Monomer–Dimer Equilibria of Yeast Hexokinase during Reacting Enzyme Sedimentation†

Jonathan P. Shill, Bruce A. Peters, and Kenneth E. Neet*

ABSTRACT: Reacting enzyme sedimentation and sedimentation velocity studies of yeast hexokinase have been undertaken in order to determine the size of the reacting form of the enzyme and to study the monomer–dimer equilibrium under conditions of assay. The utility of pH-dependent, dye-linked assays for reacting enzyme sedimentation has been demonstrated and the validity of reacting enzyme studies on systems in rapid association–dissociation has been shown. Reacting enzyme sedimentations of yeast hexokinase P_{II} utilizing pH-dependent dye-linked assays in 50% D_2O have produced the following results at pH 6.75 and 8.55. At low enzyme concentrations, it sedimented as a single boundary with an $s_{20,w}$ approaching 3.0 S at both pH values. At higher enzyme loading concentrations, near 1.0 μg/ml at pH 6.75 and 0.6 μg/ml at pH 8.55, the $s_{20,w}$ increased smoothly to 4.72 and 4.20 S, respectively. Simulation studies indicated that this increase in $s_{20,w}$ in reacting enzyme sedimentation was not an artifact of overloading with enzyme and may be interpreted as an association to dimers. The $s_{20,w}$ of the dimer at both pH values studied was about 5.6 S in the absence of substrates and 4.8 S in the presence of glucose alone. Dissociation of the dimer was prominent below 50 μg/ml in the absence of all substrates at pH 6.6. At pH 8.55, the presence of glucose plus $MgCl_2$ gave an $s_{20,w}$ of 5.7 S for the dimer. Thus, in the presence of both substrates, the interactions between subunits are strengthened to the extent that the enzyme now dimerizes at a much lower enzyme concentration. Many kinetic studies have been done where either the monomer predominates or in the region of monomer–dimer equilibrium. The sedimentation coefficients for monomers in the presence of 0.6 M NaCl at pH 8.55 were found to be 3.88 S for sedimentation velocity and 3.55 S for reacting enzyme, indicating that high salt concentrations are capable of dissociating even the reacting enzyme. Based upon the $s_{20,w}$ value of the dimer, conformational differences are suggested between reacting and nonreacting enzyme, between reacting enzyme at pH 6.75 and 8.55, between enzyme and enzyme in the presence of glucose, and between enzyme in $MgCl_2$ plus glucose and enzyme in glucose alone.

The earliest observation that substrates affect the association–dissociation equilibrium of yeast hexokinase was that either glucose or inorganic phosphate induced a decrease in the sedimentation coefficient at neutral pH (Schachman, 1960). The enzyme preparation used to observe this dissociation induced by glucose is now known to have been a proteolyzed form. Recent improvements in the purification of yeast hexokinase (Schulze and Colowick, 1969; Lazarus *et al.*, 1966; Rustum *et al.*, 1971) have yielded two hexokinase isozymes called P_I (or A) and P_{II} (or B) of relatively high specific activity and presumably with native structure because of the care taken to prevent proteolysis. P_{II}, the more active and more thoroughly studied isozyme, exists in a monomer–dimer self-association equilibrium, with a dimer molecular weight of about 100,000. Dissociation is promoted by increases in pH, ionic strength, and temperature, and by decreases in enzyme concentration (Schulze and Colowick, 1969; Derechin *et al.*, 1972).

The dissociating effect of glucose has been confirmed for hexokinase P_{II} by sedimentation velocity and light scattering studies at pH 8.0 when phosphate but not Tris was the buffer (Schulze and Colowick, 1969). Sedimentation equilibrium ultracentrifugation at high enzyme concentration demonstrated that the dissociating effect of glucose was markedly increased in the presence of MgADP at pH 8.0 (Derechin *et al.*, 1972). The authors suggested that dissociation of the enzyme occurred during catalysis. The need to know the aggregation state of the active form of yeast hexokinase under catalytic conditions was made apparent from the discovery of regulatory properties of cooperativity (Kosow and Rose, 1971) and of a slow transient in the reaction progress curve (Shill and Neet, 1971). Information of the size of the reacting form is particularly important because of the suggestion (Shill and Neet, 1974) that hexokinase may follow the slow transition model for cooperativity (Ainslie *et al.*, 1972).

In the present studies, pH-dependent dye-linked assays have been used to observe the sedimentation of the reacting enzyme at pH 6.75 and 8.55 by modifications of the reacting enzyme sedimentation method of Cohen *et al.* (1967) using 50% D_2O to stabilize the boundary (Taylor *et al.*, 1972).

Methods

Enzyme Purification and Materials. Yeast hexokinases $P_I(A)$, $P_{II}(B)$, and $P_{II}(C)$ were purified by a slight modification of the procedure of Lazarus *et al.* (1966) and Rustum *et al.* (1971). $P_I(A)$, $P_{II}(B)$, and $P_{II}(C)$ are three forms of yeast hexokinase which elute from a DE-52[1] column at different pH values (Rustum *et al.*, 1971). The enzymes were homogeneous on sedimentation velocity centrifugation and disc gel electrophoresis. These studies were performed on preparations of $P_{II}(B)$ and $P_{II}(C)$ with specific activities at pH 8.0 ranging from 500 to 800 units per milligram. One unit of activity is defined as 1 μmol of product formed per minute as assayed spec-

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received January 31, 1974.* Supported by a grant from the National Institutes of Health, U. S. Public Health Service (AM-12881). Portions of this work were taken from the Ph.D. dissertation of J. P. Shill, Case Western Reserve University, 1973. J. P. S. was supported by a Predoctoral Research Scholarship grant (PRE-24) from the American Cancer Society. B. A. P. was a predoctoral trainee of the U. S. Public Health Service. K. E. N. was supported by a Faculty Research Award (PRA-55) from the American Cancer Society.

[1] Abbreviations used are: $(EtOH)_3NHCl$, tri(hydroxyethyl)ammonium chloride; G-6-P, glucose 6-phosphate; DE-52, microgranular diethylaminoethylcellulose.

trophotometrically at 25° by coupling the reaction with glucose-6-phosphate dehydrogenase (Rose and Warms, 1967) and observing the increase in absorbance at 340 nm with the recorder set at 0.1 optical density (OD) unit full scale. ATP was from Sigma, Cresol Red from Fisher Scientific, and *p*-nitrophenol from Eastman. All supplies were of highest purity commercially available.

