The Modification of Yeast Hexokinases by Proteases and Its Relationship to the Dissociation of Hexokinase into Subunits*

IRENE T. SCHULZE and SIDNEY P. COLOWICK

From the Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37203

SUMMARY

Crystalline hexokinase prepared by the method of Darrow and Colowick consists largely of enzyme which has been modified in its chromatographic and electrophoretic behavior by yeast protease which is present during the isolation procedure. This proteolytic modification can be prevented by removal of the protease by chromatography on diethylaminoethyl cellulose; it can also be largely inhibited by phenylmethylene sulfonyl fluoride.

Preparations made by the diethylaminoethyl cellulose method contain two forms of the enzyme (P-I and P-II), both of which are more basic by chromatography and electrophoresis than is protease-modified enzyme (S forms). Form P-II shows the same catalytic activity as the Darrow-Colowick preparation, but Form P-I is less active. The two forms are equally susceptible to modification by trypsin.

Forms P-I and P-II exist mainly as tetramers of molecular weight about 100,000 at low ionic strength in the neutral pH region, as measured by ultracentrifuge, light scattering, or gel filtration. They tend to dissociate, without loss of catalytic activity, to dimers as the ionic strength, temperature, or pH is raised or when glucose is added in the presence of phosphate. They can be converted to S forms by trypsin or yeast protease only when glucose or salts are present, suggesting that the enzyme must be in the dimer state in order for modification to occur.

The protease-modified enzyme tends to exist mainly as a dimer of molecular weight around 50,000 at neutral pH. Thus, modification by trypsin seems to remove some groups which normally play a role in the association of dimer molecules to form the tetramer structure.

Mercaptoethanol causes a striking decrease in sedimentation rate when added to the enzyme at pH 9.0. This may be correlated with the recent finding that mercaptoethanol can cause inactivation of the enzyme at this pH.

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‡ Present address, The Public Health Research Institute of the City of New York, Inc., 455 First Avenue, New York, New York 10016.

Crystalline preparations of yeast hexokinase (ATP: d-hexose 6-phosphotransferase EC 2.7.1.1) made by the method of Darrow and Colowick (1) were found to contain a number of forms which were indistinguishable on the basis of enzyme activity but which could be separated by chromatography on DEAE-cellulose (2). It was concluded from previous work (3) that these forms existed in the yeast cell. Ultracentrifuge studies (4, 5) showed that these crystalline preparations exhibited a single sedimenting boundary at a pH near the isoelectric point (5.4) and a double boundary in the neutral pH range. Ramel, Barnard, and Schachman (6) have suggested that this behavior is due to the existence of two major forms of the enzyme, one of which exists as a tetramer of molecular weight approximately 94,000 at both pH 5.0 and 8.0, while the other exists as a tetramer at pH 5.0 but dissociates to dimers at pH 8.0. They also reported that the addition of glucose to hexokinase at pH 8.0 caused dissociation of the tetramers to the dimer state, so that a single sedimenting boundary was obtained.

In the course of preparing enzyme for studies on the possible relationship of the multiple forms of the enzyme to its subunit structure (4, 5), it was found that the various chromatographic forms found in highly purified preparations of hexokinase (1) appeared during purification and storage. These preparations were found to contain a protease, as Schachman had noted earlier.1 Evidence is presented here that this protease, like trypsin (3), causes alterations in the chromatographic behavior of hexokinase without causing loss of enzymatic activity. A procedure is described which removes the protease and produces stable preparations of hexokinase containing two molecular forms, both distinct from those previously obtained (2, 3). Evidence is also presented that neither of these forms arises from protease activity during purification. Since these two native forms of hexokinase differed from the enzyme which had been previously used to study the subunit structure of hexokinase (4), the effect of various agents on the molecular weight of the enzyme was reinvestigated and is reported here. The effect of the protease-induced modifications on the interactions of the subunits is also described. In addition, the relationship of the two native forms (termed P-I and P-II)
and of protease-modified enzyme (termed S form) to the forms previously described (2, 3) is discussed. Preliminary reports (7, 8) of this work and a method (9) based on this study for preparation of Forms P-I and P-II have been published. The existence of two forms of hexokinase with properties corresponding to those of P-I and P-II have been reported recently from Bar-

MATERIALS AND METHODS

Enzyme Preparations and Assays—The hexokinase used in these studies was purified from baker’s yeast by the method of Darrow and Colowick (1) or by a modification of that method with DEAE-cellulose chromatography (9). Enzyme was used at various levels of purity as indicated under “Results.” Enzyme activity was determined as described by Darrow and Colowick (1).

The protein content of crude extracts was determined by the method of Lowry et al. (12) with bovine serum albumin as standard. All other protein determinations were based on absorption at 260 and 280 nm according to the procedure of Warburg and Christian (13) as described by Layne (14).

Twice crystallized trypsin (Worthington) was used as described under “Results.”

The yeast protease activity of hexokinase preparations was assayed according to the method of Kunitz (15) and that of Schwert and Takenaka (16) with benzoyl-L-arginine ethyl ester as substrate. When the latter procedure was used, the reaction was followed for from 10 to 40 min after the addition of substrate. The amount of protein used was 0.3 to 1.0 mg per assay.

PMSF was obtained from Cyclo Chemicals. A 0.04 M solution of PMSF was made in 95% ethanol immediately before use and was added to preparations of enzyme so that the final concentration of PMSF was 0.002 M in 4.8% ethanol. No loss of hexokinase activity occurred during the addition of PMSF.

