glycerate kinase; and 10 mg of native or digested glyceraldehyde-3-P dehydrogenase. Temperature, 0°. Determination of acyl enzyme was made as in Fig. 2.

Incubation Time (min.)	Moles Acyl Groups per Mole of Glyceraldehyde-3-P Dehydrogenase	
	Native Enzyme	Digested Enzyme
0.5	2.10	0.47
2	2.04	1.26
10	2.03	1.91
50	1.66	2.29

TABLE V

EFFECT OF NATIVE ENZYME ON RATE OF ACYLATION OF DIGESTED ENZYME BY 1,3-DI-P-GLYCERATE

Conditions as in Table IV except that the incubation volume was 2 ml, containing 10 mg of digested enzyme plus native enzyme as indicated.

Incubation Time (sec.)	Moles Phosphoglyceryl Groups /Mole Digested Enzyme		
	Digested, 10 mg	Digested, 10 mg Native, 0.34 mg[a]	Digested, 10 mg Native, 0.68 mg
40	0.88	1.03	1.05
120	1.63	1.58	1.58
600	2.03	2.03	2.17

[a] Equal in over-all activity to 10 mg digested enzyme.

Sedimentation of Digested Enzyme in a Sucrose Gradient and in the Analytical Ultracentrifuge.—As shown in Figure 3, digested enzyme sedimented more slowly than native enzyme in a sucrose gradient prepared according to Martin and Ames (1961). The broader spread of the digested enzyme indicated inhomogeneity, which was confirmed by measurements of the specific activities of the enzyme in the various fractions and susceptibility to threose di-P shown in Table VI. It is apparent that the more slowly the protein sediments in the sucrose gradient the more resistant it is to the inhibitor. The inability to react with arsenate as an acyl acceptor appears to run parallel to the increase in resistance to threose di-P. Similar results were obtained in the absence of sucrose in an analytical ultracentrifuge.[2] The preparation was inhomogeneous and

[2] These experiments were carried out in collaboration with Dr. R. C. Warner.

TABLE VI

FRACTIONATION OF DIGESTED ENZYME BY CENTRIFUGATION IN A SUCROSE DENSITY GRADIENT

Fraction No.	μmoles DPNH Formed/min./mg Protein					
	Native Enzyme			Digested Enzyme		
	Acceptor					
		Thioglycerol			Thioglycerol	
	Arsenate	−TDP[a]	+TDP[a]	Arsenate	−TDP[a]	+TDP[a]
5	33.1	0.48	0.03			
6	46.7	0.50	0.03			
7	59.2	0.50	0.02	7.68	0.96	0.08
8	41.6	0.51	0.03	8.63	0.85	0.08
9	34.2	0.51	0.03	7.52	0.86	0.09
10				3.08	0.66	0.16
11				1.44	0.37	0.16
12				0.72	0.22	0.16
13				0.50	0.19	0.14
14				0.16	0.14	0.09
15				0.18	0.11	0.08
16				<0.05	0.05	0.03
Not centrifuged	65.3	0.50	0.02	1.92	0.24	0.16

[a] TDP = D-Threose-2,4-di-P (1×10^{-5} M).

the major component had a sedimentation coefficient, $s_{20,w}$, of 4.83. This value should be compared with the value of $s_{20,w}$ of 7.19 for the native enzyme sedimented under the same conditions (*cf.* legend of Table VII). As shown in Table VII, samples of a run in a separation cell again revealed differences in susceptibility to threose di-P, the sample removed from the top compartment exhibiting greater resistance to the inhibitor.