Enzyme Assays. Reacting yeast hexokinase was assayed in the ultracentrifuge at 20° by observing the spectrophotometric change in an acid–base indicator as it was titrated by H^+ produced during the glucose:ATP transphosphorylation reaction.

$$\text{glucose} + \text{ATP} \xrightarrow{Mg^{2+}} \text{glucose 6-phosphate (G-6-P}^1\text{)} + \text{ADP} + H^+$$

The pH 6.75 assay mixture, observed at 400 nm, contained in 1 ml: 2 μmol of ATP, 10 μmol of $MgCl_2$, 20 μmol of D-glucose, 0.01 μmol of *p*-nitrophenol, and 0.1 μmol of tri(hydroxyethyl)ammonium chloride ($(EtOH)_3NHCl^1$). The pH 8.55 assay mixture, observed at 560 nm, contained in 1 ml: 2 μmol of ATP, 10 μmol of $MgCl_2$, 20 μmol of D-glucose, 33 μg of Cresol Red, and 0.1, 1.67, or 3.3 μmol of glycylglycine. The reaction mixture was adjusted to the stated pH with NaOH. pH was read on a Radiometer pH meter (type PHM 26) at room temperature and corrected for 50% D_2O (Glasoe and Long, 1960).

Ultracentrifuge Techniques. The Beckman-Spinco Model E analytical centrifuge with an RTIC temperature control unit was used for all centrifugation studies.

Reacting Enzyme Sedimentation. The method of sedimentation of reacting enzyme (Cohen *et al.*, 1967), with D_2O added to stabilize the enzyme boundary (Taylor *et al.*, 1972), has been extended for use with a pH-dependent dye-linked assay of yeast hexokinase. The ultraviolet (uv) scanner of the ultracentrifuge was used to measure the reaction products formed during centrifugation as the band of enzyme, initially layered on top, passed through the assay mix. All reacting enzyme sedimentation studies were run near 20° and monitored at 400 or 560 nm of monochromatic light, with the Beckman photoelectric scanner. A charcoal-filled epon, 12-mm path length, type I double-sector band-forming centerpiece with quartz windows was run in an An-D rotor at 60,000 rpm. The use of the double sector cell is advantageous in blanking out the high background absorbance of the dye. Both sectors were filled with 0.29 ml of 50% D_2O assay mix and since both assays produce a decrease in OD, the capillary chamber of the blank sector was filled with 10 μl of an appropriate concentration of enzyme diluted in assay mix from which D_2O and ATP were omitted. The capillary chamber of the sample sector was filled with 10 μl of assay mix from which D_2O and ATP were omitted. Alternatively, the sample sector was filled with 0.30 ml of 50% D_2O assay mix and its capillary chamber was left empty. The boundary of early time tracings was stabilized when the pH of the capillary chamber solution was 0.4 pH unit below the assay mix. Volumes were delivered from Hamilton syringes. Sedimentation velocities were routinely determined from the midpoints of the scanner-traced boundaries. These were obtained either at the fastest scan speed and 25 mm/sec chart speed at 2-min intervals, or medium scan speed and 5 mm/sec chart speed at 4-min intervals. These were done with the minimum noise suppression possible for each run. These calculations were occasionally checked against sedimentation velocities calculated from the line of symmetry of difference curves (Cohen *et al.*, 1967). Observed sedimentation coefficients were obtained with a least-squares analysis of the linear log R^2 *vs.* time plots. The least-squares correlation coefficients were usually 0.98–0.99. The pH 8.55 system yielded more consistent results with less noise than the pH 6.75 system.

Correction of Observed Sedimentation Coefficients. The observed sedimentation coefficients in solvents containing D_2O were corrected for deuteration of the protein and solvent density and viscosity according to the equation of Taylor *et al.* (1972) as derived from equations of Martin *et al.* (1956) and Schachman (1959). A partial specific volume, $\bar{v}$, of 0.74 was used as calculated from amino acid composition data (Schulze and Colowick, 1969). Densities were determined at 20.0 ± 0.02° in a 2-ml Lipkin pycnometer calibrated with twice distilled water. The procedure of Daniels *et al.* (1962) was followed. The viscosities of solutions relative to water were obtained at 20.00 ± 0.02° in a 5-ml Ostwald viscometer with a flow time of 78.3 sec for twice distilled water. A correction factor for s_{obsd} of 1.31 was obtained for all dilute buffers in 50% D_2O and 1.48 for 0.6 M NaCl solutions.

Simulation Studies. All simulation studies were performed on a Hewlett-Packard Calculator Model 9100A equipped with the 9101A extended memory and the 9125A plotter.

Results

Kinetic Properties of Yeast Hexokinase. A study of enzyme activity as a function of ATP concentration confirmed that the properties of the transient and negative cooperativity (Shill and Neet, 1971, 1974) were observed under conditions identical with those to be employed in the reacting enzyme sedimentation studies in the ultracentrifuge. Yeast hexokinase $P_{II}(B)$ was assayed at pH 6.5 in a solution of 50% D_2O containing in μmol/ml: 0.01 of *p*-nitrophenol, 8 of $MgCl_2$, 20 of D-glucose, and 0.1 of $(EtOH)_3NHCl$. An equimolar mixture of $MgCl_2$ and ATP was varied between 0.4 and 20 mM. The rate of reaction was followed by the volume of 1 mM NaOH (CO_2 free), recorded automatically on a pH-Stat apparatus (Radiometer TT11-SRB2-ABUI), required to maintain constant pH. The pH-Stat was set at pH 6.5. Under these conditions with 0.2 μg/ml of yeast hexokinase $P_{II}(B)$, the progress curves exhibited a burst type of slow transient in which the rate was initially rapid and slowed down within about 1 min (data not shown, but similar to Shill and Neet, 1974). Thus, during measurements made in the ultracentrifuge, from approximately 10 to 40 min, the enzyme was in the posttransition steady state. A double reciprocal plot with variable MgATP showed negative cooperativity (data not shown, but similar to Shill and Neet, 1974). The enzyme was inhibited 20% in this assay, but the regulatory properties of the enzyme were retained. Since *p*-nitrophenol did not inhibit yeast hexokinase, the inhibition was attributed to D_2O. At pH 8.55 neither the burst type of slow transient nor the negative cooperativity of MgATP is observed.