Chromatographic and Electrophoretic Analysis—DEAE-cellulose (Brown Company, Berlin, New Hampshire, or Carl Schleicher and Schuell Company, Keene, New Hampshire) was washed with acid and alkali as described by Peterson and Sober (17), equilibrated with pH 6.0 succinate buffer (0.005 M) containing 10⁻⁴ M EDTA, and stored at 5°C as a wet pack.

All DEAE-cellulose columns used for analytical purposes were the same size (10 × 0.9 cm). The equivalent of 1 g, dry weight, of DEAE-cellulose was suspended in sufficient pH 6.0 succinate buffer to make a dilute slurry. Columns were packed at room temperature and were washed with sufficient buffer to ensure stable column size.

Chromatography was carried out in a room kept at approximately 5°C, with a modification of the chromatographic procedure of Trayser and Colowick (2). Sucinate buffers (pH 6.0 to 4.5) at 0.005 M containing 10⁻⁴ M EDTA were used throughout. Enzyme was applied to columns after dialysis against succinate buffer at pH 6.0. Columns were rinsed with sufficient pH 6.0 buffer to remove all unadsorbed proteins. The enzyme was eluted with a pH gradient from 5.4 to 4.5 followed by a NaCl gradient up to 0.1 M in pH 4.5 buffer. The volume of the gradient mixing vessel was 500 ml. A flow rate of approximately 5 ml/10 min was maintained by a using peristaltic pump. After 190 5 ml fractions had been collected, the NaCl gradient was started and approximately 70 additional fractions were collected.

Fractions eluted by the pH gradient are designated as P forms, and those eluted by the sodium chloride gradient as S forms.

Fractions containing enzyme were located by a qualitative colorimetric test (9); all active fractions were then assayed with the method of Darrow and Colowick (1). Enzyme recovery, based on a summation of the activity present in all assayed fractions, was 80 to 105%.

Polyacrylamide gel electrophoresis was carried out by the method of Raymond and Wang (18) in 0.01 M potassium phosphate buffer, pH 7.0. Horizontal gel slabs (6.5 × 30 cm), containing 5% polyacrylamide, were used. Samples were run for approximately 3.5 hours at 750 volts. Protein bands were stained with Amido black blue.

Molecular Weight Measurements—Sephadex G-100 columns (8.5 × 1.8 cm) were prepared for use in molecular weight measurements as described by Andrews (19) with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl. For some experiments the columns were equilibrated before use with buffers containing 0.1 M glucose or 0.2 M Na₂SO₄. Flow rates of approximately 25 ml per hour were used and 2 ml fractions were collected. Between periods of use, the columns were washed continuously with buffer.

Light-scattering measurements were made at room temperature with a Brice-Phoenix Universal light-scattering photometer, series 2000, equipped with a Phoenix ratio recorder. The wave length of the incident light was 496 mg. The ratio of scattered to incident light was measured at 90° only, with approximately 3.0 ml of solution in a 1-cm² cell. Values were corrected for solvent and cell scatter. All water and solutions used were filtered through fine fritted glass filters or through Millipore filters. Bovine serum albumin (Armour) to be used as the standard was filtered as a concentrated solution and was then diluted in 0.1 M NaCl. Solutions of hexokinase, adjusted to approximately 1.0 mg per ml, were filtered directly into the Brice-Phoenix cuvette through a Millipore filter attached to a 5-ml syringe. Protein concentrations were determined after light scatter measurements had been made. Approximately 10% of the protein was removed by the initial filtration of a dilute solution of enzyme through a Millipore filter disc. When repeated filtration was required, stepwise reduction in the protein concentration was avoided by using a single Millipore filter disc throughout a series of measurements. A value of 70,000 was used for the molecular weight of bovine serum albumin, and the refractive index increment of hexokinase and bovine serum albumin was assumed to be the same. The protein concentration in the unknown samples was kept low in order to minimize interactions between protein molecules. Thus, the parameter Hc/T could be taken to be approximately equal to 1/molecular weight (see Kenkare and Colowick (5)).

Sedimentation experiments were carried out in a Spinco model E analytical ultracentrifuge. The schlieren optical system was used. A Gaertner microcomparator was used to analyze the photographic plates. Sedimentation coefficients measured at 5°C and room temperature were corrected to values in H₂O at 20°C.

RESULTS

Effects of Proteases on Yeast Hexokinases

Changes in Chromatographic Behavior of Yeast Hexokinase during Purification and Storage—When yeast hexokinase is purified to constant specific activity by the procedure of Darrow and Colowick (1), the resulting crystalline preparations contain a
mixture of forms which can be separated by chromatography on DEAE-cellulose (2). When the same chromatographic procedure is applied to crude autolysates of dried yeast cells, all of the hexokinase is eluted in one fraction. This is shown in Fig. 1 which presents chromatographic patterns of hexokinase obtained at three stages in the purification procedure. The enzyme present in the crude extract (see Step 1 (1)) appears to consist of a single chromatographic form (P-I) which is readily eluted by lowering the pH. Ammonium sulfate-precipitated enzyme (after Step 2 in the purification procedure (1)) contains two poorly separated forms (P-I and P-II). In preparations brought to a constant specific activity by recrystallization, these two forms are replaced by more acidic forms (P-III and S). Only the S forms require increased salt for elution.