Fractionation of Digested Enzyme on a P-cellulose Column.—3-P-Glycerate is an inhibitor of glyceraldehyde-3-P oxidation and has been shown to protect the enzyme against inhibition by iodoacetate (Racker and Krimsky, 1958). Advantage was taken of this specific interaction to achieve separation of the digested enzyme. Native enzyme adsorbed on a P-cellulose column was readily eluted with a low concentration of 3-P-glycerate (0.01 M), whereas the digested enzyme was not. The digested enzyme remained on the column and was eluted with 0.5 M KCl. As shown in Table VIII, fractionation on the column decreased the activity with arsenate from 3% to 0.12% of the activity of native enzyme, whereas there was only a minor loss in the specific activity with thioglycerol as acceptor; in fact, these preparations reacted faster with thioglycerol than with arsenate, whereas with native enzyme the oxidation rate with arsenate is over 100 times as rapid.

TABLE VII

PROPERTIES OF DIGESTED ENZYME SEDIMENTED IN THE ANALYTICAL ULTRACENTRIFUGE

Digested enzyme was dialyzed against 300 volumes of a mixture of 0.01 M Tris-HCl buffer, *p*H 7.9; 0.005 M EDTA; 0.0008 M DPN; and then diluted to 6 mg/ml in the dialysate.

Enzyme	μmoles DPNH Formed/min./mg Protein		% Inhibition by Threose di-P (1×10^{-5} M)
	Acceptor		
	Arsenate	Thioglycerol	
Native (dialyzed)	103.8	0.43	100
Digested (dialyzed)	4.6	0.35	44
Digested centrifuged:			
Top compartment	0.06	0.10	4
Bottom compartment	1.44	0.35	49

TABLE VIII

FRACTIONATION OF DIGESTED ENZYME ON PHOSPHATE CELLULOSE COLUMN

3 mg of dialyzed native or digested enzyme was applied to a phosphocellulose column 1 × 3 cm. The columns were eluted successively with 10 ml each of 0.005 M EDTA, *p*H 7.5; 0.01 M 3-P-glycerate–0.005 M EDTA, *p*H 7.5; 0.5 M KCl–0.005 M EDTA, *p*H 7.5. Temperature, 2°.

Enzyme	μmoles DPNH Formed/min./mg Protein		
	Acceptor		
		Thioglycerol	
	Arsenate	−TDP[a]	+TDP[a]
Native	81.1	0.47	<0.02
Native, dialyzed[b]	85.9	0.62	<0.02
Native, after column	80.0	0.57	<0.02
Digested	1.55	0.25	0.23
Digested, dialyzed[b]	2.54	0.27	0.15
Digested, after column	0.10	0.20	0.20

[a] TDP = D-Threose-2,4-di-P (1×10^{-5} M). [b] Enzyme was dialyzed against 1000 volumes 0.005 M EDTA, *p*H 7.5, for 2 hours; the EDTA solution was changed at the end of the first hour.

The digested enzyme eluted from the column was completely resistant to threose di-P.

DISCUSSION

The product of the proteolytic digestion of glyceraldehyde-3-P dehydrogenase is a protein with new properties. Compared to native enzyme it has a considerably lower sedimentation constant, a lower degree of stability, a decreased affinity to DPN and DPNH, and resistance to threose di-P inhibition. But most important of all, its phosphorolytic activity is almost completely lost. These findings appear to fit the picture of a partial digestion resulting in the removal of the phosphorylating head of this double-headed enzyme. However, this simple formulation requires some amplification in view of two experimental observations: (*a*) there is a small but reproducible residual arsenolytic activity present after digestion, which is not due to undigested native enzyme; and (*b*) the enzyme can be

completely acylated by 1,3-di-P-glycerate, although at a greatly diminished rate.

There are two alternative explanations for these findings. The proteolytic enzyme may cleave the protein into an oxidizing and a phosphorylating head which are capable of limited interaction; however, most of the phosphorylating activity is lost in the process. An alternative possibility is that proteolysis results in a damaged enzyme molecule, which still can catalyze the complete process but at a greatly reduced rate. Examples of such "crippled" enzymes have been described, *e.g.*, in the case of P-glucomutase and chymotrypsin that have been exposed to photo-oxidation (Ray and Koshland, 1960). In favor of the first explanation of a separable "head" is the finding that, after adsorption of the enzyme on phosphocellulose and differential elution, over 90% of the residual arsenolytic activity is lost. In favor of a "crippled" enzyme is the failure, even in these experiments, to remove the last traces of arsenolytic activity. Attempts to demonstrate a separable phosphorylating head have thus far yielded only suggestive results.