Sedimentation of Reacting Yeast Hexokinase. The method of Darrow and Colowick (1962) for the spectrophotometric assay with Cresol Red dye was modified for use in sedimentation studies of the reacting enzyme. Other assays have been linked to pH-dependent dye indicators (Lowry *et al.*, 1954). When the dye indicator and any buffers present have the same pK_a, then the change in OD is proportional to the amount of acid or base formed. In principle, the sensitivity of the assay depends on the ratio of the buffering capacity of the dye to the other buffers present, and the total pH change during the assay depends on the total amount of buffers present. The pH 8.55 assay mix contained Cresol Red and the buffer glycylglycine which have the same pK_a of 8.35 at 25° in H_2O. The *p*-nitrophenol assay mix contained buffers of different pK_a values: *p*-nitrophenol, 7.15; $(EtOH)_3NHCl$, 7.76; H_2CO_3, 6.37; and MgATP, 4.55. Titration of the pH 8.55 assay mix from pH

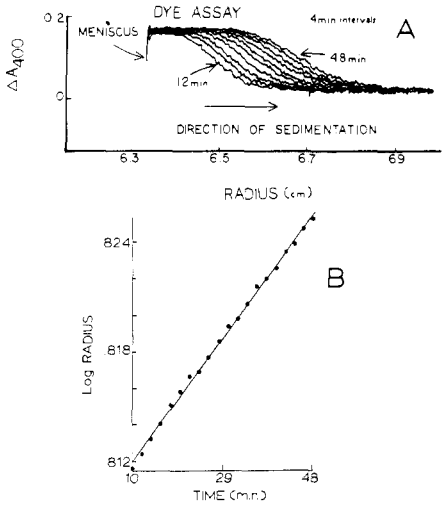

FIGURE 1: Scanner tracings and log radius *vs.* time plot of reacting enzyme. (A) Scanner tracings (change in absorbance at 400 nm *vs.* the radius (centimeters) from the center of rotation) at 4-min intervals (alternate tracings omitted for clarity) are superimposed upon a common point of reference (the outer reference hole). The enzyme loading concentration was 1 μg/ml. (B) The logarithm of the radius (centimeters) of the boundary midpoint is plotted against time (minutes). The data for this plot were taken from the sedimentation data shown in A.

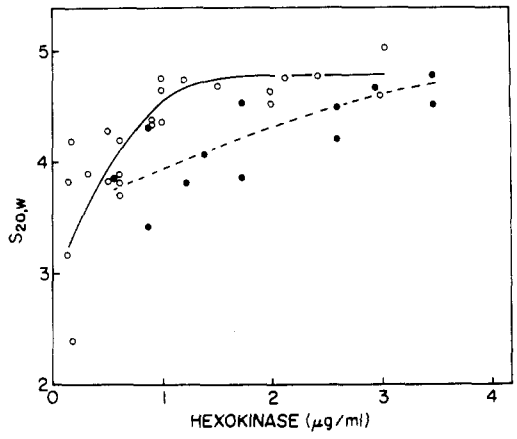

FIGURE 2: Reacting enzyme centrifugation of yeast hexokinase $P_{II}(B)$ (○) and $P_{II}(C)$ (●). The enzyme was layered on the assay mixture containing *p*-nitrophenol at pH 6.75 in 50% D_2O. The $s_{20,w}$ was determined experimentally by midpoint calculations and plotted as a function of the enzyme loading concentration (μg/ml).

8.55 and the pH 6.75 assay mix from pH 6.8 by the addition of hydrochloric acid in both cases showed a nonlinear OD response. The pH 8.55 assay mix had a ΔA_{560} of 0.0061 per nmol of HCl added per ml up to 20 nmol, while from 40 to 180 nmol, the curve was essentially linear with a ΔA_{560} of 0.0032. This deviation from linearity may be caused by the effect of 50% D_2O on the pK_a of each buffer. The pH 6.75 assay mix had a ΔA_{400} of 0.0059 per nmol of HCl added per ml for the first 20 nmol. The ΔA_{400} gradually decreased to 0.0025 between 90 and 110 nmol. The pH 6.75 assay mix was not expected to be linear because: (a) it contained buffers of different pK_a values; (b) the CO_2 concentration in water, 33 mM at 25° (1 atm), was not kept constant from run to run, and the effect of ultracentrifugation pressure on the CO_2 concentration was not determined; (c) the buffer system was changing with time during the reaction since MgATP disappears with the formation of MgADP and G-6-P; and (d) both the acidic and basic forms of *p*-nitrophenol absorb at 400 nm. The effect of these nonlinear assays on the observed $s_{20,w}$ will be discussed later.

Reacting enzyme centrifugation of yeast hexokinase has been studied over a wide range of initial loading concentrations from 0.05 to 3.5 μg/ml. The actual enzyme concentration in the sedimenting band is expected to have an average protein concentration approximately three- to fivefold lower than the loading concentration (Wampler, 1972; Vinograd and Bruner, 1966). The radial position, *r*, of the enzyme band as determined by the midpoint of the scanner traced boundary (Figure 1A) gave a linear log *r* *vs.* time plot (Figure 1B). At lower enzyme concentrations, the boundary heights were too small and the signal to noise ratio was too low to utilize the "difference curve" analysis (Cohen *et al.*, 1967), but boundary midpoints were still obtainable.

The reacting form of the enzyme in the presence of both substrates at pH 6.75 has been determined on two preparations of $P_{II}(B)$ and one preparation of $P_{II}(C)$ (Figure 2). Below an enzyme loading concentration of 1.2 μg/ml, the $s_{20,w}$ of $P_{II}(B)$ changed from approximately 3.0 to 4.7 S over a tenfold range of increasing enzyme loading concentrations. Above 1.2 μg/ml of $P_{II}(B)$, the reacting form was predominantly the dimer with an $s_{20,w}$ of 4.72 ± 0.14 S. The dependence of the $s_{20,w}$ of the reacting $P_{II}(B)$ enzyme on loading concentration suggested a reversible dissociation of the 4.72S dimer to an active species with an $s_{20,w}$ approaching 3.0 S. The reacting form of the $P_{II}(C)$ enzyme exhibited a more gradual rise in $s_{20,w}$, from approximately 3.7 S at a loading concentration of 0.6 μg/ml to a value approaching the dimer value of $P_{II}(B)$ at a loading concentration of 3.5 μg/ml.