The failure to detect Form P-II in crude extracts proved to be an artifact of chromatography. When a mixture of Form P-II and crude extract was chromatographed, Form P-II was eluted with the hexokinase in the crude extract, i.e. principally in the position of Form P-I. Thus, the appearance of the second form (P-II) during purification could be explained by the removal of contaminating proteins so that resolution of the two forms could be achieved, rather than by proteolytic modification of the hexokinase. Additional evidence is presented below that, while Forms P-III and S were formed during purification, Forms P-I and P-II were both present in crude extracts.

An examination of preparations of enzyme at various stages of purity showed that Forms P-III and S were rarely found in preparations until after the enzyme had been adsorbed to and eluted from bentonite and crystallized. This suggested that conversion to Forms P-III and S was occurring during storage of the first crystalline suspensions or during recrystallization. A suspension of first crystals was therefore stored at 5°C and at intervals crystals were sedimented, redissolved and dialyzed against appropriate buffers, and chromatographed on DEAE-cellulose. During storage, Form P-I decreased from 47 to 3%, while Forms P-III and S increased from 29 and 24% to 67 and 31% (Fig. 2, A, B, and C). Form P-II was not detected in this experiment. Fig. 2D shows the effect of incubating the sedi-

![Fig. 1. DEAE-cellulose chromatography of yeast hexokinase at three stages of purity. Chromatography was carried out as described under "Materials and Methods." The type and amount of enzyme used were as follows: supernatant fraction from autolysate of dried yeast, 620 units of hexokinase (5 units per mg); ammonium sulfate-precipitated hexokinase, 3150 units (20 units per mg); repeatedly recrystallized hexokinase, 7570 units (495 units per mg). The values on the ordinate are per cent of the total enzyme applied to each column. The fraction volume was 50 ml.](http://www.jbc.org/)

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Protease Modifications of Yeast Hexokinases

2 In previous experiments undertaken to determine which chromatographic forms of hexokinase are present in crude extract (3), the succinate buffer concentration was one-fifth of that used here and no EDTA was added. When this lower buffer concent-

3 These two forms (P-III and S) were previously called Fraction I and Fraction II (3, 20).
7.5 to 8.0. At pH 6.0 the activity was approximately 10% of that at pH 7.5. No carboxypeptidase B activity or aminopeptidase activity was detected in the preparations when hippuryl-L-arginine and leucinamide were used as substrates. High concentrations of PMSF (0.002 \( M \)) were necessary for inhibition of the protease. In this respect, as in its ability to cleave an arginine ester, this yeast protease resembles trypsin, rather than chymotrypsin (21).

The protease is adsorbed to DEAE-cellulose under the conditions described above for the adsorption of hexokinase. After the hexokinase is eluted, a protease which cleaves benzoyl-L-arginine ethyl ester can be recovered from DEAE-cellulose columns with 0.2 \( M \) NaCl in succinate buffer at pH 4.5. Further studies are required to determine whether this material is responsible for the changes in the hexokinase molecule which are described here.

**Fig. 2.** Chromatography of crystalline hexokinase on DEAE-cellulose after various periods of storage at 5°. The same suspension of first crystals was used in all experiments. The crystals were stored at 5° in the solution from which they had formed (approximately 50% saturated ammonium sulfate). At the times indicated in A through C, sedimenter crystals were dissolved in and dialyzed against pH 6.0 succinate buffer. In D, sedimenter crystals were redissolved in 10 ml of 0.02 \( M \) Tris-HCl buffer, pH 8.0, containing 0.1 \( M \) glucose and 10^{-4} \( M \) EDTA. After 150 min at 30°, the enzyme was dialyzed against succinate buffer, pH 6.0. Approximately 5000 units (22 mg) of hexokinase were applied to each column. The values on the ordinate are percentages of total enzyme applied to each column. The fraction volume was 5.0 ml.

**Fig. 3.** Chromatography of hexokinase on DEAE-cellulose at two stages of purity following treatment of crude extracts of yeast with PMSF. PMSF (final concentration, 0.002 \( M \) in 4.8% ethanol) was added to one-half of a newly made preparation of crude extract. The inhibitor-treated and the untreated preparations were then purified and crystallized. A, chromatographic behavior of the two preparations after precipitation of the enzyme by ammonium sulfate (Step 2 (I)); 3200 units (160 mg) were applied to each column. B, chromatographic behavior of crystalline hexokinase; 2000 units were applied to each column. The specific activity of the PMSF-treated preparation was 112 units per mg and that of the untreated preparation was 182 units per mg. Inset, protease activity assayed by cleavage of benzoyl-L-arginine ethyl ester. The protein concentration was 0.9 mg per ml. The values on the ordinate are the change in absorbance times 1000. The fraction volume was 5.0 ml.

**Effect of Inhibitor of Proteases on Chromatographic Behavior of Hexokinase**—Addition of PMSF to crude extract obtained by autolysis inhibited all but a trace of the yeast protease and prevented conversion to S forms from occurring during crystallization and storage. However, the trace amounts of protease which remained after PMSF treatment were sufficient to convert the enzyme to Form P-III after protecting proteins had been removed by the bentonite step. This was clearly shown with a preparation of hexokinase which was high in protease activity. As shown in Fig. 3A, partially purified enzyme obtained from PMSF-treated and from untreated crude extracts consisted only of Forms P I and P II. Moreover, the relative amounts of the two forms were the same in the two preparations. However,
crystalline enzyme (Fig. 3E) prepared from the inhibitor-treated crude extract consisted of Form P-III while that from the untreated preparation contained mostly Form S. Although the protease activity in the PMSF-treated sample was extremely low as compared with that of the untreated material (see inset), activity could be detected by casein hydrolysis (15). This activity could be eliminated by a second treatment with PMSF after the bentonite step. It was concluded from these experiments that Form P-III, as well as Form S, but not Forms P-I and P-II, was produced by protease activity during crystallization and storage.

