Physical and Chemical Properties of Rabbit Muscle Phosphofructokinase Cross-Linked with Dimethyl Suberimidate†

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ABSTRACT: Rabbit skeletal muscle phosphofructokinase was cross-linked with the bifunctional reagent dimethyl suberimidate in 0.2 M triethanolamine hydrochloride (pH 8.5) in the presence of 5 mM MgATP and 2 mM fructose 1,6-bisphosphate. The reaction mixture was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and by sucrose density gradient sedimentation in 0.1 M Tris-chloride (pH 7.0), 10 mM citrate, and 10 mM dithiothreitol. The former technique indicated that after reaction for 1 hr or longer, appreciable amounts of cross-linked dimer, tetramer, and higher aggregates are formed, but only small amounts of trimer are found. After 1 hr of the cross-linking reaction, sucrose density gradient sedimentation showed the presence of five protein aggregates, corresponding to the dimer, tetramer, hexamer, octamer, and probably decamer, with the tetramer and octamer being the major species. These results suggest that the dimer is a fundamental unit for polymerization of the enzyme. The enzyme activity decreases as the reaction proceeds; a similar rate of loss of activity is found when the enzyme is reacted with methyl octanimidate, a monofunctional reagent, which indicates that the loss of activity is not due to cross-linking *per se*. The tetrameric and octameric enzymes obtained by sucrose density gradient sedimentation have sedimentation constants of 12.3(±0.2) S and 18.6(±0.3) S and possess about 30 and 50% of the specific activity of the native enzyme at pH 8.0. Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that the tetrameric species consists of 62% of a fully cross-linked tetramer, and 38% of a tetramer composed of associated cross-linked dimers. The tetramer and octamer have 4.7 and 5.5 mol of dimethyl suberimidate reacted per 80,000 g of protein. The enzyme reacted with the monofunctional reagent shows two activity peaks, when assayed at pH 8, between the positions expected for a dimer and tetramer on sucrose density gradient sedimentation under the same conditions used for the cross-linked enzyme; the native enzyme gives a single activity peak at the position expected for a dimer. Thus modification of the lysine residues by the reaction with the imidoesters shifts the dimer–tetramer equilibrium toward the tetramer. Moreover, the cross-linked aggregates do not polymerize further at protein concentrations, pH values, and effector concentrations where the native enzyme polymerizes. Steady-state kinetic studies of the cross-linked tetramer and octamer and the methyl octanimidate modified enzyme were carried out at pH 7.0. The results obtained indicate that the positive cooperativity observed for the initial velocity–fructose 6-phosphate isotherms with the native enzyme is lost in the cross-linked enzymes, although heterotropic interactions are retained. The cross-linked species are inhibited by MgATP, which raises the fructose-6-phosphate Michaelis constants but does not alter the maximal velocities significantly. These enzymes also are activated by AMP and cAMP by decreasing the Michaelis constant for fructose 6-phosphate; the maximal velocities are essentially unchanged. In contrast to the inhibition by MgATP, citrate inhibits the cross-linked aggregates by lowering the maximal velocity, while leaving the Michaelis constant unchanged. In both cases the maximum extent of inhibition is less than with the native enzyme. With the methyl octanimidate modified enzyme, the initial velocity–fructose 6-phosphate isotherm is sigmoidal (although less so than with the native enzyme), the MgATP inhibition is almost as great as with the native enzyme, and citrate both lowers the maximal velocity and weakens the binding of fructose 6-phosphate to the enzyme as with the native enzyme. The modes of inhibition by MgATP and citrate, therefore, appear to be different for all forms of the enzyme studied.

Rabbit skeletal muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is of primary importance in the regulation of glycolysis. In the pH range 6.9–7.1 it is susceptible to activation and inhibition by a number of metabolites (Passoneau and Lowry, 1962, 1963) and exhibits sigmoidal initial velocity–fructose 6-phosphate isotherms (Hofer and Pette, 1968). The enzyme contains a single type of subunit of molecular weight 80,000, which undergoes polymerization reactions that are dependent on the protein concentration, the pH, and the presence and absence of specific ligands (Aaronson and Frieden, 1972; Leonard and Walker, 1972; Pavelich and Hammes, 1973; Lad *et al.*, 1973).

The tetramer and higher aggregates possess a high enzymatic activity and are stabilized by high protein concentrations, high pH values (above 7), and activators such as fructose 6-phosphate, AMP, and fructose 1,6-bisphosphate. The enzyme is inhibited by MgATP, which stabilizes the tetramer, and by citrate, which depolymerizes the enzyme to a dimer of low specific activity at pH 8 and essentially no activity at pH 7 (Lad *et al.*, 1973). This suggests that two different modes of inhibition exist.

In this work, the native enzyme has been cross-linked with the bifunctional reagent, dimethyl suberimidate, in order to investigate the relationships between the polymerization and regulation of the enzyme. The results obtained suggest that the dimer is a fundamental unit of aggregation. Active tetrameric and octameric cross-linked enzymes have

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been isolated. The cross-linked enzymes do not exhibit kinetic homotropic fructose 6-phosphate interactions but are activated by the allosteric effectors AMP and cAMP, which lower the Michaelis constants for fructose 6-phosphate. The inhibitor MgATP raises this Michaelis constant, while citrate decreases the maximal velocity. The kinetic properties of the cross-linked aggregates are compared with those of the native enzyme and the enzyme modified with the monofunctional reagent methyl octanimidate. The implications of these findings for the regulatory mechanism are discussed.