Reacting enzyme sedimentation at pH 8.55 has been done on $P_{II}(B)$ and $P_{II}(C)$ (Figure 3). The $s_{20,w}$ of $P_{II}(C)$ changed from approximately 3 to 4.2 S as the loading concentration was raised from 0.06 to 0.6 μg/ml. Above 0.6 μg/ml the $s_{20,w}$ was 4.17 ± 0.06 S. The dependence of the $s_{20,w}$ of the reacting $P_{II}(C)$ enzyme on loading concentration again suggested a reversible dissociation of the 4.2S dimer to a species whose $s_{20,w}$ again approached 3.0 S. The $P_{II}(B)$ exhibited an $s_{20,w}$ for the reacting enzyme of 4.20 ± 0.07 over a range of loading concentrations from 0.2 to 1.74 μg/ml. The $P_{II}(C)$ enzyme was also run in the presence of 0.6 M NaCl at pH 8.55 (Figure 3). This concentration of NaCl was found to inhibit the enzyme activity by 43%. The reacting enzyme under these conditions was presumed to be predominantly a monomer, with an $s_{20,w}$ of 3.55 ± 0.14 S. At neither pH under any condition was there any evidence of two bands or peaks of activity.

Differences have been observed between the association curves of the reacting forms of $P_{II}(B)$ and $P_{II}(C)$ (Figures 2 and 3). It appears that at both pH values, the dissociation from dimers occurs at lower loading concentrations for $P_{II}(B)$.

Overloading with enzyme can cause overestimation of the sedimentation coefficient of the reacting enzyme (Cohen *et al.*, 1967; Taylor, 1972). Experimental overloading with enzyme in reacting enzyme sedimentation causes an almost linear increase in the sedimentation coefficient with the logarithm of the enzyme loading concentration above a certain critical concentration (Cohen and Mire, 1971; B. L. Taylor and M. F. Utter, personal communication). The pH 8.55 assay system was therefore purposely overloaded with $P_{II}(C)$ to determine if the increase in $s_{20,w}$ observed up to a loading concentration of 0.6 μg/ml was really an association reaction or an artifact due to overloading with enzyme. As can be seen (Figure 4), an almost linear increase in $s_{20,w}$ with the logarithm of enzyme loading concentration was seen above 3 μg/ml. This indicated that the increase in $s_{20,w}$ below 0.6 μg/ml followed by a plateau at 4.2 S was indeed due to an association-dissociation and not

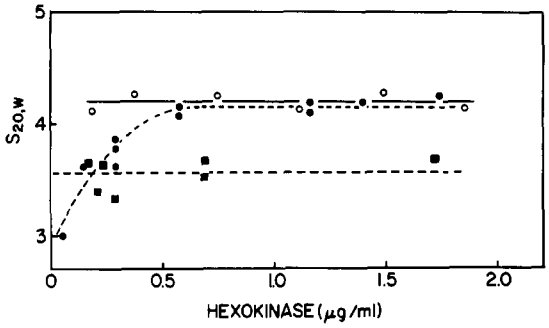

FIGURE 3: Reacting enzyme centrifugation of yeast hexokinase $P_{II}(B)$ (○), $P_{II}(C)$ (●), and $P_{II}(C)$ in the presence of 0.6 M NaCl (■). The enzyme was layered on the assay mixture containing Cresol Red at pH 8.55 in 50% D_2O. The $s_{20,w}$ was determined experimentally by midpoint calculations and plotted as a function of the enzyme loading concentration (μg/ml).

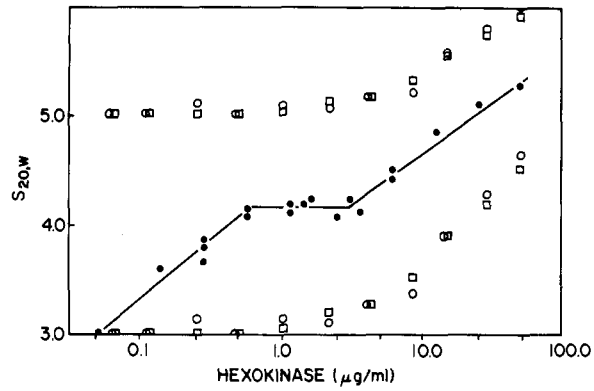

FIGURE 4: Reacting enzyme centrifugation of yeast hexokinase $P_{II}(C)$ at pH 8.55 (●) and simulated measurement of $s_{20,w}$ (□ and ○) as a function of enzyme loading concentration. The $s_{20,w}$ of $P_{II}(C)$ at pH 8.55 was determined experimentally by midpoint calculations and plotted as a function of the logarithm of enzyme loading concentration (μg/ml). Simulated measurement of $s_{20,w}$ was calculated from the maxima of the simulated distributions of specific activity (□) and from the midpoint of the simulated distributions of product (○) of Figure 5 as described in the Results section.

overloading of the pH 8.55 assay system. At 3 μg/ml the amount of substrate utilized as the enzyme band passes is estimated to be nearly 100%.

Simulation of Enzyme Overloading in Reacting Enzyme Sedimentation Studies. Cohen *et al.* (1967) have presented a theoretical analysis of the technique of reacting enzyme centrifugation demonstrating its validity under ideal conditions of a linear assay and in the absence of a change in activity across the sedimenting band which might be caused by factors including substrate depletion and/or product inhibition. In the "difference curve" analysis, the radial product distribution is obtained by plotting the difference in absorbance between successive tracings at a given radial position in the cell as a function of that radial position. The resulting bell-shaped curve corresponds to the distribution of product produced by the enzyme during the interval between scans, *i.e.*, the enzyme activity. If the absorbance is proportional to the product formed, and if the product formed is proportional to the concentration of the enzyme in the sedimenting band, the difference curve is representative of the shape and average position of the enzyme band. The sedimentation coefficient is calculated from the time-dependent centrifugal migration of the maximum or line of symmetry of the difference curves. These authors also showed that the product distributions can be numerically analyzed to correct for the sedimentation and diffusion of product, which they have estimated could introduce a 3–5% error in the sedimentation coefficient.

