Biochemistry

 Volume 20, Number 13 June 23, 1981

Catalytically Active Monomer and Dimer Forms of Rat Liver Carbamoyl-Phosphate Synthetase†

C. J. Lusty

ABSTRACT: Purified carbamoyl-phosphate synthetase of rat liver is shown to exist in a state of rapid, reversible monomer–dimer equilibrium. The allosteric activator *N*-acetyl-L-glutamate displaces the equilibrium toward monomer formation. This effect is observed over a range of initial protein concentrations of 0.02–5 mg/mL. Measurements of Stokes radii by analytical gel chromatography indicate that at concentrations less than 0.1 mg/mL at 25 °C in the presence of all the substrates the enzyme exists as a monomer of 160 000 molecular weight. A gel chromatographic method was developed to identify the active form of carbamoyl-phosphate synthetase. On the basis of analysis of the ADP boundary formed during gel chromatography, the monomer is established to be catalytically active. Active enzyme centrifugation studies confirm that the monomer is a reactive species and suggest that the dimer also functions catalytically. Under the conditions of the usual enzyme assay, carbamoyl-phosphate synthetase is mainly in the monomer form. Activation by acetylglutamate can occur at the level of the monomer and is not coupled to dissociation since the enzyme dissociates at low concentrations even in the absence of acetylglutamate. The stoichiometry of the association is observed directly in the electron microscope. The dimensions of the negatively stained particles of the enzyme in the presence or absence of substrates correspond to monomers and dimers, assuming the molecule to be a prolate ellipse. The number of monomers observed in the presence of substrate represents 86% of the total number of enzyme molecules. The average molecular weight calculated from the numbers of particles seen in negatively stained specimens of carbamoyl-phosphate synthetase is 182 000. Electron microscope studies provide independent evidence for monomer–dimer interactions and show that under the conditions examined the enzyme is mainly in the monomer form.

Carbamoyl-phosphate synthetase I catalyzes the formation of carbamoyl phosphate, the first step of arginine and urea biosynthesis, from ammonia, bicarbonate, and two molecules of ATP. Carbamoyl-phosphate synthetase activity is absolutely dependent on the presence of catalytic amounts of *N*-acetyl-L-glutamate (Hall et al., 1958). The activation by acetylglutamate has been suggested to be allosteric in nature, as evidenced by acetylglutamate-induced conformational changes of the enzyme (Guthohrlein & Knappe, 1968).

It is now generally recognized that the monomer molecular weight of mammalian carbamoyl-phosphate synthetase as measured in sodium dodecyl sulfate (Clarke, 1976; Lusty, 1978a) or guanidine hydrochloride (Lusty, 1978a) is 160 000. The question of the active form of the enzyme has still not been satisfactorily answered. In the absence of acetylglutamate, 7.5S and 11S forms of the purified rat liver enzyme have been reported to be in chemical equilibrium (Virden, 1972). In the presence of acetylglutamate, depending on the temperature, carbamoyl-phosphate synthetase has been reported to sediment at 7.5 S (at 5 °C) (Guthohrlein & Knappe, 1968; Virden, 1972) or at 11.3 S (at 30 °C) (Guthohrlein & Knappe, 1968).

In order to understand the catalytic and regulatory mechanism of the enzyme, it is of critical importance to know the physical size of the catalytic unit. The studies described here were intended to measure the size of the catalytically active rat liver carbamoyl-phosphate synthetase and to examine the effect of the allosteric activator *N*-acetylglutamate on the physical properties of the enzyme. Analytical gel chromatography at 25 and 4 °C together with sedimentation velocity measurements was employed to study acetylglutamate-induced dissociation of the enzyme. Active enzyme centrifugation and the measurement of the boundary position of the carbamoyl phosphate and ADP formed during gel chromatography were used to demonstrate that the monomer is a catalytically active species.

Experimental Procedures

Materials. *N*-Acetyl-L-glutamate, citrulline, and ornithine were Fluka products obtained from Tridom Chemical Inc. ATP and ADP were purchased from Schwarz/Mann; carbamoyl phosphate (dilithium salt), phenol red, sodium salt, and sodium azide were from Sigma Chemical Co. Glycerol was obtained from Matheson Coleman and Bell. Other biochemical reagents were of the highest purity commercially available. Sephadex G-200 and Blue Dextran 2000 were obtained from Pharmacia. The proteins used for calibration of the molecular sieving columns were obtained from Sigma Chemical Co.,

† From the Department of Biochemistry, The Public Health Research Institute of The City of New York, Inc., New York, New York 10016. *Received December 9, 1980.* This investigation was supported by Grant AM 18044 awarded by the National Institutes of Health.

0006-2960/81/0420-3665$01.25/0

Worthington Biochemicals, and Boehringer-Mannheim. Pig heart diaphorase (type III, Sigma) was further purified by chromatography on a column of calcium phosphate gel–cellulose (Williams et al., 1967). Ornithine transcarbamylase was purified from rat liver mitochondria as previous described (Lusty, 1978a; Lusty et al., 1979). The specific activity of the fractions used in the coupled assay was 100–300 units/mg of protein. Solutions and buffers were prepared as described previously (Lusty, 1978a).

Preparation of Carbamoyl-Phosphate Synthetase. Carbamoyl-phosphate synthetase was purified from rat liver mitochondria as described previously (Lusty, 1978a). The specific activity of the enzyme was 3.6 μmol of carbamoyl-P formed min^{-1} (mg of protein)$^{-1}$ at 37 °C. The enzyme was stored at −70 °C in a buffer containing Tris–acetate,[1] $\Gamma/2$ = 0.05, pH 7.6 (25 °C), 2 M ammonium sulfate, 0.3 M sodium acetate, and 2 mM dithioerythritol. The purified enzyme was stable indefinitely under these conditions. Samples of the enzyme (100 μL) were desalted by centrifugation through 1-mL columns of Sephadex G-50 equilibrated with the desired buffer, as described by Penefsky (1977).

Determination of Protein and Enzyme Activity. Protein concentration was determined from the absorbance at 280 nm, assuming that A_{280}^{1cm} is 1.0 per mg of protein. Protein concentration was also determined with Coomassie Brilliant Blue G-250 in a total volume of 3 mL according to the method of Sedmak & Grossberg (1977), using carbamoyl-phosphate synthetase as a standard.

Carbamoyl-phosphate synthetase activity was measured in the coupled reaction with ornithine transcarbamylase (Marshall et al., 1958). The reaction mixture, in a final volume of 0.5 mL, contained Bis-Tris–acetate, $\Gamma/2$ = 0.05, pH 7.2, 5 μmol of ATP, sodium salt, 7.5 μmol of magnesium acetate, 2.5 μmol of sodium acetylglutamate, 25 μmol of NH_4HCO_3, 1.5 μmol of ornithine hydrochloride, and 7 units of ornithine transcarbamylase. The reaction was started by adding 0.001–0.01 unit of enzyme. After incubation for 10 min at 37 °C, the reaction was stopped by adding 2.0 mL of acid reagent, and citrulline formation was determined according to the method of Ceriotti & Gazzaniga (1966) as previously described (Guthohrlein & Knappe, 1968). Citrulline concentration was determined from the absorbance at 464 nm, using an absorption coefficient of 37 800 M^{-1} cm^{-1} (Snodgrass & Parry, 1969). One unit of enzyme is defined as that amount which catalyzes the formation of 1 μmol of carbamoyl phosphate in 1 min at 37 °C. Specific activity is equivalent to units per milligram of protein.

Analytical Gel Chromatography. Gel chromatography was performed at 4 and 25 °C on a column (1.6 × 170 cm) of Sephadex G-200 as described by Ackers (1964). The column was equilibrated with Tris–acetate, $\Gamma/2$ = 0.05, pH 7.6 (25 °C), containing 2 or 5 mM dithioerythritol and 0.05% sodium azide. Other additions to the buffer are given in Table I and in the legends to the figures. The column was eluted at a flow rate of 13.4 mL/h maintained by a peristaltic pump, and fractions of about 2 mL were collected with an LKB RediRac with pump control. The volume of the fractions was determined by weighing and was found to be constant within ±0.02 mL. The absorbance of the column effluent was monitored at 280 nm with a Brinkmann UV monitor and recorder. Elution volumes of samples or reference proteins were determined either by assaying aliquots of the collected fractions for enzyme activity and protein or from recorder tracings of the absorbance at 280 nm of the column effluent. The volume of the enzyme sample applied to the column was 1.75 mL. Purified carbamoyl-phosphate synthetase was used without prior desalting; samples (1–20 mg) of the protein were diluted with the column elution buffer which also contained Blue Dextran (1 mg) and DNP-alanine (0.1 mg). The void volume, V_0, and the internal volume, V_i, were determined from the elution volumes of Blue Dextran 2000 and DNP-alanine. The void volume of the column varied less than 0.5 mL in each set of experiments. The partition coefficients were calculated according to the method of Ackers (1964) with the equation $K_d = (V_e - V_0)/V_i$. The Stokes radii of samples of carbamoyl-phosphate synthetase were calculated from the distribution coefficient, K_d, by use of the known value of the gel pore radius, r, according to the formula of Ackers (1964). The gel pore radius, r, was determined from the elution volumes and Stokes radii, a, of the following standard proteins: apoferritin, 79 Å (Rothen, 1944); fumarase, 52.9 Å (Cecil & Ogston, 1952); yeast alcohol dehydrogenase, 45.5 Å (Ackers, 1964; Hayes & Velick, 1954; Sytkowski, 1977); pig heart diaphorase, 43 Å (Massey et al., 1962); bovine serum albumin, 36.1 Å (Tanford, 1961). The average gel pore radii were calculated from the data obtained for the calibrating proteins under each set of experimental conditions. At 25 °C, the average value of r of Sephadex G-200 equilibrated with Tris–acetate buffer which contained substrates was 179 ± 6 Å.