Experimental Section

Materials. The ATP, ADP, fructose 6-phosphate, fructose 1,6-bisphosphate, dithiothreitol, aldolase, α-glycerophosphate dehydrogenase, triose phosphate isomerase, bovine serum albumin, yeast alcohol dehydrogenase, and beef heart catalase were purchased from Sigma Chemicals. The 1,6-dicyanohexane was purchased from Aldrich Chemical Co., sodium dodecyl sulfate (electrophoresis purity grade) was obtained from Bio-Rad Laboratories, and ultra-pure urea was purchased from SchwarzMann. Dimethyl suberimidate was synthesized from the suberonitrile (Davies and Stark, 1970). Radioactive dimethyl suberimidate and methyl octanimidate were generous gifts from Dr. L. Slobin. All other reagents were the best available commercial products. Distilled, deionized water was used in all experiments.

Phosphofructokinase. Rabbit skeletal muscle phosphofructokinase was purified by the method of Ling *et al.* (1966). The ammonium sulfate precipitate was dissolved in pH 8.0, 0.1 M potassium phosphate–1.0 mM ethylenediaminetetraacetic acid, and dialyzed against the same buffer to give a stock solution of 10–14 mg/ml. The ratio of the absorbance at 280 nm to that at 260 nm varied from 1.5 to 1.6 and was unchanged after passing the enzyme through a charcoal–cellulose (1:1) column (0.8 cm i.d. × 3 cm). The enzyme was therefore used without charcoal treatment. The enzyme concentration was determined from the absorbance at 280 nm using an extinction coefficient of 1.02 ml/(mg cm) (Parmeggiani *et al.*, 1966). The specific activity of the enzyme at 23°, 0.1 M phosphate, pH 8.0, was 110–120 units/mg. (A unit of enzyme activity is defined as the production of 1 μmol of product/min.) After a period of about 4 weeks, the specific activity of the enzyme stock solution declines significantly. Only enzyme of specific activity greater than 90 units/mg was employed to obtain the results presented here.

Assays. The production of fructose 1,6-bisphosphate was determined by using the coupling enzymes aldolase, triose phosphate isomerase, and glycerophosphate dehydrogenase and measuring the disappearance of NADH spectrophotometrically (Ling *et al.*, 1966). The large amount of ammonium sulfate present in the commercial enzymes influences the kinetic behavior of phosphofructokinase. Therefore, the auxiliary enzymes were dissolved in 0.01 M Tris-chloride (pH 8.0) with 2 mg/ml of bovine serum albumin and dialyzed overnight against the same buffer. Standard assays were run under the following conditions: assay A, pH 8.0, 33 mM Tris-chloride, 2 mM ATP, 5 mM $MgCl_2$, 2 mM fructose 6-phosphate, 0.1 mM NADH, 1 mM dithiothreitol, 0.25 unit/ml of aldolase, 3.2 units/ml of triose phosphate isomerase, and 0.1–0.3 μg of phosphofructokinase in a total volume of 3 ml; assay B, pH 6.9–7.0, varying amounts of fructose 6-phosphate and MgATP, all other reagent concentrations the same as in assay A. The assays were initiated by the addition of phosphofructokinase to the assay mixture, and the velocity of the enzymatic reaction was recorded spectrophotometrically at 340 nm using a Cary Model 14 spectrophotometer thermostated at 23°. Assay velocities were unaltered by increasing the concentration of auxiliary enzymes, and the NADH concentration was sufficiently low to avoid inhibition of the auxiliary enzymes (Newsholme *et al.*, 1970).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The general procedure of Weber and Osborn (1969) was used. A 10% acrylamide plug (1 cm) was used at the bottom of the tubes (0.5 cm i.d. × 10 cm) to ensure stability during electrophoresis. The gel was prepared by adding 1 ml of ammonium persulfate (0.15 mg/ml) to a solution containing 2.5 ml of acrylamide stock solution (32 g of acrylamide and 1 g of bisacrylamide to 100 ml), 10 ml of a buffer containing 0.1 M phosphate, 0.1% sodium dodecyl sulfate (pH 7), 50 μl of redistilled tetramethylenediamine, and distilled, deionized water to make a total volume of 20 ml. Protein samples were denatured by incubating a solution containing the protein (10–50 μg/ml), 0.1% β-mercaptoethanol, and 1% sodium dodecyl sulfate, at 37° for a period of 3 hr. The sample was then dialyzed overnight at room temperature against the electrophoresis buffer, 0.01 M sodium phosphate (pH 7.0), 0.1% sodium dodecyl sulfate, and 0.1% β-mercaptoethanol.

The electrophoresis was carried out at 3 mA/gel, and the gels were stained with Coomassie Blue. The gels were destained (Weber and Osborn, 1969) and scanned at 550 nm on a Gilford spectrophotometer with a Gilford Model 2410-S linear transport scanning accessory. A standard curve of peak area *vs.* the loaded protein concentration was determined with native phosphofructokinase to convert the observed spectrophotometric tracings of the bands to protein weight. This procedure takes into account the nonlinear variation of the stain color intensity with protein concentration.

Sucrose Density Gradient Sedimentation. Native and modified phosphofructokinase were also analyzed with sucrose density gradient sedimentation (Martin and Ames, 1961). The gradients (5–20% sucrose) were prepared using an automatic gradient mixer (Hoeffer Instruments), with solutions containing 0.1 M Tris-chloride (pH 7.0), 10 mM citrate, and 10 mM dithiothreitol. The centrifuge tube volume was 34 ml and sample volumes of 0.25–0.6 ml were layered on the gradients. Centrifugation was carried out at 4° in a Beckman Model L2-65B ultracentrifuge with an SW27 rotor for approximately 20 hr at 27,000 rpm. The tubes were then drained from the top using an Auto Densi-Flow [Buchler Instruments], and 1-ml fractions were collected. The enzymatic activity of the fractions was determined using assay A, and the protein content was determined by precipitation of the protein at 0° with 10% trichloroacetic acid, followed by the procedure of Lowry *et al.* (1951).