Overloading with enzyme can cause overestimation of the sedimentation coefficient (Cohen *et al.*, 1967; Taylor, 1972). If the sedimenting band contains a high concentration of enzyme, the trailing part of the band will have a lower enzymatic activity than the leading edge because of substrate depletion and product inhibition. The result will be a centrifugal shift of the product produced in a given time relative to the distribution of enzyme. Such a relative shift will increase with time as diffusion broadens the sedimenting band. Thus, the formation of product is not proportional to enzyme concentration and occurs predominantly at the front of the enzyme band. Therefore, the product distribution traverses a greater radial distance in a given time interval than does the enzyme distribution. Experimental overloading with enzyme in reacting enzyme sedimentation caused an almost linear increase in the sedimentation coefficient with the logarithm of the enzyme loading concentration (Cohen and Mire, 1971; B. L. Taylor and M. F. Utter, personal communication). As a result of these observations, 5–10% has been suggested as the upper limit for the substrate utilization as measured by the boundary height in the ultracentrifuge (Cohen and Mire, 1971). Their criteria for overloading, consisting of experimentally measuring the $s_{20,w}$ as a function of enzyme concentration, is applicable only for an enzyme whose aggregation state does not vary with enzyme concentration. In a rapidly associating–dissociating enzyme system, the true $s_{20,w}$ is expected to increase with increasing concentration of enzyme. If the true increase in the $s_{20,w}$ due to an enzyme association process were large compared to the apparent increase in the $s_{20,w}$ due to overloading with enzyme, the artifactual increase of overloading would not obscure the observation of an association. In order to obtain a quantitative measure of errors in the sedimentation coefficient due to overloading with enzyme, simulation studies of sedimentation of reacting enzyme were carried out.

A band of enzyme was assumed to be sedimenting centrifugally through an assay mixture in a gaussian distribution of concentration with radial distance from the center of rotation (Vinograd and Bruner, 1966)

$$f(R) = \frac{1}{\sigma\sqrt{2\pi}} \exp\left[-\frac{1}{2}\left(\frac{R-\mu}{\sigma}\right)^2\right] \qquad (1)$$

where μ is the radial (R) position of the mean and σ is the standard deviation. The enzyme-catalyzed reaction was assumed to be reversible with a single substrate and a single product. The product concentration at each point in the enzyme distribution was determined by eq 2, the product-time relationship for a

$$Et = \frac{(K_P - K_A)P}{(K_P k_f + K_A k_r)} + \frac{K_A K_P}{(K_P k_f + K_A k_r)} \times \left[1 + \frac{(k_f + k_r)A^0}{K_P k_f + K_A k_r}\right] \ln\left[\frac{(A^0)'}{(A^0)' - P}\right] \qquad (2)$$

simple reversible mechanism (Schwert, 1969). E and P are the enzyme and product concentrations, respectively; k_f and k_r are maximum turnover numbers of the forward and reverse reactions, respectively; K_A and K_P are the Michaelis constants for substrate and product, respectively; and $(A^0)'$ is defined by $(A^0 - A_{eq})$, where A^0 and A_{eq} are the substrate concentrations at initial time and at equilibrium, respectively. P can be numerically solved as a function of E when values are assigned to the constants and t is calculated for a given rate of sedimentation. The sum of A (substrate concentration) and P is equal to A^0; thus, the distribution of substrate can be determined. The ac-

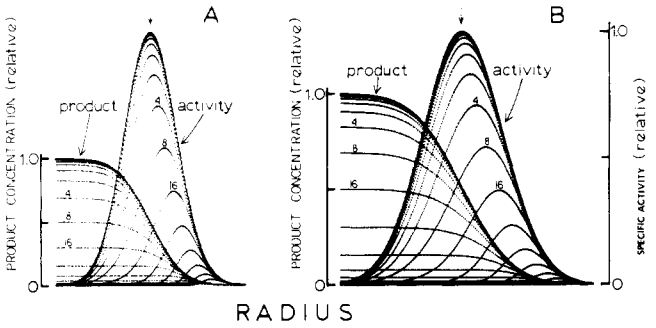

FIGURE 5: Simulated specific activity distributions and specific product distributions at increasing concentrations of enzyme. The sedimenting band of enzyme (not shown) was assumed to have a gaussian distribution of concentration with radius of centrifugation with a broadness defined by a standard deviation of σ (eq 1). The radial position of the enzyme band maximum is indicated by an arrow. The constants for eq 2 and 3 were assumed to be $K_A = K_P = 0.2$ mM, $k_f = 10k_r = 80$ μmol/min per mg, $A^0 = 2$ mM, and $K_{eq} = 10$. These constants are similar to real constants for yeast hexokinase P_{II} at pH 6.5 wherever possible. The specific activity distributions (bell-shaped) were calculated as described under Methods, and the product distributions (sigmoid-shaped) were obtained by integrating the specific activity distributions. In A, the enzyme distribution is at an early time ($\sigma = 0.5$), and in B, the enzyme distribution is broadened by diffusion at a later time ($\sigma = 0.75$). The heights of the specific activity distributions at low enzyme concentration in A and B were made the same for graphing purposes. The numbers above the simulated distributions represent the enzyme concentrations (μg/ml) for those distributions. Enzyme concentration was varied in a geometric progression by constant doubling of enzyme concentration. Each specific activity or product curve is normalized for its respective enzyme concentration so that the decreasing heights of curves with increasing enzyme concentration represent a lowering of the apparent specific activity.

tivity of the enzyme at each position in the band was determined by eq 3 (Alberty and Koerber, 1957). The calculated

$$v = \frac{[(k_f/K_A)A - (k_r/K_P)P]E}{1 + A/K_A + P/K_P} \qquad (3)$$

distributions of product and specific activity of enzyme were simulated as a function of increasing enzyme concentration in the sedimenting band (Figure 5). The distributions of specific activity relative to the position of the enzyme band (indicated by the arrow) became shifted in a centrifugal direction as the enzyme loading concentration increased above a critical value. The shift due to overloading was accompanied by a decrease in the maxima of the distributions of the relative specific activity and of the total relative product formed. Since the distributions of specific activity and product were normalized to the distributions for the lowest enzyme concentration, the decrease in maxima of the specific activity and the total product means that these parameters are no longer increasing proportionately with enzyme concentration. This deviation from linearity occurred when enzyme product formation was no longer linear with time (or when specific activity was no longer linear with enzyme concentration). As the trailing edge lost efficiency relative to the leading edge, the specific activity distributions became asymmetrical, but even when the trailing 50% of the enzyme was relatively inactive, this asymmetry was much less than we had anticipated and could easily go undetected by observation of difference curves. However, the band width of the specific activity distribution decreased considerably with overloading and possibly could be determined by inspection.