Chromatographic Stability and Properties of Crystalline Hexokinase Purified by DEAE-cellulose Chromatography—Preparations of crystalline enzyme, purified with DEAE-cellulose chromatography instead of bentonite adsorption, consisted largely of Forms P-I and P-II (only 5% of the total enzyme was Form S) and did not undergo changes in chromatographic behavior when stored for many months as a crystalline suspension in ammonium sulfate. The enzyme was also found to be unaltered after 90 min at 30°C in Tris-HCl buffer at pH 8.0, in the presence and in the absence of glucose.

When chromatography was carried out immediately after the bentonite step, the resulting preparations were free of detectable protease* and contained only Forms P-I and P-II. The bentonite step could, therefore, be retained to decrease the protein load put onto the DEAE-cellulose columns for the preparation of large amounts of hexokinase. A procedure for purifying hexokinase, with either bentonite or a second acid treatment prior to DEAE-cellulose chromatography, has been published (9). Enzyme purified by the new procedure always contained both Forms P-I and P-II, but the relative amounts of the two forms varied from preparation to preparation. The cause of this variation is at present unknown.

Preparations containing both Forms P-I and P-II require approximately 1 week for crystal formation in the absence of seed crystals. They may contain trace amounts (up to 5% of the total enzyme units) of Form S. The maximum specific activity of such preparations, usually attained after one recrystallization, is 450 to 600 units per mg. When the two forms are separated by chromatography and purified individually, the maximum specific activity of Form P-II is approximately 700 units per mg. Form P-I rechromatographed only one time may contain small amounts of Form P-II, detectable by polyacrylamide gel electrophoresis (see below). Preparations of Form P-I which are free of Form P-II have a specific activity of 100 to 150 units per mg (8). Despite their low specific activity, such preparations of Form P-I show a single, distinct band in polyacrylamide gel electrophoresis, as do preparations of P-II. Forms P-I and P-II give symmetrical peaks in the ultracentrifuge, except under conditions of pH and salt in which part of the enzyme is dissociated into subunits (see below).

Comparison of Chromatographic and Electrophoretic Behavior of Hexokinases—As shown in Fig. 4, preparations containing Forms P-I and P-II gave two distinct bands of protein, both of which migrated toward the cathode. Form P-I showed greater mobility than Form P-II, despite the fact that it was more readily eluted from DEAE-cellulose than was Form P-II (see also Gazith et al. (8)). Incubating the enzyme with trypsin brought about quantitative conversion of chromatographic Forms P-I and P-II to S forms; concomitant with that conversion, protein bands P-I and P-II were replaced by two S bands. Gazith (8, 22) has shown that the two S forms (termed S-I and S-II) can be obtained by trypsin modification of the respective P forms, with retention of the characteristic differences between Forms P-I and P-II in terms of electrophoretic behavior and chemical composition. Trypsin treatment has not led to formation of appreciable quantities of Form P-III, which is frequently observed as a product of yeast protease action.

The electrophoresis of yeast protease-modified enzyme in polyacrylamide gel showed S forms like those of the trypsin-modified enzyme, and, in some cases, slower moving bands, presumably corresponding to Form P-III.

Although no protease is detectable by the usual techniques, Gazith has noted that very slow modification can be observed when solutions made from crystalline enzyme are stored at 8°C without removal of ammonium sulfate.

Bentonite adsorption could, in fact, be used to detect defective batches of dried yeast. It was found that the hexokinase obtained from some batches of dried yeast failed to be adsorbed by bentonite. Such preparations usually consisted largely of S forms. By treating hexokinase with trypsin under conditions which lead to the formation of S forms, it was possible to show that proteases can completely destroy the ability of hexokinase to be adsorbed by bentonite.

J. Gazith, manuscript in preparation (see also Reference 22).
Effect of Glucose and Ionic Strength on Protease-induced Conversion to Form S—Either glucose or a high salt concentration was required in order for trypsin to convert forms P-I and P-II to S forms. In the case of the experiment presented in Fig. 4 both agents were present, since the ammonium sulfate which remained with the sedimented crystals was left in the solution, and glucose was added. When dialysed enzyme was used in the same type of experiment (Fig. 5A), incubation with trypsin for 1 hour at low ionic strength (0.01) failed to convert Forms P-I and P-II to S forms. Upon the addition of sodium sulfate to an ionic strength of 0.6 (Fig. 5B) rapid conversion to S forms occurred so that Forms P-I and P-II were no longer present after 30 min with trypsin. Glucose (0.1 mM) had the same effect (Fig. 5C), except that conversion proceeded more slowly, leaving some unmodified enzyme at the end of the incubation period.