In one series of experiments, the elution boundaries of the products, carbamoyl phosphate, ADP, and P_i, formed on the column during chromatography of the enzyme in the presence of added substrates were determined. In these experiments, where measurements were made of carbamoyl phosphate, ADP, and P_i in each fraction, the column effluent was quenched by mixing with an equal volume of 80 mM EDTA to prevent further conversion of substrates. The EDTA solution was separately pumped and mixed with the effluent in a three-way valve (Omnifit). The fractions were held at 0 °C in an ice bath and assayed for carbamoyl phosphate or ADP as soon as possible. The amount of carbamoyl phosphate in 0.2-mL aliquots of each fraction was determined by measuring the citrulline formed after enzymatic conversion of carbamoyl phosphate with ornithine and an excess of ornithine transcarbamylase. P_i was extracted from 1.0-mL samples of each fraction by adding 2 mL of cold 1 N H_2SO_4, 0.75 mL of 10% ammonium molybdate, and 1 mL of isobutyl alcohol/benzene (1:1) followed by vigorous shaking for 20 s on a Vortex mixer. The concentration of P_i in aliquots (0.2–0.5 mL) of the organic layer was determined colorimetrically by reduction with $SnCl_2$ (Lindberg & Ernster, 1956). ADP formation was determined in the following manner. To aliquots (0.2–1.0 mL) of the column fractions was added 0.1 mL of 2 N HCl to denature the enzyme. After the samples stood for 10 min at 0 °C, they were neutralized with 0.1 mL of 2 M Tris, and 0.1 mL of 0.5 M magnesium acetate was added to reduce the concentration of EDTA. ADP concentration was determined by adding aliquots (0.1–1.0 mL) of the samples to 2.9–2.0 mL of a reaction mixture that contained 0.1 M Tris–acetate, pH 7.6, 0.06 M KCl, 0.02 M $MgSO_4$, 0.5 mM phosphoenolpyruvate, 0.15 mM NADH, and 30 μg/mL lactic dehydrogenase (500 units/mg). The net decrease in absorbance at 340 nm was determined after adding 50 μg of pyruvate kinase (200 units/mg). All of the assay values of the ADP and P_i formed were corrected for nonenzymatic degradation of ATP, which was less than 2%.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane; DNP-alanine, dinitrophenylalanine.

When the amount of carbamoyl phosphate synthesized on the column was plotted vs. the volume of column effluent, the leading boundary formed by the carbamoyl phosphate corresponded to the protein boundary. Analysis of P_i, however, showed that P_i was in excess of carbamoyl phosphate, indicating that most of the carbamoyl phosphate was being hydrolyzed during development of the column. Based on the plateau values of carbamoyl phosphate and P_i, 68% of the carbamoyl phosphate was broken down. This was confirmed by measurements of ADP levels in the plateau region. ADP concentration equaled the sum of the carbamoyl phosphate and P_i concentrations. Since ADP was stable during the course of the experiment, and nonenzymatic breakdown of ATP was negligible, the ADP boundary was utilized to determine the elution volume of the reacting enzyme species in gel chromatography.

Sedimentation Velocity Measurements. Sedimentation velocity experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with ultraviolet optics and an automatic scanner. Sedimentation velocity coefficents were measured over a wide range of initial protein concentrations by band sedimentation and boundary sedimentation in 12- and 30-mm double-sector centerpieces. The enzyme was desalted on small columns of Sephadex G-50 equilibrated with Tris–acetate, pH 7.6, $\Gamma/2 = 0.1$, containing 2 mM dithioerythritol. Immediately prior to the experiment, samples for sedimentation analysis were prepared by dilution of the protein with the desired solvent. The compositions of the solvents are given in Table I and the legends to the figures. Sodium azide was not added to solutions for sedimentation analysis. Protein concentration was determined from the absorbance at 280 nm, or in the case of dilute samples by the Coomassie Blue method. In the boundary sedimentation experiments, samples were centrifuged in the An-D rotor at 48 230 rpm. Absorbance scans at 280 nm (100–500 mV/cm) were recorded by using minimum suppression at the fastest scan speed and 25 mm/s chart speed at 2- or 4-min intervals. In the experiments where the addition of ATP to the solvent contributed to the absorbance at 280 nm, the scanner was operated at 290–298 nm, depending on the protein concentration of the sample.

In band sedimentation experiments, samples were centrifuged in 12-mm Vinograd type double-sector centerpieces. The enzyme (5 μL) in 5% glycerol containing 1 mM Tris and 5 mM dithiothreitol was layered onto 0.35 mL of a sedimentation column containing 10% glycerol, Tris–acetate, $\Gamma/2 = 0.1$, pH 7.8, and 1 mM dithioerythritol plus and minus substrates. After acceleration to 60 000 rpm, absorbance scans at 280 nm (100–200 mV/cm) were recorded at the fastest scan speed and 25 mm/s chart speed at 1-min intervals. All sedimentation velocity experiments were conducted at 25 ± 0.1 °C.

The duration of the experiments was 20–60 min. For each experiment, a plot of the log of recorder deflection vs. the distance from the axis of rotation was linear. Observed sedimentation coefficients were calculated from the rate of movement of the midpoints of the sedimenting boundaries by least-squares fit of the data and were corrected for the densities and viscosities of the solvents. The partial specific volume of carbamoyl-phosphate synthetase was taken to be 0.74; this value was calculated from the amino acid compostion of the protein (Clarke, 1976). Since at high concentrations of glycerol the buoyancy term $(1 - \bar{v}\rho)$ will increase, $\bar{v}_2$ was increased by 0.006 and 0.010 mL/g (Lee et al., 1979) in the experiments with 10% and 20% glycerol, respectively. When the corrections for $\bar{v}_2$ and the measured viscosities of glycerol were applied, the sedimentation coefficients of fumarase (a nondissociating protein) measured in solutions of 10% and 20% glycerol were in excellent agreement with the literature values.

Reacting Enzyme Sedimentation. Active enzyme centrifugation studies were performed according to the method of Cohen et al. (1967) (Cohen & Mire, 1971; Claverie et al., 1975; Cohen & Claverie, 1975; Claverie, 1976). The experimental conditions and precautions necessary to obtain valid results with this method have been discussed by a number of investigators (Taylor et al., 1972; Kemper & Everse, 1973; Shill et al., 1974). During sedimentation, carbamoyl-phosphate synthetase activity was followed spectrophotometrically by coupling the enzyme reaction with phenol red. The acid produced during carbamoyl phosphate synthesis or, in the absence of NH_4^+, during acetylglutamate-dependent, bicarbonate-dependent ATP hydrolysis was measured by the change in the absorbance of phenol red at 560 nm. Glycerol (10%) was added to stabilize the sedimenting boundaries. For the overall reaction, the assay mixtures contained 10% glycerol, Tris–acetate, pH 7.8, $\Gamma/2 = 0.001$, 10 or 20 mM ATP, 15 or 25 mM magnesium acetate (added in 5 mM excess of ATP), 5 mM acetylglutamate, 20 mM potassium acetate, 10–40 mM NH_4HCO_3, 3 mM dithioerythritol, and 25 μM phenol red ($pK_a = 7.9$). The pH of the reaction mixture was adjusted to pH 7.8 with dilute NaOH. The ATPase reaction was measured in reaction mixtures that contained 10% glycerol, Tris–acetate, pH 7.8, $\Gamma/2 = 0.001$, 10 or 20 mM ATP, 9 or 18 mM manganese acetate, 10–20 mM $KHCO_3$, 30–40 mM potassium acetate, 5 mM acetylglutamate, 3 mM dithioerythritol, and 25 μM phenol red. The reaction mixtures adjusted to pH 7.8 had an initial A_{560} of about 1. In preliminary experiments, it was found that the decrease in absorbance at 560 nm was first order with respect to the amount of H^+ added to the reaction mixtures. This relation is presumably due to pK differences between the dye and the components of the assay mixtures that acted as buffers (Lowry et al., 1954; Segel, 1975), mainly ammonium bicarbonate, but also $MgATP^{2-}$. The $\Delta A_{560}/\mu$mol of H^+ varied, depending on the concentration of $MgATP^{2-}$ and NH_4HCO_3. At 4 mM $MgATP^{2-}$ and 5 mM NH_4HCO_3, the ΔA_{560} was 0.5/μmol of H^+. The corresponding values for NH_4HCO_3 concentrations of 10 and 20 mM were 0.28 and 0.1 per μmol of H^+, respectively. It was determined that for each set of conditions ΔA_{560} provided a true measurement of the amount of carbamoyl phosphate formed. The assay conditions and the range of enzyme concentrations were chosen so that in the cuvette H^+ production was linear for 90 min, and the final pH of the reaction mixtures was not less than pH 7.2, still in the optimum range of enzyme activity and in the buffering range of phenol red. The total ΔpH was dependent on the composition of the reaction mixture. Absorbance changes at 560 nm were nearly proportional to the amount of enzyme added up to $\Delta A_{560} = 0.26$. The active enzyme centrifugation method, however, could be used only in a narrow range of protein concentrations (2–6 μg of enzyme) when the overall enzyme reaction was measured. Valid sedimentation data could be obtained over a wider range of enzyme concentrations by measuring the partial reaction, acetylglutamate-dependent, bicarbonate-dependent ATPase (Lusty, 1978b).