Simulation of the sedimentation coefficient as a function of overloading with enzyme requires simulation of the product and velocity distributions at more than one time and radial position in the cell. As the sedimentation progresses with time, broadening of the enzyme distribution occurs due to diffusion. This broadening, as reflected in the value for σ (Figure 5), was adjusted in accordance with experimentally observed difference curves for yeast hexokinase P_{II}(B) at pH 6.75. The enzyme distributions were separated in time and radial distance for true $s_{20,w}$ values of 3.0 and 5.0 S. The radial position of the maximum of each specific activity curve and the midpoint of each product curve were automatically calculated in the simulation program. The apparent $s_{20,w}$ was then calculated from the maxima of the distributions of the specific activity and from the midpoints of the distributions of product at increasing concentrations of enzyme (Figure 4). Only negligible differences were seen in the $s_{20,w}$ calculated by midpoints and by specific activity (difference) curves. The calculated sedimentation coefficient was invariant at low enzyme concentrations and increased at high enzyme concentrations for both $s_{20,w}$ values. The slopes of the overloaded portion of the log enzyme *vs.* $s_{20,w}$ plots decreased with increased initial $s_{20,w}$ (Figure 4). At 100% substrate utilization, the 3.0- and 5.0S plots have increased to 4.25 (42%) and 5.76 S (15%), respectively. For the 3.0S plot, which had the greatest slope, small errors in the sedimentation coefficient (less than 5%) occurred when 5% of the substrates were depleted, but errors of 15% did not occur until 50% of the substrates were depleted (about 3 μg/ml for hexokinase P_{II} at pH 6.75). A 15% error in the sedimentation coefficient would not obscure the observation of a 50–75% increase expected for dimerization of the enzyme.

Simulation studies were done to check the dependency of the $s_{20,w}$ on the assumed σ value. $s_{20,w}$ values were calculated from $\sigma = 0.5$ to $\sigma = 1.0$ for an appropriate time interval. Increasing the final σ from 0.75 to 1.00 had the effect of decreasing the slope of the overloaded portion of the plot. At 100% substrate utilization, the 3.0S plot value was decreased from 4.25 to 4.02 S by increasing the final σ from 0.75 to 1.00.

An additional problem in dye-linked assays is the nonlinearity of the assay system mainly due to pH changes and pK differences. Simulated specific activity distributions and product distributions for low enzyme concentrations have been empirically corrected from an experimental absorbance *vs.* enzyme–time curve (typical for our studies), and both simulated curves were corrected downward because of the nonlinearity of the A_{400} *vs.* enzyme–time curve. The resulting increase in calculated $s_{20,w}$ for a 3.0S enzyme was less than 8%. Thus, nonlinear assays of this magnitude (which includes the nonlinearity of the dye system itself, previously discussed) would have a relatively small effect on the observed $s_{20,w}$.

Sedimentation Velocity Studies. Sedimentation velocity studies of yeast hexokinase were done in the presence of 50% D_2O in order to facilitate comparison with reacting enzyme centrifugation studies. The dye indicators were omitted, and at pH 8.55, glycylglycine was maintained at 1.7 mM, but at pH 6.6, the $(EtOH)_3NHCl$ was raised to 0.03 M to stabilize the pH. At pH 6.6, above 50 μg/ml, in the absence of substrates, or in the presence of glucose, yeast hexokinase P_{II}(B) sedimented as a dimer, 5.57 ± 0.22 and 4.69 ± 0.12 S, respectively (Figure 6). In both cases, dissociation occurred below 50 μg/ml. In the region of dissociation, the moving boundary in the ultracentrifuge did not give evidence for separable moving boundaries, *i.e.*, monomers separable from dimers. This indicated that the monomer–dimer equilibrium was rapid compared to the time of centrifugation. Glucose did not appear to significantly shift the dependence of this dissociation on protein concentration. The decrease in the dimer sedimentation coefficient in the presence of glucose is consistent with a conformational change of the enzyme (Roustan *et al.*, 1973). The dimer

value in the presence of glucose, 4.7 S, was identical with the dimer value obtained for the reacting enzyme (Table I). The simultaneous presence of both substrates (Figure 2) compared to the presence of glucose alone or the absence of substrates (Figure 6) had a strong associative effect, shifting the association-dissociation to 50-fold lower protein concentration. However, in the presence of both substrates, dissociation to active monomers can still occur at very low enzyme concentrations.

There was no evidence for dissociation of $P_{II}(B)$ and $P_{II}(C)$ above 50–90 μg/ml at pH 8.55 in 50% D_2O in the absence of substrates, in the presence of the single substrate, 20 mM glucose, or in the presence of 20 mM glucose plus 8 mM $MgCl_2$. A constant $s_{20,w}$ was observed from 50 to 600 μg/ml in each case. The dimer, 5.67 ± 0.16 S, in the absence of substrates had almost the identical $s_{20,w}$ as at pH 6.6. The presence of 20 mM glucose alone decreased the $_{20,w}$ to 4.88 ± 0.18 S, again almost identical with the value obtained at pH 6.6. The addition of 8 mM $MgCl_2$ in the presence of glucose caused an increase in $s_{20,w}$ to 5.74 ± 0.17 S, a value almost identical with the dimer value in buffer alone. The addition of 0.6 M NaCl to any of the previous solutions caused extensive dissociation to monomers of 3.88 ± 0.13 S.

Discussion

There is an increase in the $s_{20,w}$ of reacting yeast hexokinase P_{II} observed at low enzyme concentrations (less than 3 μg/ml) at both pH 6.75 and 8.55 (Figures 2 and 3) which is not due to artifactual increases due to overloading of the system. We conclude that an association-dissociation of reacting monomers and reacting dimers occurs. Since the boundary does not give evidence for separate moving peaks, this reversible equilibrium must be rapid. Most enzyme assays are done under conditions where the enzyme is predominantly in the monomer form. This includes conditions under which many of the regulatory properties of hexokinase P_{II} at pH 6.75 are observed (Shill and Neet, 1974).

Since hexokinase P_{II} without substrates is essentially completely dissociated at the microgram level (see Figure 8 of Derechin *et al.*, 1972), the question arises as to what factor(s) causes the strong association seen in the reacting enzyme (Figures 2 and 3). At pH 8.7, an association constant of 400 has been reported (Derechin *et al.*, 1972) for P_{II} in glycine (I = 0.15), plus the products 1 mM MgADP and 10 mM G-6-P. If this were the association constant, it can be calculated from the concentration of enzyme that the percentage dimer by weight would be less than 2.5%. Therefore, even if all of the substrates have been converted to product, one would still not expect to observe a dimer sedimentation coefficient such as observed at pH 8.55. These results indicate that the combined effect of the substrates themselves, and not the products, is causing the observed dimerization. At pH 6.75, one would require a hexokinase association constant greater than 4×10^5 in the presence of products to obtain 85% dimer by weight at the lowest enzyme concentration where a dimer sedimentation coefficient is observed in the reacting enzyme sedimentation. This association constant represents a 1000-fold increase over the pH 8.7 value, which seems unlikely. Furthermore, at this enzyme loading concentration, 1.2 μg/ml, less than 20% of the MgATP has been converted to MgADP. Therefore, the presence of both substrates also enhances the dimerization at pH 6.75.