A requirement for either high ionic strength or glucose applied as well to conversion by yeast protease. This is shown in Table I. Conversion to S forms occurred only in the presence of glucose or sodium sulfate and was prevented by preliminary treatment with PMSF. In the absence of glucose or sodium sulfate, a loss of activity was observed when yeast protease was present (Sample I). This was accompanied by partial disappearance of protein bands P-I and P-II without concomitant appearance of new bands. Glucose (Sample 3) and sodium sulfate (Sample 5), when added to enzyme which contained yeast protease, partially protected the hexokinase from inactivation. This effect was more pronounced after 48 hours at 5°C (not shown in Table I) when 72% of the original activity was present in tube 3, 48% in tube 5, and only 9% in tube 1. The yeast protease activity of those samples was equivalent to an incubation mixture containing approximately 0.03% trypsin (by weight of hexokinase).

Subunit Interactions of Unmodified and Protease-modified Hexokinases

The experiments presented thus far suggested that Forms P-I and P-II were resistant to modification in media of low ionic strength because certain sites were unavailable to trypsin and to yeast protease. They also suggested that the addition of salt or glucose promoted conversion to S forms by inducing conformational changes which made these sites available. In view of the work of Schachman (4) and Kenkare and Colowick (5), the changes induced by glucose or salt were presumed to involve a dissociation of hexokinase from the tetramer to the dimer state. The remainder of this report deals with the effect of glucose, salt, pH, and temperature on the dissociation of unmodified and protease-modified hexokinases.

Effect of Ionic Strength on the Molecular Weight of Forms P-I and P-II—Table II shows the effect of ionic strength on the molecular weight of unmodified hexokinase (Form P-II). Measurements were made in 0.05 M phosphate buffer at pH 7.0 by light scattering. Addition of sodium sulfate in small increments resulted in a progressive decrease in molecular weight from 82,000 at an ionic strength of 0.1 to 68,000 at an ionic strength of 0.37. Similar results were obtained when potassium chloride was used in place of sodium sulfate. Addition of sodium sulfate to hexokinase in Tris buffer at pH 8.0 brought about a reduction in molecular weight similar to that observed in phosphate buffer at pH 7.0.

An effect of ionic strength on molecular weight was also observed in ultracentrifugation studies and by Sephadex gel filtration carried out according to the procedure of Andrews (19). In 0.02 M Tris-HCl buffer containing 10^{-4} M EDTA at pH 8.0, a mixture of Forms P-I and P-II containing 5% S forms showed a major component with an s_{20,w} of 5.5 and a small amount of a

![Fig. 5. Polycrylamide gel electrophoresis showing the effect of salt and glucose on the conversion of hexokinase to S forms by trypsin. Crystalline hexokinase containing Forms P-I and P-II was dissolved in and dialyzed against 0.02 M Tris-HCl buffer, pH 8.0, with 10^{-4} M EDTA. Aliquots containing 7.0 mg of protein per ml were incubated at 30°C with 0.01% trypsin (by weight of hexokinase) in (A) Tris buffer alone, (B) Tris buffer containing 0.2 M sodium sulfate, and (C) Tris buffer containing 0.1 M glucose. At 1, 15, 30, and 60 min, aliquots of each sample were chilled in ice, the pH was adjusted to approximately 5.0 with HCl, and PMSF was added to 0.002 M. Samples were frozen at -50°C until they were subjected to electrophoresis as described under "Materials and Methods."](http://www.jbc.org/)

<table>
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<tr>
<th>Sample</th>
<th>Previously treated with PMSF</th>
<th>Addition during dialysis and storage</th>
<th>Protease activity</th>
<th>Per cent of original hexokinase activity</th>
<th>Conversion to S forms</th>
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second, slower moving component. In Tris buffer containing sodium sulfate (ionic strength, 0.65) a single component with an $s_{20,w}$ of 3.9 was observed. These sedimentation coefficients correspond to molecular weights of 99,000 and 39,000 for hexokinase.* A molecular weight of 89,000 was also obtained by Sephadex gel filtration in Tris buffer containing sodium sulfate (ionic strength, 0.65). These results support the idea that an equilibrium exists between tetramers and dimers, and that dissociation is fostered by the addition of salt.

**Effect of Phosphate on Dissociation of Hexokinase by Glucose**—Although glucose clearly promoted the modification of hexokinase by proteases, attempts to show a corresponding shift toward dimers upon the addition of glucose to hexokinase in Tris buffer failed consistently. This is shown in Table III which presents data from sedimentation and light-scattering experiments in which glucose was added to hexokinase in Tris buffer and in phosphate buffer. A reduction in the molecular weight upon addition of glucose to hexokinase could be detected only when phosphate ions were present. However, a slight effect of glucose alone on the dissociation equilibrium, too small to be detected here, could account for the observed promotion of protease-induced modification (see “Discussion”).

The dissociating effect of glucose in phosphate buffer was also observed in Sephadex gel filtration studies carried out at room temperature. The molecular weight of hexokinase in 0.05 M phosphate buffer containing 0.1 M NaCl was approximately 76,000; in the same medium containing 0.1 M glucose, the molecular weight was 55,000.

These experiments indicate a phosphate ion effect on the glucose-promoted dissociation of hexokinase. That phosphate ions alone promote dissociation of hexokinase into dimers was reported earlier by Schachman (4). Kenkare and Colowick (5) observed that phosphate ions were more effective than acetate ions in promoting dissociation of hexokinase into dimers at pH 5.0 and 6.1. As is shown below, we have observed similar effects of phosphate buffer as compared to Tris buffer in light-scattering experiments while studying the effect of pH on the molecular weight of Forms P-I and P-II.