Sedimentation coefficients were determined from the rate of sedimentation of the midpoint of the substrate depletion boundary (Taylor et al., 1972; Kemper & Everse, 1973) or the midpoints of the difference curves constructed from successive sedimentation boundaries (Cohen & Mire, 1971; Taylor et al., 1972). Values of s_{obsd} calculated by the two procedures

agreed within ±0.5 S. In some experiments where very low enzyme concentrations were employed, the trailing plateau that should be observed behind the sedimenting enzyme protein failed to form. In these experiments, s_{obsd} was calculated by the method of difference curves. In all cases, the values of s_{obsd} were corrected for the densities and viscosities of the reaction mixtures. The correction factor was 1.22. No correction was made for the increase in the buoyancy term, since the correction (+0.2 S) was within the error inherent in the active enzyme centrifugation method (±0.5 S) (Cohen et al., 1967). Even a 10% underestimation of the sedimentation coefficient would not change the interpretation of the experiments.

The densities and viscosities of the solvents and reaction mixtures were determined experimentally at 25.0 ± 0.05 °C with a Model DMA 02 C densitometer (Anton Paar, Graz). The viscosities of the solvents relative to water were measured at 25.0 °C in 5-mL Ostwald viscometers.

Electron Microscopy. Carbamoyl-phosphate synthetase was desalted just prior to the electron microscope experiments by Sephadex centrifugation. Samples of the enzyme (1 mg/mL) in Tris–acetate, pH 7.6, $\Gamma/2 = 0.05$, 2 mM dithioerythritol, and 0.5% glycerol were diluted to final concentrations of 10–100 μg/mL with the desired solvents. The enzyme preparations were immediately applied by the drop method (Horne, 1967; Haschemeyer, 1970) to thin carbon films on 400-mesh copper grids. Samples (5–10 μL) of the enzyme were placed on the grids with a micropipet and allowed to stand for 1 min. The excess was drained to a thin film by touching with a wetted tissue. A drop (5 μL) of 0.3% phosphotungstic acid (titrated to pH 7 with KOH) was added to the grid and allowed to stand for 2 min. The excess stain was drained as above, and the grid was allowed to air-day. Several other negative stains (uranyl acetate, sodium silicotungstate, and ammonium molybdate) were tried, but the best contrast was achieved with potassium phosphotungstate. The underlying assumption of the staining procedure used is that the molecules undergo minimal morphological changes after the initial absorption to the carbon film (Haschemeyers, 1970). In some experiments, a monolayer of octanol was put on the surface of the stain droplet to improve spreading and deposition of the stain (Gordon, 1972). About 0.5 μL of a solution of octanol in hexane (0.015% w/v) was delivered to the surface of the stain from a Hamilton microsyringe. Negatively stained preparations of carbamoyl-phosphate synthetase were examined with a Phillips EM 300 electron microscope equipped with an anticontamination device and operated with a 30-μm objective aperture at an accelerating voltage of 80 kV. Images were recorded for 2 s on Kodak Electron Image Film (No. 4463) in a through-focus series at an instrumental magnification of 40 500–52 000. Although the photographs taken in the near-focal region had less contrast, the granularity of the phase image was reduced to about 10 Å (Haschemeyer, 1970). The magnification of the microscope was calibrated with a carbon grating replica (54 864 lines/in.). Measurements of the negatively stained particles were taken from photographic enlargements of the electron micrographs by using a Bausch and Lomb 7× magnifying glass calibrated in divisions of 0.1 mm.

Results

Sedimentation Velocity Measurements of Carbamoyl-Phosphate Synthetase. The molecular weight of native carbamoyl-phosphate synthetase is dependent on the protein concentration and on the composition of the solvent. Sedimentation velocity measurements of carbamoyl-phosphate synthetase were made at 25 °C in Tris–acetate, pH 7.6, $\Gamma/2 = 0.1$, which contained 2 mM dithioerythritol. A single sedimentation boundary was observed in all of the experiments. As the initial concentration was increased, the boundary region narrowed and the boundary curves became sharper, a feature characteristic of concentration-dependent sedimentation (Vinograd & Bruner, 1966a). The calculated sedimentation coefficients (Figure 1) rose steeply with initial concentration, passed through a maximum at 0.3 mg/mL, and then decreased with increasing concentration as a consequence of hydrodynamic effects. The marked concentration dependence of the sedimentation coefficients of the enzyme in the range 0.02–0.3 mg/mL was not observed in previous studies (Virden, 1972), where all the measurements were made at concentrations of carbamoyl-phosphate synthetase above 1 mg/mL. In the concentration range of 1–1.5 mg/mL, the sedimentation coefficients determined in the present study are in excellent agreement with those previously reported (Virden, 1972) (see Figure 1). The concentration dependence of the sedimentation coefficients and the single, integral sedimentation boundaries are in accordance with the behavior of a monomer–dimer system in rapid chemical equilibrium predicted by the Gilbert theory (Gilbert, 1955, 1959). The distinctive shape of the plot of sedimentation coefficient vs. concentration is also characteristic of association–dissociation (Gilbert, 1960; Gilbert & Gilbert, 1961). The sedimentation coefficient of the monomeric molecule can be obtained by extrapolation of the downward part of the curve to zero concentration. Since the height of the maximum is a function of kg, the product of the protein dissociation constant (k) and the hydrodynamic constant [here defined as g, cf. Gilbert (1955, 1959, 1960) and Gilbert & Gilbert (1961)], extrapolation of the ascending part of the curve to zero concentration does not give the sedimentation coefficient of the dimer but rather gives an estimate of the sedimentation velocity somewhere between the monomer and dimer (Gilbert, 1960; Gilbert & Gilbert, 1961). When the observed sedimentation coefficients in Figure 1 are extrapolated to zero concentration, a value of $s^0_{20,w}$ of 7.65 ± 0.1 is obtained for the monomer. With the assumption that the monomer is an anhydrated sphere and that the dimer is a prolate ellipsoid with a volume equal to twice that of the monomer, and a major semiaxis equal to the diameter of the monomer, the sedimentation coefficient of the dimer was estimated from the relation $s_P = s_M n^{2/3}/1.044$ (Gilbert, 1959) taking into account the changes in weight ($2^{2/3}$) and shape

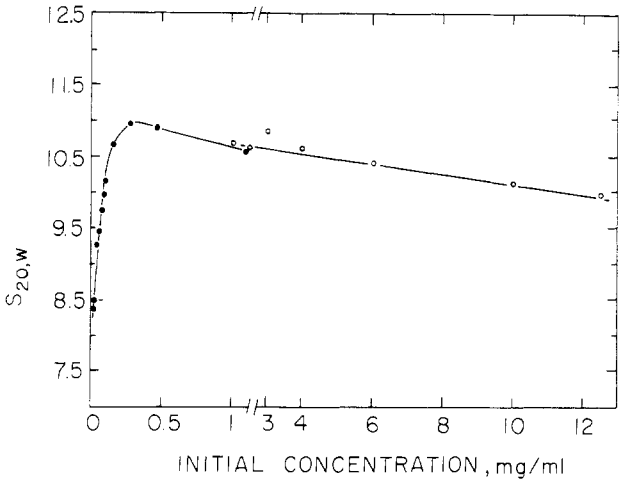

FIGURE 1: Sedimentation velocity of carbamoyl-phosphate synthetase in Tris–acetate, pH 7.6, ionic strength 0.1, containing 2 mM dithioerythritol at 25 °C. The sedimentation coefficients represented by (○) are taken from the data of Virden (1972).