The sedimentation constants of the various cross-linked species were determined using the following external standards: aldolase (10 mg/ml), $s_{20,w}^0$ = 8.0 S (Zavodsky and Biszku, 1967), beef heart catalase (1.2 mg/ml), $s_{20,w}^0$ = 11.4 S (Sund *et al.*, 1967), and yeast alcohol dehydrogenase (2 mg/ml), $s_{20,w}^0$ = 7.2 S (Hogeboom and Kuff, 1954). For sucrose density gradient sedimentation with these standards, the 5–20% sucrose gradient was made up in 0.01 M Tris-chloride (pH 7.5) and 0.25 ml of a solution containing the enzyme in the same buffer at the concentrations indi-

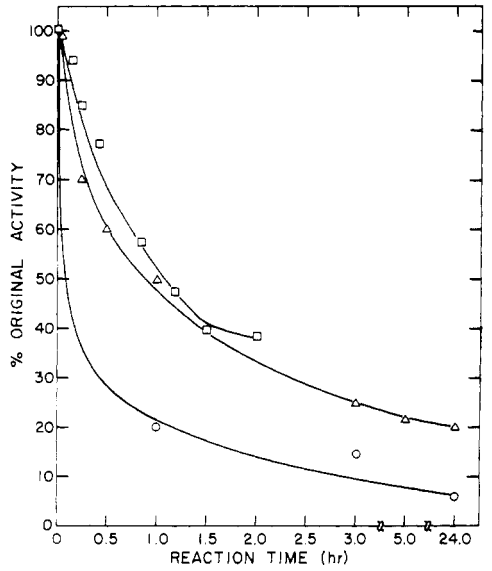

FIGURE 1: A plot of the per cent of original activity and per cent of enzyme monomer during the course of the reaction of enzyme with dimethyl suberimidate (activity △, monomer ○) and methyl octanimidate (activity, □). The reaction was initiated by the addition of solid dimethyl suberimidate (3 mg/ml) or methyl octanimidate (0.15 mg/ml) to the enzyme (0.25 mg/ml) in 0.2 M triethanolamine hydrochloride (pH 8.5), 5 mM MgATP, and 5 mM fructose 1,6-bisphosphate at 4°. The enzyme activities were measured by dilution of a small aliquot of the reaction mixture into assay mixture A. The per cent of monomer was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Procedural details are given in the Experimental Section.

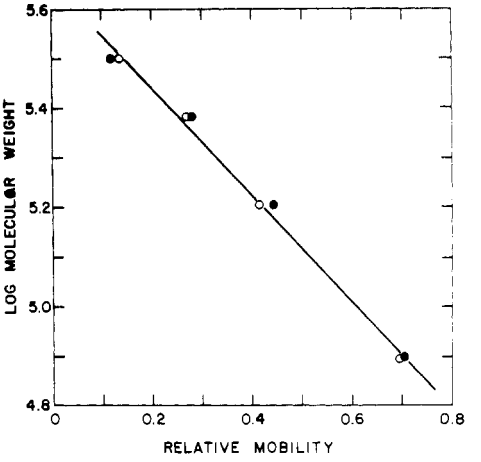

FIGURE 2: A plot of the logarithm of the molecular weight of phosphofructokinase aggregates *vs.* the relative mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis: (○) cross-linking at pH 8.5; (●) cross-linking at pH 8.0. Detailed reaction conditions are given in the Experimental Section.

cated. The assays for these standards were as described in the Biochemica Catalogue (Boehringer-Mannheim 1970 edition). The $s_{20,w}{}^0$ values were estimated using the relationship $s_{20,wU}{}^0/s_{20,wR}{}^0 = d_U/d_R$ where the subscripts U and R designate the unknown and reference substances, respectively, and d is the distance from the meniscus to where the material has sedimented (Martin and Ames, 1961). Molecular weights were estimated from the relationship $s_{20,wR}{}^0/s_{20,wU}{}^0 = (MW_R/MW_U)^{2/3}$ where MW is the molecular weight (Martin and Ames, 1961).

Cross-Linking Experiments. Phosphofructokinase was incubated at a concentration of 0.25 mg/ml in 0.2 M triethanolamine hydrochloride (pH 8.0 or 8.5) with 5 mM ATP, 5 mM $MgCl_2$, and 2 mM fructose 1,6-bisphosphate at 4°. The reaction was initiated by the addition of solid dimethyl suberimidate to a final concentration of 3 mg/ml (11 mM), and the reaction was stopped by the addition of 2 M ammonium chloride to a final concentration of 50 mM. After 5 min the pH of the quenched reaction mixture was lowered to pH 7.0 by the addition of potassium dihydrogen phosphate. The loss of activity with time was followed by withdrawing aliquots from the reaction mixture and assaying them at pH 8.0 (assay A). In order to assess the degree of cross-linking of the protein, the reaction was stopped at 1, 3, and 24 hr, and samples were withdrawn for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The modified enzyme aggregates were also characterized using sucrose density gradient sedimentation. The reaction of the enzyme with 0.15 mg/ml (0.8 mM) methyl octanimidate was carried out under the same conditions as the reaction with dimethyl suberimidate.

The number of moles of dimethyl suberimidate reacted with phosphofructokinase was estimated by using radioactive dimethyl suberimidate (^3H, 1.84 Ci/mol) in the reaction mixture. After 1 hr of reaction, an aliquot of the reaction mixture was analyzed by sucrose density gradient sedimentation. The fractions corresponding to the major components of enzyme activity were pooled and dialyzed against 0.1 M Tris-chloride (pH 7.0), 10 mM citrate, and 10 mM dithiothreitol. The protein was precipitated with ice-cold trichloroacetic acid and the amount of protein present was determined by the method of Lowry *et al.* (1951). Small aliquots of these fractions (10–25 μl) were dissolved in 10 ml of scintillation fluid (Bray, 1960) and the radioactivity was determined with a Beckman Model LS-255 scintillation counter. Since the specific activity of the cross-linking agent is known, the number of moles of cross-linking agent reacted per mole of protein can be calculated directly.