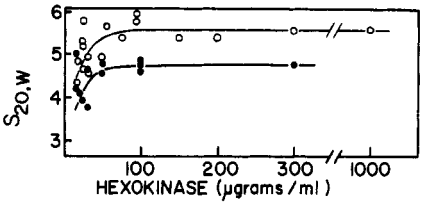

FIGURE 6: Boundary sedimentation velocity of yeast hexokinase P_{II} in the presence and absence of glucose. With the open circles (○) the enzyme was dialyzed overnight at 2 mg/ml against a solution of 0.06 M $(EtOH)_3NHCl$ at pH 6.75 at 4°. Aliquots of the dialyzed enzyme were then diluted to obtain the enzyme concentration of the ordinate in 0.03 M $(EtOH)_3NHCl$ and 50% D_2O at pH 6.6. Ultracentrifugation was run near 20°. With the closed circles (●), the conditions were the same, except after dialysis D-glucose was added to a final concentration of 20 mM.

The apparent contradiction of the sedimentation equilibrium studies (Derechin *et al.*, 1972) and our reacting enzyme sedimentation studies with regard to the predominant molecular weight species during catalysis are explicable because the enzyme is most likely in a different conformation in these two types of experiments. Only the hexokinase-glucose-MgATP ternary complex undergoes a slow conformational change to a form which has different kinetic properties (Shill and Neet, 1971, 1974). Other enzyme complexes such as nonreactive combinations of substrates and products, or complexes predominating at equilibrium, do not undergo this transition. Thus, the two conformations, equilibrium and reacting steady state, not only have different kinetic properties, but apparently differ markedly in their self-association properties.

Different sedimentation coefficients were obtained for the dimer reacting enzyme at pH 6.75 and 8.55 (Table I). Prelimi-

TABLE I: Sedimentation Coefficients of Yeast Hexokinase under Various Conditions.[a,b]

	Reacting Enzyme Sedimentation	
Conditions	pH 6.75	pH 8.55
Standard (see Methods)	4.72 ± 0.14 S	4.20 ± 0.07 S
Standard + 0.6 M NaCl		3.55 ± 0.14 S
	Sedimentation Velocity	
Conditions	pH 6.6	pH 8.55
Buffer[c]	5.57 ± 0.22 S	5.67 ± 0.16 S
Buffer + 20 mM glucose	4.69 ± 0.12 S	4.88 ± 0.18 S
Buffer + 20 mM glucose + 8 mM $MgCl_2$		5.74 ± 0.17 S
Buffer + 0.6 M NaCl		3.88 ± 0.13 S

[a] Sedimentation coefficients are averages (± standard deviation) of the plateaus of Figures 2, 3, and 6, and unpublished observations.[2] [b] The sedimentation coefficient of the reacting enzyme under standard conditions at pH 8.55 is significantly different ($p <$ 0.005) from the reacting enzyme under standard conditions at pH 6.75, the reacting enzyme in the presence of 0.6 M NaCl, and the sedimentation velocity in the presence of 0.6 M NaCl. [c] 0.03 M $(EtOH)_3NHCl$ at pH 6.6 or 1.7 mM glycylglycine at pH 8.55.

nary studies done with the Cresol Red assay system indicate that the drop in $s_{20,w}$ from 4.7 to 4.2 S occurs in a smooth transition but mainly between pH 7.6 and 8.0. The difference is unlikely to be caused by preferential solvation because of the low concentration of buffers in the assay systems. Furthermore, the same sedimentation coefficient for the lower pH region was obtained with both dye systems. The difference in $s_{20,w}$ is most likely due to a conformational change in the enzyme.

Reacting enzyme sedimentation, which can be used to study an enzyme-catalyzed reaction in which there is a difference between the absorption spectrum (uv or visible) of the substrates and products (Cohen *et al.*, 1967), recently has been extended using the coupled enzyme assay (Cohen and Mire, 1971; Taylor *et al.*, 1972). The use of the coupled enzyme assay is limited to systems in which the enzyme being studied sediments faster than the coupling enzyme. Since many enzyme-catalyzed metabolic reactions are accompanied by the production or uptake of a proton, a pH-dependent dye-linked assay permits considerable extension of the number of enzymes that can be studied by reacting enzyme sedimentation. We have now demonstrated with experimental and theoretical methods with yeast hexokinase that this type of system can yield useful information, even though there are several inherent sources of error.

An additional problem in this case is that the enzyme-time curve is different for monomers and dimers, because they have somewhat different specific activities at pH 6.75 (Shill and Neet, 1974). The steady-state velocity is about twice as great for the dimers. In the region of association-dissociation for a rapid equilibrium, the sedimenting band could have dimers predominating in the center of the band and monomers predominating in the leading and trailing edges where the enzyme concentration is lowest. If the monomers are much more active than the dimers, difference curves could show two separate peaks, or, *vice versa*, hypersharpening of the band. No evidence for this has been observed within the error of the difference curves. To a first approximation, such an effect would not cause error in the radial position of the midpoint of the boundary. Dilution of the band during sedimentation should produce further dissociation. However, all log R^2 *vs.* time plots have been linear suggesting that such an effect is not large.

Sedimentation velocity studies suggest that the dimer form of the enzyme undergoes a conformational change in the presence of glucose at both pH 6.60 and 8.55 (Table I). This change is probably not due to nonideality effects, because at pH 8.55 there is good agreement between sedimentation velocity and equilibrium sedimentation data (unpublished observations[2]). The good agreement between the plateau values at the two pH values suggests that little or no nonideality occurs at pH 6.6 either. Sedimentation equilibrium studies at either pH showed no detectable differences between enzyme and enzyme plus 20 mM glucose (unpublished observations[2,3]), which indicates that the lowering in sedimentation coefficients by glucose is not due to dissociation. This is in agreement with other studies which showed that glucose had a dissociating effect in phosphate, but not in Tris buffers (Schulze and Colowick, 1969). Addition of 8 mM $MgCl_2$ in addition to 20 mM glucose raised the $s_{20,w}$ of the dimer back to 5.7 S, the original $s_{20,w}$ of the enzyme in buffer at pH 8.55 (Table I). Equilibrium results again show no detectable dissociation and no difference from the curves in buffer alone and buffer plus glucose.[2] Therefore, $MgCl_2$ must be having a direct effect upon the enzyme causing either return to the original conformation or a change to a new conformation with sedimentation properties similar to the enzyme in buffer alone.