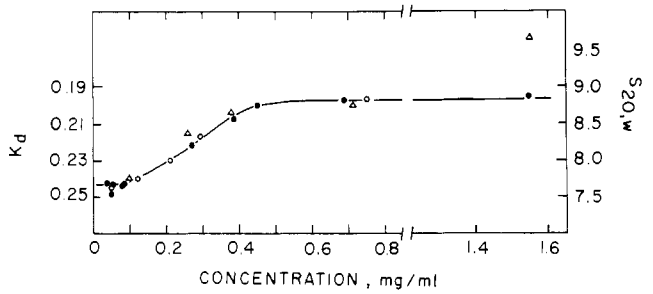

FIGURE 2: Concentration dependence of K_d of carbamoyl-phosphate synthetase in the presence of substrates at 25 °C. The enzyme (1.7 mL) was chromatographed in the presence of Tris–acetate, pH 7.6, $\Gamma/2 = 0.05$, 2 or 20 mM ATP, 7 or 25 mM magnesium acetate, 50 mM NH_4HCO_3, 3 mM sodium acetylglutamate, 2 mM dithioerythritol, and 0.05% sodium azide. Concentration is the concentration measured in the peak maximum: (●) 2 mM ATP; (○) 20 mM ATP. (△) Sedimentation coefficients of the enzyme measured by boundary sedimentation in the same reaction mixture (2 mM ATP) at 25 °C. In these experiments, concentration refers to initial concentration. The details of gel chromatography and centrifugation are given under Experimental Procedures.

[1/1.044] (Perrin, 1936) of the monomer and dimer. A value of 11.6 ± 0.15 S is calculated for the dimer. The earlier reported value of 10.8 S (Virden, 1972) for the dimer is probably an underestimate. The actual determination of an accurate value of the sedimentation coefficient for the carbamoyl-phosphate synthetase dimer presents a problem, since it can be calculated that after correction for hydrodynamic effects (Virden, 1972), even at an initial concentration of 30 mg/mL, the rat liver enzyme is not completely polymerized. Attempts to maximize dimer formation or to stabilize the dimer by the addition of high concentrations of salts (e.g., ammonium sulfate plus sodium acetate) resulted in the formation of oligomers ($n \geq 4$) (Lusty, 1978a). However, the evidence for a chemical equilibrium between monomer and dimer is rather compelling. Simple dilution of the enzyme from a concentration of 0.25 to 0.025 mg/mL resulted in a decrease in the sedimentation coefficient from 10.8 to 8.3 S. Only a single boundary without any suggestion of a shoulder or inflexion is observed in both boundary sedimentation and gel elution patterns over a wide range of protein concentrations. Furthermore, the equilibrium can be perturbed by substrates and the allosteric activator, *N*-acetylglutamate.

Influence of Substrates and N-Acetylglutamate on Monomer–Dimer Transitions. The apparent molecular weight of carbamoyl-phosphate synthetase was determined at 25 °C in the presence of substrates by molecular sieve chromatography and sedimentation velocity measurements. The concentration dependence of the partition coefficient, K_d, of the enzyme in the presence of saturating amounts of acetylglutamate, NH_4HCO_3, and 2 or 20 mM $MgATP^{2-}$ is shown in Figure 2. Also shown are the sedimentation coefficients of the protein, determined under the same experimental conditions by boundary sedimentation. In the gel chromatography experiments, concentration refers to milligrams per milliliter of protein in the peak. At concentrations of less than 0.1 mg/mL in the peak, dissociation appeared to be complete. Extrapolation of the measured K_d to zero concentration provides a value of K_d from which a Stokes radius for the monomer of 48.0 ± 0.8 Å was calculated. By use of the Stokes–Einstein equation, the value of $D^0_{20,w}$ calculated for the monomer from the Stokes radius is $(4.46 \pm 0.07) \times 10^{-7}$ cm^{-2} s^{-1}.

At low protein concentrations (<0.1 mg/mL), the enzyme eluted as a nearly symmetrical peak. As the initial concentration was increased, the leading edge of the peak became steeper, and the trailing side was slightly skewed, indicating that the equilibrium was displaced toward dissociation as the protein moved down the column. Since the experiments were carried out with small (1.7 mL) sample volumes relative to the column volume, the elution volume (V_e) is not a quantitative weight average of the monomer and dimer in the peak. Thus, at high concentrations, K_d deviates from the line described by the sedimentation coefficients. Although the data do not allow estimation of a dissociation constant for the monomer–dimer interconversion, the concentration dependence of K_d is consistent with a reversible equilibrium and indicates that the addition of substrates displaces the equilibrium in favor of monomer. This conclusion is supported by the sedimentation data presented in Figures 1 and 2. Comparison of the curves of sedimentation coefficient as a function of concentration in the presence and absence of substrates shows that the addition of substrates leads to dissociation and results in a lower maximum and a change in the shape of the curve. For example, at a concentration of 0.10 mg/mL, the sedimentation coefficient of the enzyme in the presence of substrates is 7.8 S compared to a value of 10.1 S determined in the absence of substrates.

In order to correlate the physical size of carbamoyl-phosphate synthetase with catalytic activity, and to determine the effects of substrates on the degree of association of the enzyme under conditions approximating those of the usual enzyme assay, the molecular weight determinations were made at low protein concentrations (<100 μg) in the presence of various substrates and cofactors. Apparent molecular weights were calculated from the Stokes radii and sedimentation coefficients also determined under the same experimental conditions by boundary centrifugation. The results of the experiments are presented in Table I. The protein concentrations reported in the table are those measured in the peak maxima and correspond to the initial concentrations used for sedimentation analysis. In the absence of substrates, the enzyme behaved as an equilibrium mixture with apparent molecular weights of 250000 and 195000 at protein concentrations of 0.075 and 0.018 mg/mL, respectively. The enzyme was completely dissociated to the monomer in the presence of acetylglutamate alone. Further additions of ATP, Mg^{2+}, and NH_4HCO_3 had little effect on the size and physical properties of the enzyme. Increasing the concentration of $MgATP^{2-}$ up to 20 mM did not reverse the dissociation. When acetylglutamate was omitted, the addition of only the substrates ATP, Mg^{2+}, and NH_4HCO_3 resulted in a partial depolymerization as evidenced by a decrease in the molecular weight of the enzyme to that of a mixture with an apparent molecular weight of about 206000. Glycerol up to 20% did not stabilize the dimer form of the enzyme. Unlike the bacterial carbamoyl-phosphate synthetase (Matthews & Anderson, 1972; Abdelal & Ingraham, 1975; Trotta et al., 1974), whose polymerization is induced by ornithine and potassium phosphate, this effect was not seen with the rat liver enzyme at a concentration of 0.1 mg/mL.

Frictional coefficients for the monomer were calculated from the average Stokes radii. The value of 1.33 obtained is in the range of other globular proteins. There was no substantial effect of temperature on the elution volume of carbamoyl-phosphate synthetase in solutions of Tris–acetate, pH 7.6, $\Gamma/2 = 0.1$, containing 2 mM dithioerythritol; the Stokes radius determined at 4 °C was the same as that measured at 25 °C.

Size of the Catalytic Unit. The finding that at concentrations less than 0.1 mg/mL, the Stokes radius of the enzyme chromatographed in the presence of substrates is the same as that of monomeric carbamoyl-phosphate synthetase suggested

Table I: Stokes Radii ($\bar{a}$) and Molecular Weight of Carbamoyl-Phosphate Synthetase in the Presence of Various Salts and Substrates at 25 °C[a]

additions to the elution buffer	concn (mg/mL)	f/f_o	$\bar{a}$ (Å)	$s_{20,w}$ (S)	$\bar{M}_{w,app}$
none	0.075	1.42	59.4	9.70 ± 0.08	251 000 ± 2 000
	0.018	1.39	53.4	8.4 ± 0.1	195 000 ± 2 000
3 mM acetylglutamate	0.062		48.4		
	0.070		47.4		
	0.100	1.33	48.6	7.67 ± 0.33	160 500 ± 8 000[b]
3 mM acetylglutamate, 2 mM ATP, 7 mM Mg^{2+}, and 50 mM NH_4HCO_3	0.105		47.4	7.78 ± 0.33	162 000 ± 7 000[b]
	0.133	1.33	48.3		
	0.050		47.9		
	0.038		48.7		
3 mM acetylglutamate, 20 mM ATP, 25 mM Mg^{2+}, and 50 mM NH_4HCO_3	0.050		48.5		
	0.085		48.5		
2 mM ATP, 7 mM Mg^{2+}, and 50 mM NH_4HCO_3	0.105	1.38	54.0[c]	8.78 ± 0.44	206 000 ± 10 000
		1.32	50.7		194 000 ± 10 000
20% glycerol	0.10	1.48	59.4 ± 1	8.45 ± 0.21	218 000 ± 6 400
3 mM acetylglutamate, 2 mM ATP, 7 mM Mg^{2+}, 50 mM NH_4HCO_3, and 20% glycerol	0.090	1.35	50.3	8.07 ± 0.34	176 000 ± 7 000
0.1 M potassium phosphate, pH 7.6, and 10 mM ornithine	0.09	1.39	58.1	9.77 ± 0.06	247 000 ± 2 000