Results

Enzyme Cross-Linking with Dimethyl Suberimidate. Dimethyl suberimidate is a bifunctional reagent, specific for lysine residues (Means and Feeney, 1971; Hunter and Ludwig, 1962). The reaction was carried out at pH 8.5, where an appreciable amount of cross-linking takes place without extensive hydrolysis of the reagent (Davies and Stark, 1970). The time dependence of the enzyme activity after mixing the enzyme and cross-linking reagent is shown in Figure 1. After 3 hr only about 25% of the original activity remains. Reaction of the enzyme with the monofunctional reagent, under the same conditions, shows a similar rate of loss of activity (Figure 1). Aliquots of the reaction mixture were also analyzed at various times by sodium dodecyl sulfate polyacrylamide gel electrophoresis. A number of bands corresponding to various cross-linked aggregates were obtained. The fastest moving band in the reaction mixture has the same mobility as found for the native enzyme and was therefore assumed to have a molecular weight of approximately 80,000 (Pavelich and Hammes, 1973). Slower moving bands were assumed to be oligomers having molecular weights corresponding to the dimer, trimer, and tetramer, respectively. A plot of the logarithm of the molecular weight *vs.* the relative mobility is linear as expected (Figure 2). Protein bands corresponding to aggregates of higher molecular weight than the tetramer could not be identified in this way, because the relative mobilities of these aggregates are too small to be measured accurately and the variation of

TABLE I: Cross-Linked Enzyme Species at Various Reaction Times.[a]

Reaction Time (hr)	Per Cent				
	Monomer	Dimer	Trimer	Tetramer	Higher
0	100				
1	21	34	6	10	29
3	17	40	4	16	23
24	4	30	0.2	21	45

[a] Phosphofructokinase (0.25 mg/ml) and dimethyl suberimidate (3 mg/ml) were incubated at 4° in 0.2 M triethanolamine (pH 8.5) with 5 mM MgATP and 2 mM fructose 1,6-bisphosphate. The samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Details are given in the Experimental Section.

the mobility with the logarithm of the molecular weight is not linear in this molecular weight region (Weber and Osborn, 1969).

The relative amounts of the various cross-linked aggregates present at different reaction times were estimated from spectrophotometric scanning of the gels as described in the Experimental Section (Table I). As expected the amount of monomeric subunit decreases steadily with increasing reaction time. The amount of trimer is quite small at all times, while the amounts of tetramer and higher aggregates increase with time and the dimer content decreases slightly.

The per cent monomer of the enzyme (indicative of non-cross-linked protein) is shown in Figure 1 as a function of the reaction time. The cross-linking reaction has proceeded to 80% completion after 1 hr (corresponding to about 50% loss of activity). The modified enzymes obtained after 1 hr of reaction with dimethyl suberimidate and with methyl octanimidate were further characterized as described below.

Purification and Characterization of Cross-Linked Aggregates. Attempts to isolate the various cross-linked aggregates by column chromatography with Agarose (Bio-Gel 0.5, 1.5, and 5 M) and Sephadex G-200 were unsuccessful. Separation by using polyacrylamide gel electrophoresis also failed. However, sedimentation of the reaction mixture in a 5–20% sucrose gradient yielded a good separation of aggregates. The protein and activity profiles of a typical sucrose gradient sedimentation are shown in Figure 3. The buffer used for the separation was 0.1 M Tris-chloride (pH 7.0), 10 mM citrate, and 10 mM dithiothreitol, which dissociates the native enzyme to a dimer and reduces the possibility of the cross-linked species forming aggregates. Five protein peaks are obtained, as shown in Figure 3. The sedimentation coefficients of these peaks correspond to dimer (I), tetramer (II), hexamer (III), octamer (IV), and probably decamer (V). Peaks II and IV, which have the highest specific activity, were further characterized. The sedimentation coefficients ($s_{20,w}^0$) of peaks II and IV were determined to be 12.3(±0.2) S and 18.6(±0.3) S, respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the octamer (peak IV) showed about 90% of the protein in one band; higher molecular weight aggregates (up to 10%) and small amounts of smaller aggregates (<2%) were also present. The tetramer protein peak (II) showed two distinct

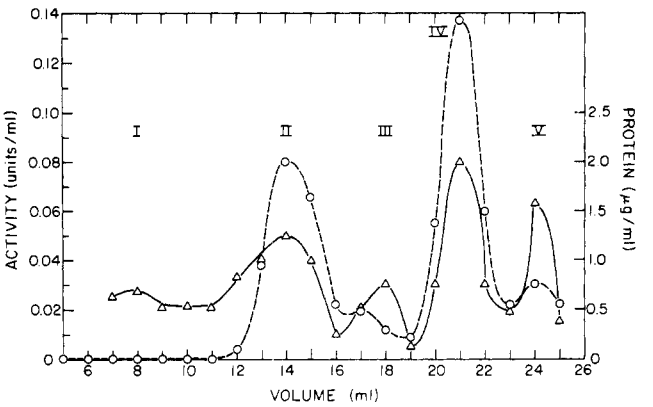

FIGURE 3: The sucrose density gradient sedimentation profile of the cross-linked enzyme. The enzyme activity (○) in units/ml is the left ordinate and the μg/ml of protein (△) is the right ordinate; the abscissa is the volume measured from the top of the gradient. The reaction of the enzyme with dimethyl suberimidate was stopped after 1 hr, and the reaction mixture (0.25 ml) was applied on a 5–20% sucrose density gradient (34 ml) in 0.1 M Tris-chloride, 10 mM citrate, and 10 mM dithiothreitol (pH 7.0) and centrifuged for 20 hr at 27,000 rpm in a Beckman L2-65B ultracentrifuge with an SW27 rotor. The enzyme activity was determined with assay A, and the protein concentration was determined by the method of Lowry *et al.* (1951), after precipitation of the protein with ice-cold 10% trichloroacetic acid.