**Molecular Weights of Protease-modified and Unmodified Hexokinases**—Sephadex gel filtration studies were carried out to determine whether the trypsin-induced modification altered the tetramer-dimer equilibrium. As indicated above, Forms P-I and P-II were not converted to S forms by trypsin when they were incubated for 1 hour at 30° in Tris buffer at a low ionic strength (see Fig. 5A). Gel filtration carried out at the end of the incubation indicated that the molecular weight was approximately 88,000 in 0.05 M phosphate, pH 7.0, containing 0.1 M NaCl. In the same buffer, enzyme which had been converted to S forms by incubation with trypsin in medium of high ionic strength (see Fig. 5B) had a molecular weight of 52,000. Enzyme treated with trypsin in glucose and partially converted to S forms (see Fig. 5C) showed two peaks of hexokinase activity by gel filtration, one corresponding to a molecular weight of 52,000 and the other to a molecular weight of approximately 55,000. These studies indicated that, at pH 7.0, Forms P-I and P-II were largely in the tetramer state while the S forms were dimers. They prompted a more detailed study of the effect of pH on the molecular weight of protease-modified and unmodified enzyme.

In Fig. 6, sedimentation patterns of Forms P-I, P-II, and S at 5° are compared at pH 5.5, pH 7.0, and pH 9.0. While the composition (Kaji, unpublished data) and an intrinsic viscosity value of 4.5 cc per g obtained by Kenkare and Colowick (5). Both of these values were obtained with enzyme now known to have been protease-modified. For the molecular weight calculations a value of $2.16 \times 10^4$ for the $\beta$ function of Scheraga and Mandelkern (23) was used.

### Table II

**Effect of ionic strength on molecular weight of hexokinase as determined by light scattering**

Crystalline Form P-II (specific activity, 700 units per mg) was dissolved and dialyzed in 0.05 M potassium phosphate buffer, pH 7.0. The ratio of scattered to incident light was measured at 90° before and after 30-μl additions of a filtered solution of 1.5 M NaSO₄. The protein concentration, determined after the last light-scattering measurement had been made, was 0.83 mg per ml. The initial concentration and the concentration at each ionic strength were calculated from this value.

<table>
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<th>Ionic strength</th>
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<td>0.010</td>
<td>82,000</td>
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<tr>
<td>0.015</td>
<td>77,000</td>
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</tr>
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</table>

### Table III

**Effect of phosphate on dissociation of hexokinase by glucose**

Forms P-I and P-II were used. Enzyme was dialyzed against the buffer indicated in the table. Glucose was added to the concentration indicated immediately before centrifugation or, in light-scattering experiments, after the ratio of scattered to incident light in the absence of glucose had been measured (see “Materials and Methods”). The protein concentration used for ultracentrifugation studies was 7.0 to 8.0 mg per ml; approximately 1.0 mg per ml was used in light-scattering experiments.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Glucose concentration</th>
<th>Temperature</th>
<th>$s_{20,w}$ (From light scattering)</th>
<th>Mol wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (0.02 M)-10⁻⁴ M EDTA, pH 8.0</td>
<td>0.1</td>
<td>6</td>
<td>5.4</td>
<td>96,000</td>
</tr>
<tr>
<td>Phosphate (0.05 M)-0.1 M KCl, pH 7.0</td>
<td>0.1</td>
<td>23</td>
<td>4.1</td>
<td>63,000</td>
</tr>
<tr>
<td>Phosphate (0.05 M), pH 7.0</td>
<td>0.01</td>
<td>5</td>
<td>4.5</td>
<td>73,000</td>
</tr>
</tbody>
</table>

* Based on an assumed value of $2.16 \times 10^4$ for the $\beta$ function of Scheraga and Mandelkern (23) and an intrinsic viscosity of 4.5 cc per g (5).

* Mercaptoethanol, 0.02 M, added immediately before light-scattering measurements.
FIG. 6. Sedimentation properties of unmodified and trypsin-modified hexokinase. Crystals of Forms P-I, P-II, and S were sedimented and redissolved in 0.05 M potassium phosphate at pH 7.0. After overnight dialysis against the same buffer at pH 5.5, an aliquot of the enzyme was centrifuged at a speed of 59,780 rpm. The remainder of the dialyzed enzyme was then adjusted to pH 7.0 and to pH 9.0 with KOH and was centrifuged. Pictures were taken 88 min after the rotor had attained nominal speed. The bar angle was 70°; the temperature of all runs was 5°. The protein concentration was approximately 8 mg per ml. The specific activities of the preparations were as follows: P-I, 200 units per mg; P-II, 700 units per mg; and S, 555 units per mg.

three forms of the enzyme had similar sedimentation coefficients at pH 5.5, the S forms sedimented more slowly at pH 7.0 than did Form P-I and the major component of Form P-II. Molecular weights calculated from these data are 104,000 at both pH values for Forms P-I and P-II, 101,000 for Form S at pH 5.5, and 59,000 for Form S at pH 7.0. Increasing the pH from 7.0 to 9.0 had no further effect on Form S, but did increase the amount of the slower moving component present in Form P-I and in Form P-II. The sedimentation coefficient of this second component was approximately 3.8, indicating partial dissociation into dimers at pH 9.0.