[a] The composition of the elution buffer was Tris-acetate, pH 7.6, $\Gamma/2 = 0.05$, 2 or 5 mM dithioerythritol, and 0.05% sodium azide. The sedimentation coefficients were determined by boundary centrifugation in 12- or 30-mm double-sector centerpieces. The protein concentration observed in the peak after elution was used in the corresponding sedimentation analysis. The s values given are the mean ± root mean square of the standard deviation obtained from least-squares analyses of three to five determinations. The apparent molecular weight was calculated from the equation $M_w = 6\pi Nas/(1 - \bar{v}\rho)$. The frictional coefficient, f/f_o, was calculated from the equation $f/f_o = a/[3\bar{v}M_{w,app}/(4\pi N)]^{1/3}$. [b] The mean value of a obtained from the experiments shown was used to calculate $\bar{M}_{w,app}$. [c] In this experiment, the peaks of protein and enzyme activity were not coincident. The values of 54.0 and 50.7 Å were calculated from the K_d of the protein and enzyme activity peaks, respectively.

that the monomer is catalytically active. This was verified by analyzing the elution boundary of the ADP formed during gel chromatography of the enzyme in the presence of all of the substrates, as described under Experimental Procedures. The sample (0.050 mg/mL) was applied to the column in a volume of 100 mL, so that the protein eluted as a broad band with a concentration in the plateau equal to the initial concentration. The elution profile in Figure 3 shows that the elution boundaries of the protein were nearly symmetrical. In gel chromatography when a large sample volume is used to maintain a plateau concentration, the midpoints (corresponding to V_e) of the leading and trailing boundaries correspond to the weight average of the protein in the plateau, and plots of the first derivative of the trailing boundary are comparable to patterns obtained by sedimentation analysis (Winzor & Scheraga, 1963). Plots of the first derivatives of the boundaries show that the trailing boundary is slightly more diffuse than the leading boundary (Figure 3), indicating that the protein is not associating (Winzor & Scheraga, 1963). The partition coefficients, K_d, obtained from the elution volumes of the leading and trailing boundaries of the protein correspond to Stokes radii of 48.4 Å, the same as that of the monomer. Figure 3 also shows the leading boundary of the ADP formed on the column, and a plot of its first derivative. The midpoint of the ADP boundary corresponds precisely to the midpoint of the protein boundary. It can be calculated that the amount of ADP formed on the column during chromatography is the amount expected, assuming the enzyme to be fully active. The coincidence of the ADP and protein boundaries indicated that the monomer is enzymatically active.

The gel chromatographic method described here is a useful method to determine the size of catalytically active species, since the theoretical basis is the same as that of active enzyme centrifugation (Claverie, 1976). The gel filtration method has the advantage that the leading boundary of the protein and

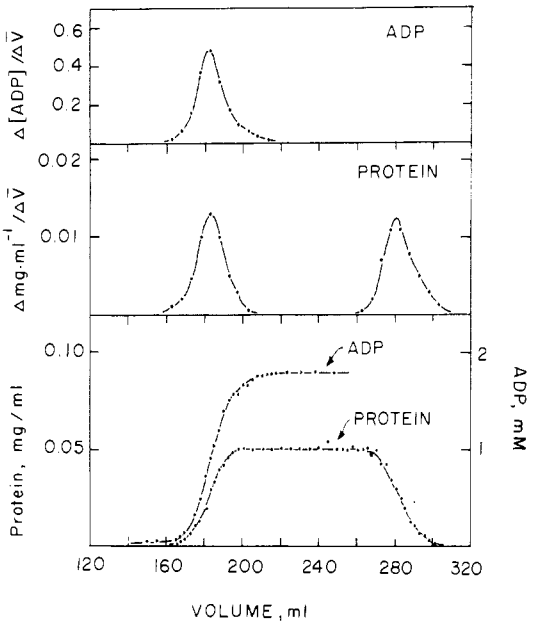

FIGURE 3: Gel chromatography of carbamoyl-phosphate synthetase in the presence of substrates. Analysis of ADP boundary formed during chromatography. The enzyme (4.8 mg) in 100 mL of a buffer containing Tris-acetate, pH 7.6, $\Gamma/2 = 0.05$, 2 mM ATP, 7 mM magnesium acetate, 3 mM sodium acetylglutamate, 50 mM NH_4HCO_3, 2 mM dithioerythritol, and 0.05% sodium azide was applied to a column (1.6 × 170 cm) of Sephadex G-200, equilibrated with the same buffer. The column was developed at 25 °C at a flow rate of 12.2 mL/h, as described under Experimental Procedures. Aliquots of the fractions were analyzed for ADP and protein as described under Experimental Procedures. All of the protein applied to the column was recovered in the fractions corresponding to the peak. The abscissa in the two *upper panels* is $\bar{V}$.

the leading boundary of the products of the reaction formed during chromatography are obtained in the same analysis. A

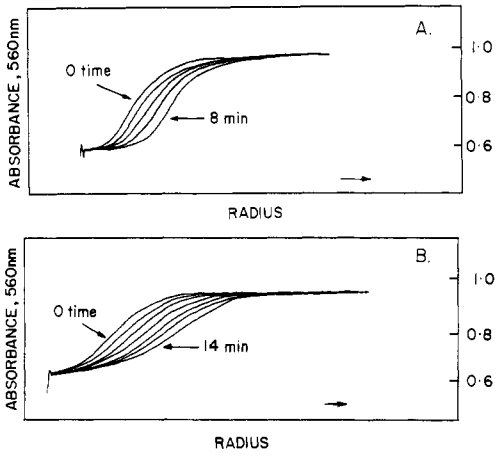

FIGURE 4: Tracings of a series of absorbance scans taken during active enzyme sedimentation of carbamoyl-phosphate synthetase. (A) The decrease in absorbance at 560 nm of phenol red was used to observe H^+ production during overall carbamoyl-phosphate synthesis. The enzyme (2.34 μg) in a volume of 5 μL was sedimented through a solution column containing 5 mM ATP, 10 mM magnesium acetate, 20 mM potassium acetate, 5 mM sodium acetylglutamate, 20 mM NH_4HCO_3, 3 mM dithioerythritol, and 25 μM phenol red at 60 000 rpm, at 25 °C. (B) H^+ production observed during bicarbonate-dependent ATP hydrolysis in the absence of ammonia. The enzyme (22.7 μg) in a volume of 5 μL was layered on a sedimentation solution column containing 10% glycerol, 10 mM ATP, 10 mM manganese acetate, 5 mM sodium acetylglutamate, 10 mM $KHCO_3$, 30 mM potassium acetate, 3 mM dithioerythritol, and 25 μM phenol red and centrifuged at 60 000 rpm, at 25 °C. Absorbance scans at 560 nm were taken at 1-min intervals; other conditions were scan speed, fastest; chart speed, 25 mm/s; suppression, $^1/_4$; 100 mV/cm; 0–1.0 optical density range. Successive scanner traces are superimposed on the zero time scanner trace. The interval between traces is 2 min because alternate traces have been omitted. The arrow indicates the direction of sedimentation.

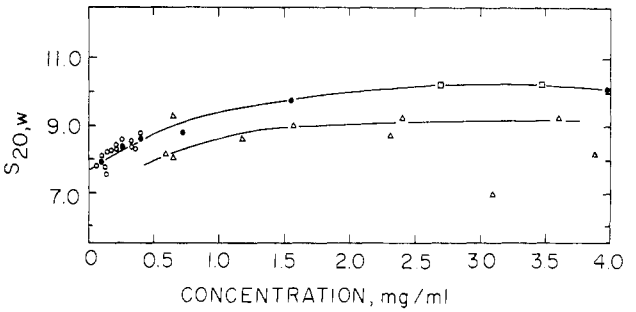

FIGURE 5: Concentration dependence of the sedimentation velocity of carbamoyl-phosphate synthetase in the presence of substrates at 25 °C. Concentration dependence of the sedimentation velocity of the reacting species was determined by active enzyme centrifugation. (●) Sedimentation coefficients determined by boundary sedimentation of the enzyme in Tris–acetate, pH 7.6, $\Gamma/2 = 0.05$, 3 mM sodium acetylglutamate, 2 mM ATP, 7 mM magnesium acetate, 50 mM NH_4HCO_3, and 2 mM dithioerythritol. (□) Boundary sedimentation experiments showing the sedimentation velocity of the enzyme in Tris–acetate, pH 7.6, $\Gamma/2 = 0.05$, 5 mM sodium acetylglutamate, 2 mM ATP, 1.8 mM manganese acetate, 20 mM $KHCO_3$, 10 mM potassium acetate, and 2 mM dithioerythritol. Concentration refers to initial concentration. (○) Sedimentation velocity of the active species determined by measuring H^+ production during carbamoyl phosphate synthesis. The enzyme (2–7 μg in 5 μL) was layered onto solution columns containing 10% glycerol, 1 mM Tris–acetate, pH 7.8, 4–20 mM ATP, 9–25 mM magnesium acetate, 5 mM sodium acetylglutamate, 10–40 mM NH_4HCO_3, 2 mM dithioerythritol, and 25 μM phenol red. (△) Sedimentation velocity of the active species determined by measuring H^+ production during acetylglutamate- and bicarbonate-dependent ATP hydrolysis in the absence of ammonia. The enzyme (7–80 μg in 5 μL) was layered onto solution columns containing 10% glycerol, 1 mM Tris–acetate, pH 7.8, 10 or 20 mM ATP, 9 or 18 mM manganese acetate, 20 mM $KHCO_3$, 10 mM potassium acetate, 5 mM sodium acetylglutamate, 3 mM dithioerythritol, and 25 μM phenol red. The pH of the reaction mixtures was 7.8. In the active enzyme centrifugation experiments, concentration is the concentration (0.3 c_i) calculated to be in the band maximum midway through the experiment ($t = 5$ min). The conditions of centrifugation was as described in the legend of Figure 4.

disadvantage of the method is that the product distribution in the trailing boundary is obscured due to sieving, and analysis of the first derivative of the trailing boundary to look for evidence of more than one reacting species would probably not be informative. Despite this drawback, evidence for interactions can still be obtained from analysis of the trailing boundary of the protein peak.