bands in sodium dodecyl sulfate polyacrylamide gels. These correspond to dimer (38%) and tetramer (62%). This analysis indicates that protein peak II is heterogeneous in composition: it consists of a fully cross-linked tetramer and associated cross-linked dimers. Efforts to resolve this peak further into enzymatically active cross-linked dimers and tetramers by sucrose density gradient sedimentation in the presence of urea (0–2.5 M) were unsuccessful. Separation of these components did not occur even at a urea concentration of 2.5 M when 70% of the original enzyme activity is lost.

The amount of dimethyl suberimidate reacted with the enzyme in protein peaks II and IV was determined with radioactive dimethyl suberimidate as described in the Experimental Section: 4.75 and 5.5 mol of dimethyl suberimidate per 80,000 g of protein were found in peaks II and IV, respectively. The physical and chemical properties of protein peaks II and IV are summarized in Table II.

The native enzyme shows aggregation behavior which is sensitive to the concentration of the enzyme as well as to the pH and ligands present (Aaronson and Frieden, 1972; Pavelich and Hammes, 1973; Lad *et al.*, 1973). However, sucrose gradient sedimentation of the cross-linked aggregates in 0.1 M phosphate (pH 8.0) gave the same protein and enzyme activity profiles as obtained using 0.1 M Tris-chloride (pH 7.0), 10 mM citrate, and 10 mM dithiothreitol. Also a sixfold concentration of the modified enzyme did not significantly alter the profiles. The concentrated protein, however, precipitated gradually with time; therefore the protein in the reaction mixture was not normally concentrated.

Sucrose density gradient sedimentation of the native enzyme in 0.1 M Tris-chloride (pH 7.0), 10 mM citrate, and 10 mM dithiothreitol yields a single sharp activity peak, when assayed at pH 8 (assay A), with $s_{20,w}^0$ = 8.3(±0.3) S, which corresponds closely to that expected for the molecular weight of the dimer. Under the same conditions the enzyme modified with methyl octanimidate displayed a sucrose density gradient sedimentation profile with two activity peaks, one at the position expected for a dimer and the other at a position between those expected for a dimer and tetramer (Figure 4).

TABLE II: Properties of Cross-Linked Aggregates.

	Protein Peak II[a]	Protein Peak IV[a]	Technique
$s_{20,w}^0$ (S)	12.3(±0.2)	18.6(±0.3)	Sucrose density gradient sedimentation
Composition	38% dimer 62% tetramer	90% octamer 8% larger than octamer <2% smaller than octamer	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Specific activity[b] (units/mg)	45	62	Steady-state kinetics
Moles of dimethyl suberimidate reacted per 80,000 g of enzyme	4.75	5.50	Radioactive dimethyl suberimidate

[a] See Figure 3. [b] Average of four preparations, assay A.

Kinetic Studies of Modified Enzymes. Steady-state kinetic experiments were carried out with the tetrameric and octameric enzymes at pH 7.0, using assay B. When the fructose 6-phosphate concentration was held constant at 1 mM, the initial velocity varied with the MgATP concentration as shown in Figure 5. For both the tetrameric and octameric enzyme the maximal activity was observed at a MgATP concentration of approximately 0.3 mM and the inhibition, about 30% of the maximal activity, was essentially complete at MgATP concentrations in the 2–4 mM range (Figure 5). The dependence of the initial velocity on the fructose 6-phosphate concentration was studied at MgATP concentrations where maximal activity (0.3 mM) and essentially complete inhibition (4 mM) occur. At both concentrations the variation of the initial velocity with fructose 6-phosphate concentration obeys Michaelis–Menten kinetics for the cross-linked tetramer and octamer. This is in contrast to the native enzyme which displays sigmoidal initial velocity–fructose 6-phosphate isotherms (Hofer and Pette, 1968). The maximum velocities and fructose 6-phosphate Michaelis constants for the cross-linked aggregates were determined from a weighted least-squares analysis of plots of the reciprocal initial velocity *vs.* the reciprocal fructose 6-phosphate concentration and are given in Table III. Similar kinetic experiments were also carried out in the presence of activators, AMP (3.5 mM) and cAMP (3 mM), and in the presence of the inhibitor citrate (10 mM). Again Michaelis–Menten behavior was observed, and the steady-state parameters are included in Table III.

For the native enzyme, the maximal velocities and the fructose 6-phosphate concentrations at which the initial velocity is one-half of its maximal value, $K_{1/2}$, are also presented in Table III at both inhibitory and noninhibitory MgATP levels in the presence and absence of activators and citrate. Since the initial velocity–fructose 6-phosphate isotherms are sigmoidal, Michaelis constants cannot be determined.