Fig. 7 shows the effect of pH and buffer composition on weight average molecular weights of the three forms of hexokinase at room temperature as measured by light scattering. Measurements were made in phosphate buffer (Fig. 7A) with and without mercaptoethanol and in Tris buffer (Fig. 7B) with mercaptoethanol. In phosphate buffer containing mercaptoethanol, Forms P-I and P-II gave identical responses to changes in pH; these results were in striking contrast to those obtained with S forms, whether produced by yeast protease or derived from P forms by trypsin treatment. Increasing the pH from 5.5 to 7.0 resulted in a reduction in molecular weight of S forms from 110,000 to 59,000, while similar treatment of Forms P-I and P-II reduced the molecular weights to approximately 85,000. If one assumes that this reduction in molecular weight reflects only a dissociation into dimers, at pH 7.0, 85% of the S forms and 30% of the P forms were dissociated. At pH 7.4, 50% of Forms P-I and P-II was dissociated.

Similar experiments with Tris buffer showed that the S forms gave a pH response identical with that observed in phosphate buffer (Fig. 7B). However, dissociation of Forms P-I and P-II in Tris buffer required that the pH be adjusted to approximately 8.8 (rather than 7.4) in order to obtain 50% dissociation.

Stepwise increases in pH above pH 10.0 in either buffer consistently resulted in large increases in light scattering by all three forms of the enzyme.
forms of hexokinase. This increase was presumed to result from nonspecific aggregation. When the pH was adjusted from pH 7.0 to approximately 11.0 in one step, molecular weights of approximately 50,000 were obtained. No indication of dissociation to monomer (mol wt 25,000) was obtained.

Effect of Temperature on Dissociation of Hexokinase—Data from light-scattering experiments with phosphatase buffer showed that Forms P-I and P-II were extensively dissociated at pH 9.0 at room temperature, while ultracentrifugation studies with the same buffer indicated that the enzyme was mainly in the tetramer state at pH 9.0 at 5°. Table III shows a similar discrepancy at pH 7.0; i.e., molecular weight values obtained from light-scattering data at room temperature are consistently lower than those calculated from sedimentation data obtained at 5°.

Since sedimentation analyses were carried out at protein concentrations around 8.0 mg per ml, whereas approximately 1.0 mg per ml was used for light-scattering measurements, the effect of protein concentration on the rate of sedimentation of P-I at pH 9.0 was measured. The $s_{20,w}$ of the major component was 0.1, and no increase in the amount of the slower moving component was observed; thus, the enzyme remained largely in the tetramer state at low protein concentrations at 5°.

This discrepancy between molecular weight values obtained from light-scattering data and from sedimentation coefficients appears to be due to the difference in the temperature at which the two measurements were made (Table IV, Part A). The $s_{20,w}$ calculated from a single experiment at pH 9.0 was 4.6 S at 23° as compared with 6.0 S at 5°. Data from gel filtration studies at pH 7.0 also indicate that dissociation of unmodified enzyme is promoted by an increase in temperature. At an ionic strength of 0.2, a mixture of Forms P-I and P-II had a molecular weight of 88,000 at 5° and 76,000 at 23°. Since values for the molecular weight of the S forms calculated from light scattering and from sedimentation data are in good agreement at pH 5.5, 7.0 and 9.0, it can be concluded that temperature, like phosphate ions, has an effect on dissociation of Forms P-I and P-II only.

Effect of Mercaptoethanol on Sedimentation Coefficient of Unmodified Hexokinase—Although mercaptoethanol had little effect on the dissociation of Form P-II as measured by light scattering (Fig. 7A), it drastically reduced the sedimentation constant of Forms P-I and P-II when added to phosphate buffer at pH 9.0 (Table IV, Part B). This effect of mercaptoethanol correlates with the recent finding of Gooding and Colowick (24) that hexokinase is rapidly inactivated by mercaptoethanol at pH 9.0. Further studies are required to determine whether these changes in sedimentation rates and in activity reflect a dissociation into monomers or result from conformational changes in the dimer. The inability to show monomer formation in the presence of mercaptoethanol by light scattering would suggest that dissociation does not occur.

DISCUSSION

The experiments presented here show conclusively that most if not all of the previously described (2, 3) isozymes of hexokinase are modified forms of the enzyme. In view of the role of proteases in the production of those isozymes, the possibility that only one of the two forms reported here is native must be considered. However, various lines of evidence indicate that neither Form P-I nor P-II is produced by proteolysis. Interconversion of Forms P-I and P-II has failed consistently. Inhibition of the yeast protease early during purification does not alter the relative amounts of the two forms. Other methods of extraction, including disrupting cells by freezing with liquid nitrogen and by stirring with glass beads, with and without an inhibitor of protease, gave rise to extracts which showed more heterogeneity than those obtained by autolysis of dried yeast. In addition, Gazith has observed that the amino acid composition of Form P-I is different from that of Form P-II and that trypsin treatment of each P form produces a specific S form which is similar in amino acid composition to that of the "parent" molecule (8, 22). Even more convincing evidence that one P form does not arise from the other comes from recent studies in this laboratory which show that both forms are always present in crude extract. (a) Following polyaacrylamide gel electrophoreses of crude extracts, bands corresponding to Forms P-I and P-II have been identified by hexokinase activity stains (experiments by Dr. M. Goil). (b) Dr. Frances Womack has shown that Forms P-I and P-II are antigenically distinct; by using specific antisera she has been able to detect both Forms P-I and P-II in crude extracts by complement fixation. (c) Since the two forms of hexokinase differ in their relative ability to phosphorylate fructose and glucose (10), the presence of both forms in crude extract can be shown by measuring the reaction with both sugars (experiments by Mr. J. Schmidt). Form P-I phosphorylates fructose 2.6 times the rate at which glucose is phosphorylated, while, with Form P-II, this ratio is only 1.3. The fructose to glucose ratios obtained with crude extracts are intermediate between these two values. The presence of both isozymes in crude extract eliminates the possibility that one is produced from the other by proteolysis during purification. These observations, along with the fact that two isozymes of yeast hexokinase which appear to be identical with Forms P-I and P-II have been purified by another method (10), support the conclusion that both Forms P-I and P-II are native isozymes.