Active Enzyme Sedimentation. The studies on the active form of carbamoyl-phosphate synthetase with the gel chromatographic method had the disadvantage that 11–12 h were required before catalytic activity could be assayed. It was therefore important to demonstrate the catalytically active monomer in experiments of short duration which more nearly approximate the conditions of a normal enzyme assay. Moreover, it was of interest to determine whether the monomer exists at enzyme concentrations approaching those of rat liver mitochondria (Clarke, 1976; Lusty, 1978a; Raijman & Jones, 1976) and whether the dimer is also enzymatically active. This was attempted by active enzyme centrifugation studies in which the active enzyme boundary was observed within a few minutes following contact of the enzyme with the reaction mixture. The sedimentation of carbamoyl-phosphate synthetase could be followed by measuring H^+ production resulting from ATP utilization either in net carbamoyl phosphate synthesis or in the acetylglutamate- and bicarbonate-dependent ATPase. The latter partial reaction is especially useful since it allows the sedimentation properties of the active enzyme to be estimated over a wide range of initial protein concentrations. In the first experiments, the sedimentation coefficients of the active species were determined by following H^+ production during carbamoyl phosphate formation. For technical reasons, this proved difficult. The changes in the absorbance at 560 nm were proportional to the amount of enzyme added only over a narrow range of 2–6 μg of protein. Increasing the amount of protein led to spuriously high sedimentation coefficients. This was due to depletion of substrates by the leading edge of the sedimenting enzyme band, as seen when tracings of the sedimenting band (recorded at 280 nm) were laid over the substrate–product boundaries. Substrate buffering effects set a lower limit on the amount of enzyme that could produce a change in absorbance during sedimentation. The substrate–product boundaries of sedimenting bands of enzyme, 2.3 μg in the overall reaction and 23 μg in the acetylglutamate- and bicarbonate-dependent ATPase, are shown in Figure 4. The sedimentation coefficients of the active enzyme species calculated from the midpoints of the sedimenting boundaries are 8.7 and 8.6 ± 0.25 S, respectively. The concentration dependence of the sedimentation coefficients of carbamoyl-phosphate synthetase measured by active enzyme sedimentation is summarized in Figure 5. The *upper curve* shows the sedimentation coefficients of the enzyme determined by boundary sedimentation as a function of initial concentration. The *open circles* represent the sedimentation coefficients of the enzyme determined by active enzyme centrifugation by measuring H^+ produced during carbamoyl phosphate synthesis. Within the concentration range 2–6 μg of protein, all of the sedimentation values fell on the line described by boundary sedimentation. In the active enzyme sedimentation experiments, concentration refers to the average protein concentration in the sedimenting band, which was taken to be 0.3 of the initial concentration. This factor was determined from the area of the sedimenting band

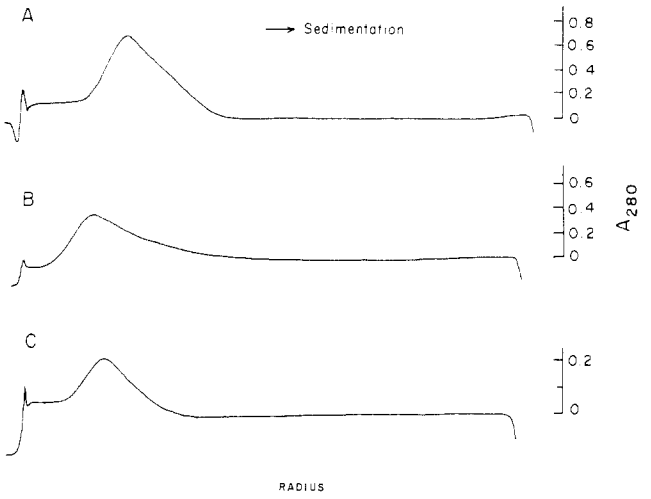

FIGURE 6: Tracings of absorbance scans at 280 nm taken during band sedimentation of carbamoyl-phosphate synthetase in 10% glycerol in the presence and absence of substrates. (A) Without added substrates. The enzyme (28 μg) in a volume of 15 μL was sedimented through a solution column containing 20% glycerol, Tris–acetate, pH 7.8, $\Gamma/2$ = 0.1, and 1 mM dithioerythritol. The absorbance at 280 nm was recorded after sedimentation at 56 000 rpm, at 25 °C, for 10 min. (B) With added substrates. The enzyme (15.4 μg) in a volume of 5 μL was centrifuged through a solution column containing 10% glycerol, 20 mM NH_4HCO_3, 20 mM potassium acetate, 25 mM magnesium acetate, 5 mM sodium acetylglutamate, and 1 mM dithioerythritol. The absorbance scan at 280 nm was recorded after sedimentation at 60 000 rpm, at 25 °C, for 9 min. (C) The enzyme (6.6 μg) was centrifuged exactly as described in (B). The absorbance scan was recorded after centrifugation at 60 000 rpm for 8 min. Sedimentation is from *left* to *right*.

measured on scanner traces (midway through the run) and corresponds to the value given by Vinograd & Bruner (1966b). The curvature of the plots in Figure 5, however, was not significantly affected by the precise value of the dilution factor within the range 0.2–0.4 c_i. The *lower curve* in Figure 5 shows the concentration dependence of the sedimentation coefficients determined by active enzyme centrifugation with the ATPase assay.

The shape of the sedimenting boundaries (Figure 6) in the band sedimentation experiments indicated that sedimentation was concentration dependent. As the band moves, dilution of the leading edge causes the maximum to move more slowly than the leading edge, which gives appearance of broadened leading and sharpened trailing boundaries (Vinograd & Bruner, 1966a). Depending on the magnitude of the product of the hydrodynamic constant and the concentration in the band maximum, gc_m, it was found that the sedimentation velocity of the maximum will be lower than the actual sedimentation coefficient (Vinograd & Bruner, 1966a). This effect could account for the disparity in the sedimentation coefficients determined by band sedimentation and boundary sedimentation shown in the two curves in Figure 5. The sedimentation data in Figure 5 suggest that the reacting species sediments with the same concentration dependence as the protein measured by moving boundary sedimentation. Measured during carbamoyl phosphate synthesis, the active species sedimented with a velocity of 7.8 S at a concentration of 0.12 mg/mL, and 9.3 S at a concentration of 0.36 mg/mL. Extrapolation of the sedimentation coefficients to zero concentration gives a $s^0_{20,w}$ value of 7.6, which corresponds to the sedimentation coefficient of the monomer. The concentration dependence of the sedimentation coefficients of the active species measured during the ATPase reaction also corresponds closely to those determined by boundary sedimentation in the concentration range 0.6–5 mg/mL. The increase in the sedimentation coefficient of the active enzyme species over the concentration range 0.1–4 mg/mL is consistent with concentration-dependent sedimentation of catalytically active monomers in equilibrium with dimers. Dissociation of the enzyme in the presence of substrates appears to be rapid and reversible,[2] since single sedimenting boundaries were observed in both boundary sedimentation and band sedimentation experiments. By analysis of the sedimenting substrate–product boundaries obtained by active enzyme centrifugation, it should be possible to obtain some information about the enzymatic activity of the dimer as well as the monomer. By consideration of the most likely situation, that of a reacting monomer–reacting dimer, if the equilibrium is rapid relative to the rate of sedimentation, a single sedimenting substrate–product boundary will be observed; on the other hand, if the equilibrium is slow relative to sedimentation, two separate moving boundaries should be observed. The invisible sedimenting monomer and dimer species will be evident from difference curves constructed from the product boundaries (Cohen & Claverie, 1975). In the case of a reacting monomer–inactive dimer, only a single substrate–product boundary will be observed, but this boundary will exhibit a long leading edge, indicating the presence of an invisible (inactive) and fast sedimenting component. Unless the activities of the monomer and dimer differed significantly, the case active monomer–less active dimer would be difficult to distinguish in these experiments.