The kinetic properties of the enzyme reacted for 1 hr with methyl octanimidate were determined after dialysis of the enzyme overnight against 0.1 M phosphate (pH 8.0), 0.1 mM ethylenediaminetetraacetic acid. The specific activity of the modified enzyme was 40 units/mg at pH 8.0 and 21 units/mg at pH 7.0. The inhibition of the modified enzyme by MgATP (Figure 5) was much greater than with the cross-linked enzymes and similar to the native enzyme. The initial velocity–fructose 6-phosphate isotherms were sigmoidal with a Hill coefficient of approximately 2–2.6 at in-

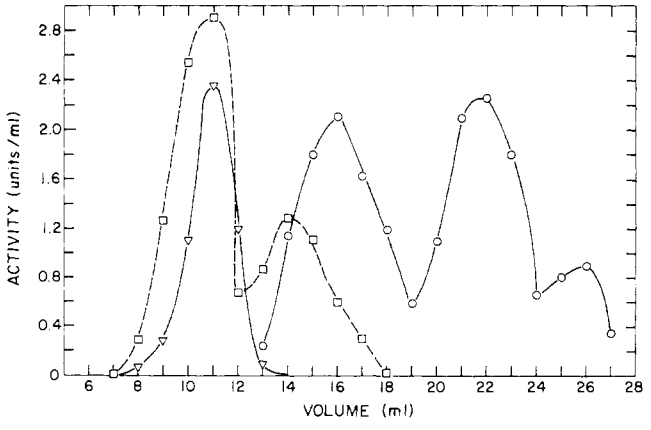

FIGURE 4: A plot of the enzyme activity in units/ml *vs.* the volume measured from the top of the sucrose density gradient centrifuged for 20 hr at 27,000 rpm in a Beckman L2-65B ultracentrifuge with an SW27 rotor: native enzyme, ∇; the enzyme reacted with dimethyl suberimidate, ○; and the enzyme reacted with methyl octanimidate, □. Other experimental conditions are the same as in Figure 3.

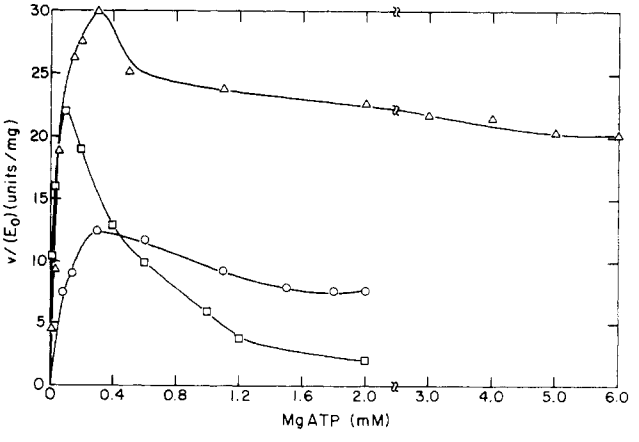

FIGURE 5: A plot of the initial velocity divided by the enzyme concentration, $v/(E_0)$, in units/mg *vs.* the MgATP concentration at a fructose 6-phosphate concentration of 1 mM, for the cross-linked tetramer (○, protein fraction II, Figure 2), the cross-linked octamer (△, protein fraction IV, Figure 2), and the methyl octanimidate modified enzyme (□) at 23°. Assay B, as described in the Experimental Section, was used.

TABLE III: Steady-State Kinetic Parameters of Phosphofructokinase.[a]

Effector	Native		Methyl Octanimidate Modified Enzyme		Protein Peak II		Protein Peak IV	
	$K_{1/2}$ (mM)	V_{max} (units/mg)	$K_{1/2}$ (mM)	V_{max} (units/mg)	K_m (mM)	V_{max} (units/mg)	K_m (mM)	V_{max} (units/mg)
Noninhibitory MgATP[b]								
None	0.3	52.5	0.2	16.6	0.64	12.4	0.40	28.3
AMP[c]	0.056	52.5	0.035	17.3	0.59	13.4	0.26	28.3
cAMP[d]	0.063	52.5	0.030	17.5	0.53	13.3	0.24	28.3
Citrate[e]	1.9	10.2	3.5	2.8	0.63	5.9	0.38	11.0
Inhibitory MgATP[f]								
None	3.7	52.5	3.2	16.6	2.2	15.9	2.01	35.0
AMP	0.48	52.5	1.6	17.3	0.71	15.2	1.03	31.0
cAMP	0.55	52.5	1.43	17.5	0.92	13.3	1.03	31.0
Citrate[e]	12.5	21.2	22.5	7.7	2.2	9.5	2.28	9.4

[a] Initial velocities were determined at 23°, pH 7.0, assay B. For the cross-linked aggregates the reciprocal initial velocity was plotted against the reciprocal fructose 6-phosphate concentration, and the Michaelis constants for fructose 6-phosphate and the maximal velocities were obtained by a weighted least-squares analysis of the data. In the case of the native enzyme and the enzyme modified with the monofunctional reagent, Michaelis–Menten behavior is not observed, and $K_{1/2}$ indicates the concentration of fructose 6-phosphate for which the velocity is one-half of the maximal velocity. The maximal velocities were estimated from the value of the initial velocities at high fructose 6-phosphate concentrations. [b] 0.3 mM MgATP for cross-linked enzyme, 0.1 mM for methyl octanimidate modified enzyme, and 0.13 mM for native enzyme. [c] 3.5 mM AMP for cross-linked enzyme, and 3.0 mM for native enzyme and methyl octanimidate modified enzyme. [d] 3.0 mM cAMP for cross-linked enzyme and methyl octanimidate modified enzyme, and 2.0 mM for native enzyme. [e] 10 mM. [f] 4.0 mM MgATP for cross-linked enzyme and methyl octanimidate modified enzyme, and 2.7 mM for native enzyme.

hibitory concentrations of MgATP and 1.2–1.5 at noninhibitory MgATP concentrations. The corresponding Hill coefficients for the native enzyme are 3.3–4.0 and 1.1–1.6, respectively. (The range of Hill coefficients is because the Hill coefficient varies somewhat depending on the activator or inhibitor present.) The maximal velocities and $K_{1/2}$ values for the methyl octanimidate modified enzyme are given in Table III at both inhibitory and noninhibitory MgATP levels in the presence and absence of activators and citrate.