A rather complicating feature is the described variability of inhibition by threose di-P and the inhomogeneity of the digested protein as manifested in sedimentation experiments. These findings suggest that the product of digestion is actually a family of closely related proteins. Alternatively, a single protein species may exist in non-covalent combination with various fragments of proteolysis. There is some experimental evidence in favor of this latter possibility; it was observed in recent experiments that prolonged dialysis of the unfractionated digested enzyme against 0.66 M $(NH_4)_2SO_4$ considerably increased the susceptibility of the enzyme to threose di-P and also increased somewhat the residual arsenolytic activity. It seems likely, therefore, that small fragments of protein can combine with the digested enzyme and alter its properties.

Glyceraldehyde-3-P dehydrogenase catalyzes the step of oxidative phosphorylation in glycolysis and has therefore served many investigators as a model for studies of oxidative phosphorylation in mitochondria. Two observations recorded in this paper should be of interest in this connection.

The first observation is that exposure of glyceraldehyde-3-P dehydrogenase to chymotrypsin inactivated the phosphorolytic activity, whereas oxidation was unimpaired or accelerated. This finding stimulated experiments on proteolytic digestion of mitochondria, which resulted in the solubilizing of a component (coupling factor 3) which stimulates the phosphorylation process (Racker, 1962).

The second observation that may have some bearing on studies of oxidative phosphorylation is that the digested enzyme no longer exhibits a high degree of inhibition by DPNH. One can readily visualize that this inhibition phenomenon is akin to the well-known restraint of oxidation in "tightly coupled" mitochondria, referred to as respiratory control. Similarly, there is control in glycolysis, where the oxidation of glyceraldehyde-3-P is kept in check on the one hand by the restricted availability of P_i and ADP and on the other hand by DPNH inhibition. It is of interest, therefore, to discuss the properties of the native enzyme which give rise to limited acyl enzyme formation and which are altered by proteolytic digestion. The key to the mechanism of the inhibition of acylation was obtained in experiments showing that the rapidly declining rate of glyceraldehyde-3-P oxidation under the usual test conditions (0.3 mM DPN) can be converted to zero-order rates by addition of a large excess of DPN (5 mM) or by removal of DPNH with pyruvate and lactate dehydrogenase (Racker *et al.*, 1959). The reason for the inhibitory effect of DPNH, which was rather unexpected in view of the previously reported high dissociation constant of DPNH compared to DPN (Velick, 1958), can be found in the experiments showing decreased affinity to DPN after acylation of the enzyme. Furthermore, the arsenolysis of 1,3-di-P-glycerate (Racker and Krimsky, 1952) and the P_i-acetyl phosphate exchange (Harting and Velick, 1954) require DPN, as does the reduction of 1,3-di-P-glycerate (Hilvers and Weenen, 1962). Although these studies have not eliminated the possibility of a slow phosphorolysis of the acyl-E-DPNH, it appears that a rapid rate can occur only after DPNH becomes oxidized. Thus the scheme of the reaction sequence of glyceraldehyde-3-P oxidation to 1,3-di-P-glycerate requires insertion of an obligatory step, namely, oxidation of DPNH to DPN, as shown below.

Step 1: Glyceraldehyde-3-P + DPN-Enzyme $\rightleftharpoons$ Acyl-E-DPNH

Step 2: DPNH + pyruvate $\rightleftharpoons$ DPN + lactate

Step 3: Acyl-E-DPN + $P_i \rightleftharpoons$ 1,3-di-P-glycerate + DPN-E

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