Inspection of the sedimenting product boundaries in Figure 4 shows that H^+ production in both the overall reaction and in the ATPase generates single, integral boundaries with no hint of a shoulder or inflexion. Difference curves constructed from the boundary in Figure 4A (0.12 mg/mL in the band maximum, at a time corresponding to the midpoint of the run) were nearly symmetrical and fell to the base line without evidence of inflexion or trailing. Difference curves constructed from the sedimenting boundary in the ATPase reaction (1.38 mg/mL in the band maximum) were similar to the shape of the sedimenting band shown in Figure 6. The trailing boundary was sharpened in the same way, and spreading of the leading boundary was observed, as a consequence of changing s during sedimentation. The concentration dependence of the sedimenting boundaries makes interpretation difficult, but, at the same time, it is clear that the sedimentation of the active species accurately reflects the sedimentation of the total protein. Thus, the results are consistent with an enzymatically active dimer, but, since we have been unable to find conditions to stabilize the dimeric form, this must remain a tentative conclusion.

Electron Microscopy. Electron microscopy studies were undertaken to directly observe the stoichiometry of association of carbamoyl-phosphate synthetase in the presence and absence of acetylglutamate and substrates, and to obtain some information about the size and geometry of the subunits. Micrographs of negatively stained preparations of the enzyme (25 μg/mL) in Tris–acetate, pH 7.6, $\Gamma/2$ = 0.05, and 1 mM dithioerythritol (with and without substrates) showed images of molecules of two distinctly different sizes, which correspond in dimensions to monomers and dimers. The photograph (Figure 7) shows carbamoyl-phosphate synthetase in the presence of added substrates. It appears that the monomer is asymmetric, with average dimensions of 69 ± 8 Å along the minor axis and 80 ± 7 Å along the major axis. The average dimensions of the dimers were 72 ± 12 and 122 ± 22 Å along the minor and major axes, respectively. The axial ratios of

[2] The sedimentation experiments were of short duration (10–20 min), so that irreversible denaturation of the enzyme was not a problem.

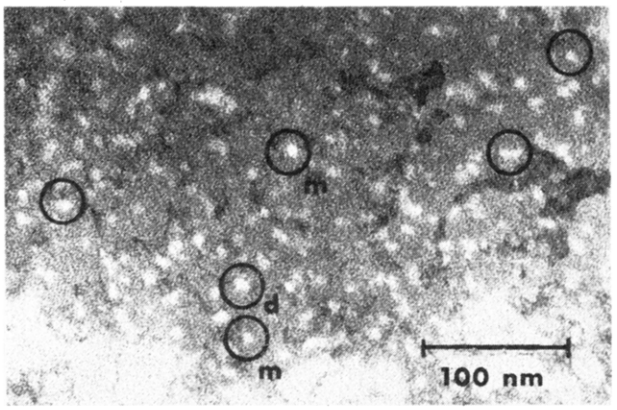

FIGURE 7: Electron microscope images of rat liver carbamoyl-phosphate synthetase (25 μg/mL) in Tris–acetate, pH 7.6, Γ/2 = 0.05, 3 mM acetylglutamate, 10 mM ATP, 15 mM magnesium acetate, 50 mM NH_4HCO_3, 2 mM dithioerythritol, and 0.5% glycerol. The preparation was negatively stained with phosphotungstate. Micrograph shows isolated molecules corresponding in dimensions to monomers and dimers, 187 200×.

monomers and dimers of the given dimensions are 1.16 and 1.69, respectively. In addition to monomers and dimers, occasional particles measuring 69 × 180–210 Å were seen. These were distinctly larger than monomers and dimers. The larger particles did not appear to be artifactual aggregates on the grids. Particles with dimensions larger than these of monomers and dimers were also observed when the enzyme was diluted in buffer containing 1 M ammonium sulfate. Images of these particles measured 125 × 172 Å; no substructure was discernible. Other larger structures were also seen. Particles of the enzyme dissociated with acetylglutamate in the absence of other substrates possessed elongations giving the appearance of tails. Small 50-Å particles appeared to be end-on views. These shapes, however, were too indistinct to contribute much information about the structure of the enzyme. It could be that monomers dissociated with acetylglutamate alone undergo significant changes in size and shape.

The molecular weights of the monomer and dimer correlate reasonably well with the dimensions of the negatively stained particles. The exact shape of carbamoyl-phosphate synthetase cannot be discerned in the micrographs. With the assumption that the enzyme is an ellipsoid with dimensions of 69 × 80 Å, molecular weights of 162 000 and 188 000 are calculated for prolate and oblate ellipses, respectively. The diameter of an approximately spherical molecule with a molecular weight of 160 000 and a partial specific volume of 0.74 would be nearly 72 Å. This value is close to the average diameter of the 69 × 80 Å particle. Molecular weights of 269 000 and 456 000 are obtained from the dimensions of the dimer, assuming prolate and oblate ellipses, respectively. The latter particle weight is much larger than that found by physical measurements, which implies that the dimers are prolate ellipsoids.

The average molecular weight of the enzyme can be calculated from the numbers of particles seen in negatively stained preparations of carbamoyl-phosphate synthetase (Table II). Since in the absence of substrates dissociation to monomers occurs by dilution (Table I, Figure 1), it is not possible to make a conclusion about the effect of substrates on dissociation of the enzyme. In all of the micrographs examined, the monomer was predominant. Direct visualization of the enzyme in the electron microscope was limited by the amount of enzyme that could be applied to the grid. At concentrations of protein greater than 100 μg/mL, fields contained so many molecules

Table II: Apparent Number-Average Molecular Weight of Carbamoyl-Phosphate Synthetase Calculated from the Number of Monomers and Dimers Observed in Electron Micrographs[a]

conditions	no. of particles	dimensions (Å) 60–80	dimensions (Å) 120–160	$\bar{M}_{n,app}$
without substrates				
25 μg/mL	60[b]	47	12	196 000
100 μg/mL	104	82	22	203 000
with substrates				
25 μg/mL	121	104	17	182 500
100 μg/mL	146	118	28	191 000

[a] The enzyme was diluted in Tris–acetate, pH 7.6, Γ/2 = 0.05, containing 2 mM dithioerythritol with or without substrates (10 mM ATP, 15 mM magnesium acetate, 3 mM sodium acetylglutamate, and 50 mM NH_4HCO_3) to the concentrations indicated in the table. Specimens were prepared for electron microscopy and stained with 0.3% potassium phosphotungstate as described under Experimental Procedures. [b] In this experiment, one large particle (69 × 222 Å) was also observed and has been included in the calculations.

that images could not be identified as individual monomers or dimers. Under the conditions of the experiments in the table, which favor monomer formation, the molecular weights calculated from the numbers of monomers and dimers in a representative field are in accord with the results of ultracentrifugation, gel filtration, and active enzyme centrifugation. At a concentration of 25 μg/mL in the presence of substrates, the average molecular weight of the enzyme was 182 500. The number of monomers represented 86% of the total number of enzyme molecules. The remaining 14% were dimers. Thus, electron microscope studies provide independent evidence, apart from physical studies, to support the idea that the enzyme exists as a mixture of monomers and dimers and that the enzyme is predominantly in monomer form at low protein concentrations.

Discussion

In the studies described in the present paper, it is shown that at 25 °C in Tris–acetate buffers rat liver carbamoyl-phosphate synthetase consists of monomers and dimers in rapid equilibrium. In the concentration range of 20–250 μg/mL, the equilibrium is perturbed by slight differences in the composition of the solvent. Addition of the positive allosteric effector *N*-acetylglutamate causes carbamoyl-phosphate synthetase to dissociate under the conditions normally used in the kinetic assay. The resultant monomer form of the enzyme is catalytially active. In the present studies, even at high initial concentrations (2–5 mg/mL) of protein, the presence of saturating concentrations of acetylglutamate favors monomer formation. Even though these concentrations are still below the concentrations (80–160 mg/mL) calculated to exist in the mitochondria (Clarke, 1976; Lusty, 1978a; Raijman & Jones, 1976), they are high enough to suggest that the monomer form could exist in vivo. Dissociation if it occurs in vivo would be limited by the intramitochondrial concentration of acetylglutamate, which is $\sim 10^{-4}$ M (Shigesada & Tatibama, 1971).

Active enzyme centrifugation analyses also indicated that the dimer is catalytically active. Although attempts were made to estimate the specific activity of the dimer relative to that of the monomer, this proved technically difficult. In the range 0.002–1 mg/mL, increasing the protein concentration resulted in only a small (15–20%) decrease in the specific activity of the enzyme. It is possible to state, however, that the monomer is maximally active. It is of interest that the glutamine-requiring carbamoyl-phosphate synthetase of *Escherichia coli* has also been reported to be catalytically active as both a monomer and an oligomer (Anderson, 1977; Powers et al.,

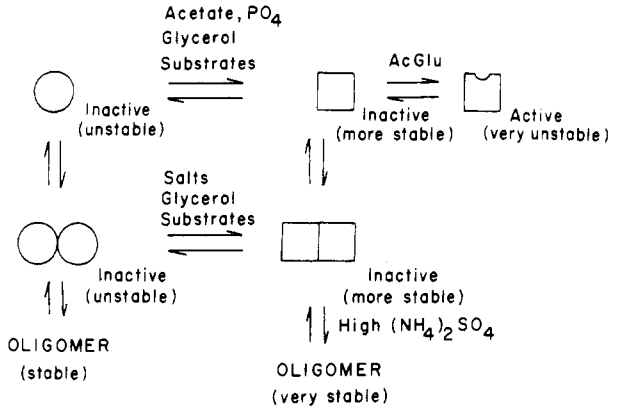

FIGURE 8: Simplified scheme to explain the effect of substrates and acetylglutamate on oligomeric structure and enzyme stability.