The estimated uncertainty in the kinetic parameters listed in Table III is approximately ±15%.

Discussion

The reaction of rabbit muscle phosphofructokinase with dimethyl suberimidate leads to the formation of several active cross-linked aggregates. In the case of the tetrameric and octameric species about 5 mol of the reagent react/mol of monomeric enzyme. Because only small amounts of these aggregates could be obtained a direct determination of the number of cross-links could not be made (*cf.* Hartman and Wold, 1967). If the reacted bifunctional reagent is present entirely as cross-links, about 30% (10/33) (Parmeggiani *et al.*, 1966) of the lysine residues have been modified; if the reagent binds only monofunctionally, about 15% (5/33) of the lysine residues have been altered. With ribonuclease A it has been found that about one-third of the cross-linking reagent, dimethyl adipimidate, reacts bifunctionally (Hartman and Wold, 1967). This suggests that about seven lysine residues have been modified per polypeptide chain (80,000 molecular weight), which results in a reduction of the specific activity to about one-half that of the native enzyme at pH 8 (assay A). Since the monofunctional reagent causes a similar drop in the specific activity, the loss of enzymatic activity seems to be due to the modification of lysines, rather than to cross-linking *per se*. Apparently the monofunctional reagent is a more potent inactivator of the enzyme than the cross-linking reagent since the same amount of inactivation is produced at a lower reagent concentration.

The composition of the cross-linked aggregates is an indication of the stability of specific aggregates present in solution. The protein concentration in the reaction mixture is sufficiently low for cross-linking due to transient collisions between protein molecules to be negligible. Analysis of the reaction mixture with sodium dodecyl sulfate polyacrylamide gel electrophoresis establishes the amounts of monomer, dimer, trimer, and tetramer present; higher aggregates are also present, but the molecular weights of these cannot be reliably established by this method. The results in Table I indicate that at long reaction times very little monomer or trimer is present. The data suggest that in the molecular weight range up to the tetramer, the dimer is the fundamental unit of aggregation. (A possibility which cannot be excluded is that the partial cross-linking of large aggregates present in solution also produces small aggregates.) The results obtained by sucrose density gradient sedimentation indicate the presence of dimer, tetramer, hexamer, octamer, and probably decamer, the latter four being enzymatically active (Figure 3). However the dimer, hexamer, and decamer are present in small amounts. Sodium dodecyl sulfate electrophoresis of protein peak II, which sediments as a tetramer, shows that both cross-linked dimer and cross-linked tetramer are present, while protein peak IV contains essentially only cross-linked octamer. This suggests that the dominant mode of protein aggregation involves dimer, tetramer, and octamer, although the unit of polymerization beyond the tetramer could be either the dimer or the tetramer.

Sucrose density gradient sedimentation of the native enzyme in 0.1 M Tris-chloride, 10 mM citrate, and 10 mM dithiothreitol indicates only a dimer ($s_{20,w}^0 = 8.3(\pm 0.3)$ S) is present in appreciable amounts. The dimer has a low specific activity at pH 8 and is essentially inactive at pH 7 (Lad *et al.*, 1973). Previous ultracentrifuge (Paetkau and Lardy, 1967) and gel filtration (Lad *et al.*, 1973) studies have suggested that the dimeric species is unstable. However, the dimer is stable after 20 hr of sedimentation in a sucrose density gradient; perhaps sucrose significantly stabilizes the dimer. Ultracentrifuge studies at alkaline pH values and high protein concentrations (Leonard and Walker, 1972; Aaronson and Frieden, 1973) have led to conjecture about the existence of an octamer (18 S species), but its existence has not been conclusively established. The fact that relatively large amounts of an active cross-linked octamer are present suggests that the octamer is a stable species.

The modified enzyme which sediments as a tetramer in the sucrose density gradient (protein peak II, Figure 3) contains both dimeric and tetrameric cross-linked aggregates. However, the tetramer formed from cross-linked dimers is very stable; dissociation does not occur even in 2.5 M urea; this is in contrast to the native enzyme which dissociates to stable dimers under the conditions used in the sucrose density gradient sedimentation experiments (Figures 3 and 4). Modification of the enzyme with the monofunctional reagent yields an enzyme which gives a two peak activity profile on sedimentation in a sucrose gradient that suggests the existence of a dimer–tetramer equilibrium. The hydrophobic octanimidate chains on the lysine residues apparently enhance protein association, either through a conformational change of the protein, or through the interaction of the hydrocarbon chains either with each other or with the protein. Similar enhanced stability of polymers has been reported for phosphorylase *b* modified with glutaraldehyde or butyraldehyde (Wang and Tu, 1969, 1970). In contrast to this behavior, the reaction of pyridoxal phosphate with the lysine residues of phosphofructokinase promotes dissociation, rather than association, of the enzyme (Uyeda, 1969).

At pH 7.0 the tetrameric and octameric species of cross-linked enzymes (protein peaks II and IV, Figure 3) are quite active, with maximal activities about 25 and 50% of the native enzyme. However, the native enzyme and the methyl octanimidate modified enzyme display cooperative initial velocity–fructose 6-phosphate isotherms, while the cross-linked enzymes follow simple Michaelis–Menten kinetics. The cross-linked aggregates are activated by AMP and cAMP, as is the native enzyme and the methyl octanimidate modified enzyme, indicating that heterotropic interactions are retained. The activation in all cases involves an increase in the apparent affinity of the enzyme (a decrease in $K_{1/2}$ or K_m) for fructose 6-phosphate. The maximum velocity remains essentially unaltered in all cases.