The resistance of Forms P-I and P-II in solutions of low ionic strength to conversion by proteases makes it appear as though the sites to be modified by these enzymes are not available under such conditions. The role of glucose and salt in potentiating trypsin conversion suggests that agents which foster dissociations make the necessary sites available to trypsin. Thus, agents such as sodium sulfate, which bring about virtually complete dissociation to dimers, permit rapid conversion to occur in the presence of trace amounts of trypsin. On the other hand, glu-
case, an agent with a much smaller effect on molecular weight, causes slow conversion to S forms. Glucose, when added to sodium sulfate, does not hinder conversion, indicating that the slow conversion observed with glucose is not due to a slower attack of trypsin on the glucose-dimer complex. One is, therefore, tempted to conclude that only the dimer form of hexokinase is susceptible to this effect of trypsin and that the rate of conversion is dependent on the concentration of dimers. Since the rate of conversion with glucose is only 1 to 2% of that observed by trypsin would probably occur. Since trypsin can pull the sodium sulfate, does not hinder conversion, indicating that the rate of conversion to S forms. Glucose, when added to phosphate buffer, this difference was reduced by 1.5 pH units, since phosphate ions reduce the pK of dissociation of the forms without affecting that of the S forms. These results indicate that trypsin alters the sites involved in tetramer formation, presumably by removing specific groups from the molecule.

The use of the term dimer to describe the active form of hexokinase implies that it is composed of two subunits, as was suggested earlier by the experiments of Ramel, Stellwagen, and Schachman (27), with sodium dodecyl sulfate, and by experiments in this laboratory (6) with acid and alkali. In the present work, however, no definitive evidence for dissociation to monomers (mol wt 25,000) has been obtained. Although we have observed a decrease in sedimentation coefficient at pH 9.0 in the presence of mercaptoethanol and at pH 11.0 which could be due to partial dissociation to monomers, the changes observed could well result from substantial changes in the conformation of the dimer. Our inability to show dissociation of the dimer by light scattering supports the latter interpretation. A recent report from Barnard’s laboratory (11) has indicated that the low sedimentation coefficient obtained in acid is not due to dissociation. The use of the term dimer for the active form of the enzyme has been retained on the basis of analytical data (see References 8 and 22 and Footnote 7). The number of tryptic peptides and COOH-terminal amino acids found suggest the presence of phosphate, thereby shifting the tetramer-dimer equilibrium.

Although glucose clearly promotes the modification of hexokinase by trypsin, it also clearly protects the enzyme against inactivation by excessive amounts of trypsin as well as against spontaneous inactivation (20, 26). These apparently conflicting effects of glucose can be explained if modification involves action on dimers, whereas inactivation involves action on an unfolded molecule. It is of interest, therefore, that sodium sulfate, another agent which causes dissociation of the tetramer and promotes modification, also affords some protection against inactivation by yeast protease. Thus, both of these agents appear to function in two ways; in addition to promoting dissociation of the tetramer so that proteolytic modification can occur, they protect the enzyme from inactivation by proteolysis, presumably by stabilizing the dimer in its native conformation.

The findings in this paper are compatible with those from earlier molecular weight studies carried out at pH 7.0 on highly purified enzyme prepared by the Darrow-Colowick procedure, i.e., on preparations consisting of the protease-modified Forms P-III and S. Such preparations were found to contain two sedimenting components at pH 7.0 (4–6). Ramel et al. (6) reported that the two components represented two forms of the enzyme, one of which had a molecular weight approximately twice that of the other at neutral pH. It appears that their high molecular weight form is equivalent to our protease-modified Form P-III and that their low molecular weight form corresponds to the S forms described here in that it tends to exist in the dissociated (dimer) form at neutral pH, but can reassociate to form tetramers if the pH is lowered. Thus, on the basis of earlier studies (5, 6), it appears that Form P-III, shown here to be clearly different from unmodified enzyme ( Forms P-I and P-II) in chromatographic behavior, exhibits dissociation properties similar to those of Forms P-I and P-II. The sites involved in subunit interaction were apparently unaltered, despite changes in the over-all acidity of the molecule.

The molecular alteration involved in the formation of S forms results in more drastic changes in the acidity of the molecule and in changes in its dissociation properties. Thus, S forms exhibit a lower pK for the dissociation process than do Forms P-I and P-II. When comparisons were made in Tris buffer, the pH at which 50% dissociation of S forms had occurred differed by 3 pH units from that required for 50% dissociation of the P forms. In phosphate buffer, this difference was reduced by 1.5 pH units, since phosphate ions reduce the pK of dissociation of the forms without affecting that of the S forms. These results indicate that trypsin alters the sites involved in tetramer formation, presumably by removing specific groups from the molecule.

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