The sedimentation coefficients of reacting dimers at both pH values appear to be different from those of the dimer in buffer alone, and may represent a conformational change in the presence of both substrates. The values of the reacting dimer sedimentation coefficients are most similar to those of the P_{II} dimer plus glucose at each pH. Perhaps glucose by itself can induce nearly the correct form for catalysis. These and other differences in the $s_{20,w}$ of the dimers may relate to the different types of dimers observed in crystallographic studies of hexokinase (Steitz *et al.*, 1974; Anderson *et al.*, 1974) which would have different frictional coefficients and therefore different $s_{20,w}$ values.

The kinetic and regulatory properties of yeast hexokinase P_{II} have been shown to be markedly pH dependent with negative cooperativity and slow transients only appearing below pH 7 (Kosow and Rose, 1971; Shill and Neet, 1974). These properties must have some basis in different molecular structures at pH 6.7 and 8.5. The studies presented here show no major differences in association behavior at the two pH values.

Acknowledgments

The authors wish to thank Dr. B. L. Taylor for many helpful discussions and Mr. Charles Starling for technical assistance.

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Isolation and Partial Characterization of an Acid Carboxypeptidase from Yeast†

Robert W. Kuhn,‡ Kenneth A. Walsh, and Hans Neurath*

ABSTRACT: The purification and characterization of a carboxypeptidase from baker's yeast, *Saccharomyces cerevisiae,* are described. The purified enzyme has been characterized with respect to molecular weight (62,000), amino acid composition, amino terminal sequence, enzymatic properties, and sensitivity to group-specific inhibitors. The enzyme is a glycoprotein composed of a single polypeptide chain, which contains 13 half-cystine residues, 2.6% amino sugars, and 12.7% neutral hexoses. The enzyme releases a variety of amino acids, including proline, from the carboxyl terminus of proteins. Unlike pancreatic carboxypeptidases, the yeast enzyme is not inhibited by chelating agents but is inhibited by diisopropyl phosphorofluoridate, *p*-mercuribenzoate, mercuric chloride, *N*-tosyl-L-phenylalanine chloromethyl ketone, phenylglyoxal, iodoacetamide, and by photooxidation in the presence of rose bengal. The enzyme retains full activity in 4 M urea and is useful for determining carboxyl-terminal sequences of proteins and peptides.

Two distinct classes of carboxypeptidases have been described. One class, typified by the pancreatic carboxypeptidases, exhibits a neutral to alkaline pH optimum and requires for activity divalent cations of the IIB transition series (Vallee and Riordan, 1969; Petra, 1970). The second class, termed "acid carboxypeptidases" (Zuber and Matile, 1968), displays maximal activity in the acid range and is inhibited by Dip-F.[1] In contrast to the metallocarboxypeptidases, acid carboxypeptidases release from the carboxyl terminus of proteins the imino acid proline, in addition to several other amino acids. Acid carboxypeptidases have been isolated from yeast (Hata *et al.,* 1967a,b), citrus peel (Zuber, 1964), citrus leaves (Zuber and Matile, 1968; Sprossler *et al.,* 1971; Tschesche and Kupfer, 1972), Aspergillus (Ichishima, 1972), bean leaves (Wells, 1965), cotyledons of germinating cotton seedlings (Ihle and Dure, 1972a,b), germinating barley (Visuri *et al.,* 1969), and have been observed in a variety of plant tissues (Zuber and Matile, 1968). Cathepsin A from bovine spleen also displays several characteristics of acid carboxypeptidases (Logunov and Orekhovich, 1972).

In contrast to the extensively studied metallocarboxypeptidases, relatively little is known about the structure and mechanism of action of the acid carboxypeptidases. In order to extend our knowledge of these enzymes, we have isolated an acid carboxypeptidase from baker's yeast. This enzyme was first described by Hata *et al.* (1967a) under the name of "protease C" and certain of its chemical and enzymatic properties have been reported (Hata *et al.,* 1967b; Aibara *et al.,* 1971; Hayashi *et al.,* 1970). The present paper describes a method of isolation of the enzyme and its characterization. While this work was in progress, Hayashi *et al.* (1973) described the isolation and some characteristic properties of this enzyme which are in close agreement with those reported herein.

Experimental Section

Materials

Compressed baker's yeast was obtained through the courtesy of Standard Brands, Inc.

N-Acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) was purchased from Fox Chemical Co. All carbobenzoxy dipeptides were obtained from Cyclo Chemical Corp. The B-chain of oxidized bovine insulin (lot W-1300) was from Schwarz/Mann.

DEAE-cellulose was purchased from Schleicher and Schuell, Inc. and DEAE-Sephadex A-50 and Sephadex G-150 from Pharmacia Fine Chemicals.

The protein standards used for sodium dodecyl sulfate gel electrophoresis were all obtained from Worthington Biochemical Corp. except phosphorylase *b* which was a gift from Dr. Edmond H. Fischer.

Dip-F was purchased from Pierce Chemical Co. The reagent was diluted to 1 M in water-free 2-propanol and stored over molecular sieves (Matheson Coleman and Bell).

Tetranitromethane, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, and Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) were obtained from Aldrich Chemical Co., Inc. *N*-

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received April 25, 1974.* Taken in part from a dissertation submitted by R. W. Kuhn to the Graduate School of the University of Washington in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A preliminary report has been published (Kuhn *et al.,* 1972). This work was supported by research grants from the National Institutes of Health (GM 15731) and the American Cancer Society (BC-91). R. W. K. was supported by a training grant from the National Institutes of Health (GM 00053) to the Department of Biochemistry.

‡ Present Address: Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77025.

[1] Abbreviations used are: Dip-F, diisopropyl phosphorofluoridate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Cbz-Phe-Leu, benzyloxycarboxylphenylalanylleucine; Cbz-Gly-Leu, benzyloxycarboxylglycylleucine, $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.