All of the enzyme species studied are inhibited by MgATP, which causes a decrease in the apparent affinity of the enzyme for fructose 6-phosphate (*i.e.*, $K_{1/2}$ or K_m increases). However, the extent of inhibition is much greater in the native enzyme and the methyl octanimidate modified enzyme. Citrate, on the other hand, inhibits all of the cross-linked species by lowering the maximal velocity. In the cases of the native enzyme and methyl octanimidate enzyme, $K_{1/2}$ is also greatly increased. An interpretation of this increase in $K_{1/2}$ is complicated by the polymerization of the enzyme caused by the binding of fructose 6-phosphate to the enzyme (Lad *et al.*, 1973). The modes of inhibition of the native enzyme by MgATP and citrate are not the same. This has been previously suggested by the fact that while MgATP stabilizes the tetrameric enzyme, citrate dissociates it rapidly into dimers (and probably monomers), which are essentially inactive at pH 7 and have a very low specific activity at pH 8 (Lad *et al.*, 1973). Citrate inhibits the cross-linked enzyme 50% maximally; a similar but probably larger conformational change leads to dissociation and essentially complete inactivation (when assayed at pH 7) of the native enzyme and the methyl octanimidate modified enzyme. However, the native tetrameric enzyme (and presumably the tetrameric methyl octanimidate modified enzyme) is also partially inhibited by citrate (Table III, V_{max} in 10 mM citrate).

The homotropic and heterotropic interactions between substrate and activator sites on the methyl octanimidate enzyme are less strong than in the native enzyme. In the cross-linked aggregates the homotropic interactions between fructose 6-phosphate sites are essentially abolished, while the heterotropic interactions are somewhat less than in the methyl octanimidate modified enzyme. With sheep heart phosphofructokinase, which is similar to the rabbit skeletal muscle enzyme, homotropic interactions are destroyed by photooxidation of the enzyme with Methylene Blue, or by acylation with ethoxyformic anhydride (Setlow and Mansour, 1970). However, MgATP inhibition also does not occur, and other heterotropic effects are less with these modified enzymes.

While the qualitative behavior of the tetrameric and octameric cross-linked enzymes is similar, the octamer is more active, and has higher maximal velocities and slightly lower Michaelis constants at noninhibitory MgATP concentrations, and requires a higher concentration of MgATP to produce complete inhibition. Thus, the octamer may be frozen into a more active conformation than the tetramer. Unfortunately this comparison is complicated by the existence of two types of tetramers, one type having all subunits cross-linked, the other consisting of cross-linked dimers.

The differences in the kinetic properties of the cross-linked aggregates and the native enzyme and the methyl octanimidate modified enzyme, and their response to allosteric ligands, permit some inferences to be made about the regulatory mechanism. Since the homotropic interactions can be destroyed while heterotropic interactions are retained, a simple two-state conformational model for the regulation of the native enzyme tetramer, such as that of Monod *et al.* (1965), is not sufficient. Multiple conformational transitions probably occur. Furthermore, at least two different mechanisms of inhibition exist, one utilized by MgATP, the other by citrate. The MgATP inhibition involves a conformational change which stabilizes the tetrameric enzyme, whereas citrate can induce a very different conformational change that causes dissociation of the tetramer. These diverse and complex regulatory mechanisms are accomplished by a single type of subunit, and the molecular details of the regulatory mechanism remain to be established.

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A Homogeneous, Thermostable Deoxythymidine Kinase from *Bacillus stearothermophilus*†

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ABSTRACT: A deoxythymidine kinase has been purified from *Bacillus stearothermophilus* 16,000-fold over the activity of the extract. The molecular weight of the kinase determined by molecular sieve chromatography (0.005 M Tris–glycine (pH 8.3), 4° was found to be 52,000. A molecular weight estimation by polyacrylamide gel electrophoresis (10% acrylamide, pH 10.5) revealed isomers of molecular weight 54,000 and 108,000. A polypeptide chain analysis revealed a single protein band of molecular weight 28,500. The Michaelis–Menten constant (K_m) for deoxythymidine was $4.8 \pm 0.8 \times 10^{-5}$ M at 75° and $5.3 \pm 0.6 \times 10^{-7}$ M at 37°. The K_m for Mg-ATP^{2-} was $4.3 \pm 0.2 \times 10^{-4}$ M at 75° and remained constant at $3.8 \pm 0.4 \times 10^{-4}$ M at 37°. Deoxythymidine triphosphate (dTTP) functioned as a negative effector. The inhibition constant (K_i) for dTTP was 23×10^{-6} M at 75° and 9.3×10^{-6} M at 39°. Exposure of the enzyme at 75° for 60 min resulted in no loss of activity. When exposed at 100° for 30 min, a 40% loss of activity was observed. The catalytic power of the kinase increased from 1.25 μmol of dTMP hr^{-1} μg^{-1} at 32° to 220 μmol at 90°.

The role of deoxythymidine kinase in regulation of DNA synthesis is yet to be established, however, an increased activity of the enzyme has always been reported preceding or concomitant with the onset of DNA synthesis. The phenomenon has been observed in a variety of extracts from plants (Hotta and Stern, 1963; Wanka *et al.*, 1964), regenerating rat liver (Bollum and Potter, 1959; Bresnick *et al.*, 1970), virus infected cells (McAuslan, 1963; Hatanaka and Dulbecco, 1967; Kit *et al.*, 1970), adrenal gland stimulated by ACTH, insect epithelium stimulated by ecdysone (Masui and Garren, 1970; Brookes and Williams, 1965), and mammalian cells during the S phase (Stubblefield and Mueller, 1965). The enzyme is also very active in mammalian tumors (Sneider *et al.*, 1969; Bresnick and Thompson, 1965). In general, the activity of the enzyme appears to be very responsive to stimuli which ultimately result in DNA synthesis.

So far, only partial purification of this enzyme has been achieved due to its instability. The method of preparation of

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