1980). Since rat liver carbamoyl-phosphate synthetase exists primarily as the monomer at low protein concentrations, allosteric activation of the enzyme by acetylglutamate can occur at the level of the monomer, apparently unmediated by subunit interactions. In this regard, also, the mammalian carbamoyl-phosphate synthetase resembles the bacterial enzyme. Carbamoyl-phosphate synthetase from *Pseudomonas aeruginosa* PA01, which like the *E. coli* enzyme is regulated by UMP and ornithine, exists as a monomer in the presence or absence of ligands,[3] suggesting that catalytic and allosteric behavior is unrelated to the polymeric state and can occur at the monomeric level.

A summary of our present knowledge of the oligomeric structure of rat liver carbamoyl-phosphate synthetase and its relation to catalytic activity and enzyme stability is shown in Figure 8. The enzyme can exist in at least three oligomeric forms, which can assume two conformations, involving sulfhydryl groups and differing in stability (Lusty, 1978a; Novoa & Grisolia, 1964). One conformation is attained in dilute buffer and the absence of acetylglutamate and substrates. Under these conditions, the enzyme undergoes concentration-dependent monomer–dimer interconversions, all forms being unstable (Lusty, 1978a; Novoa & Grisolia, 1964). The addition of glycerol, salts, or substrates stabilizes both dimer and monomer and shifts the equilibrium toward the monomer (Guthohrlein & Knappe, 1968; Lusty, 1978a; present paper). In the presence of high concentrations of $(NH_4)_2SO_4$ (plus sodium acetate), the enzyme in the more stable conformation has a tendency to form oligomers ($n \geq 4$). Under these conditions also, both monomer and oligomer are catalytically inactive in the absence of acetylglutamate (Lusty, 1978a,b). The interaction of acetylglutamate with the monomer form, possible also with the dimer, yields the catalytically active and most unstable configuration (Lusty, 1978a; Novoa & Grisolia, 1964).

Carbamoyl-phosphate synthetase has all of the properties predicted by Monod et al. (1965) for positive V-type allosteric enzymes. Thus, the allosteric ligand exerts a direct effect on the catalytic activity, rather than on the affinity of the enzyme for its substrates, and cooperative substrate interactions should not be observed. The model further predicts that the allosteric ligand will act as a specific dissociative or associative agent. Carbamoyl-phosphate synthetase does not exhibit cooperative substrate interactions (Lusty, 1978a,b). In addition, acetylglutamate, as shown in this study, appears to act as a specific allosteric dissociating agent.

The interaction of allosteric ligands with proteins that undergo association–dissociation has been described in terms of the following two mechanisms (Kegeles & Cann, 1978). In the first, the allosteric modifier is assumed to bind preferentially to the dimeric enzyme to form an intermediate enzyme complex. If the interactions between the subunits are weak, the induced change in conformation is sufficient to cause dissociation of the complex to monomers. According to the second mechanism, the dissociation constant of the protein is increased due to a preferential binding of the allosteric ligand to the monomer. Although the two mechanisms cannot be distinguished by sedimentation analysis, at least for systems in rapid equilibrium (Kegeles & Cann, 1978), much of the results obtained with carbamoyl-phosphate synthetase suggests that acetylglutamate has a stronger binding affinity for the monomer. Interaction of the protein with acetylglutamate may lead to the formation of the catalytic site. The activation of the catalytic site by acetylglutamate could result either from the activation of a group on the enzyme that is part of the catalytic process or from a conformational rearrangement of active-site substructures that generates the catalytic site.

The gel chromatographic method used in the present studies to demonstrate the active form of carbamoyl-phosphate synthetase could be more generally applied to identify the active form of enzymes, especially those which cannot be assayed spectrophotometrically and, therefore, are not adaptable to active enzyme centrifugation analysis.

Acknowledgments

I am indebted to Dr. Jeanne Stowe Poindexter for her help in the electron microscope studies and to James Hagenzeiker for his skillful assistance. I also thank Eric H. Nietsch and Richard N. Ingram for their expert and enthusiastic assistance during the course of these studies.

References

Abdelal, A. T. H., & Ingraham, J. L. (1975) *J. Biol. Chem. 250*, 4410–4417.
Ackers, G. K. (1964) *Biochemistry 3*, 723–730.
Anderson, P. M. (1977) *Biochemistry 16*, 583–586.
Cecil, R., & Ogston, A. G. (1952) *Biochem. J. 51*, 494.
Ceriotti, G., & Gazzaniga, A. (1966) *Clin. Chim. Acta 14*, 57–62.
Clarke, S. (1976) *J. Biol. Chem. 251*, 950–961.
Claverie, J.-M. (1976) *Biopolymers 15*, 843–857.
Claverie, J.-M., Dreux, H., & Cohen, R. (1975) *Biopolymers 14*, 1685–1700.
Cohen, R., & Mire, M. (1971) *Eur. J. Biochem. 23*, 267–275.
Cohen, R., & Claverie, J.-M. (1975) *Biopolymers 14*, 1701–1716.
Cohen, R., Giraud, B., & Messiah, A. (1967) *Biopolymers 5*, 203–225.
Gilbert, G. A. (1955) *Discuss. Faraday Soc. No. 20*, 68–71.
Gilbert, G. A. (1959) *Proc. R. Soc. London, Ser. A 250*, 377–388.
Gilbert, G. A. (1960) *Nature (London) 186*, 882–883.
Gilbert, L. M., & Gilbert, G. A. (1961) *Nature (London) 192*, 1181.
Gordon, C. N. (1972) *J. Ultrastruct. Res. 39*, 173–185.
Guthohrlein, G., & Knappe, J. (1968) *Eur. J. Biochem. 7*, 119–127.
Hall, L. M., Metzenberg, R. L., & Cohen, P. P. (1958) *J. Biol. Chem. 230*, 1013–1021.
Haschemeyer, R. H. (1970) *Adv. Enzymol. Relat. Areas Mol. Biol. 33*, 71–118.
Hayes, J. E., Jr., & Velick, S. F. (1954) *J. Biol. Chem. 207*, 225–244.
Horne, R. W. (1967) *Methods Virol. 3*, 521–574.

[3] Ahmed Abdelal, personal communication.

Kegeles, G., & Cann, J. R. (1978) *Methods Enzymol. 48*, 248–270.
Kemper, D. L., & Everse, J. (1973) *Methods Enzymol. 27*, 67–82.
Lee, J. C., Gekko, K., & Timasheff, S. N. (1979) *Methods Enzymol. 61*, 26–49.
Lindberg, O., & Ernster, L. (1956) *Methods Biochem. Anal. 3*, 1–22.
Lowry, O. H., Roberts, N. R., Wu, M.-L., Hixon, W. S., & Crawford, E. J. (1954) *J. Biol. Chem. 207*, 19–37.
Lusty, C. J. (1978a) *Eur. J. Biochem. 85*, 373–383.
Lusty, C. J. (1978b) *J. Biol. Chem. 253*, 4270–4278.
Lusty, C. J., Jilka, R. L., & Nietsch, E. R. (1979) *J. Biol. Chem. 254*, 10030–10036.
Marshall, M., Metzenberg, R. L., & Cohen, P. P. (1958) *J. Biol. Chem. 233*, 102–105.
Massey, V., Hofmann, T., & Palmer, G. (1962) *J. Biol. Chem. 237*, 3820–3828.
Matthews, S. L., & Anderson, P. M. (1972) *Biochemistry 11*, 1176–1183.
Monod, J., Wyman, F., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118.
Novoa, W. B., & Grisolia, S. (1964) *Biochim. Biophys. Acta 85*, 274–282.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Perrin, F. (1936) *J. Phys. Radium 7*, 1.
Powers, S. G., Meister, A., & Haschemeyer, R. H. (1980) *J. Biol. Chem. 255*, 1554–1558.
Raijman, L., & Jones, M. E. (1976) *Arch. Biochem. Biophys. 175*, 270–278.
Rothen, A. (1944) *J. Biol. Chem. 152*, 679–693.
Sedmak, J. J., & Grossberg, S. E. (1977) *Anal. Biochem. 79*, 544–552.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 85–89, Wiley, New York.
Shigesada, K., & Tatibana, M. (1971) *J. Biol. Chem. 246*, 5588–5595.
Shill, J. P., Peters, B. A., & Neet, K. E. (1974) *Biochemistry 13*, 3864–3871.
Snodgrass, P. J., & Parry, D. J. (1969) *J. Lab. Clin. Med. 73*, 940–950.
Sytkowski, A. J. (1977) *Arch. Biochem. Biophys. 184*, 505–517.
Tanford, C. H. (1961) *Physical Chemistry of Macromolecules*, pp 359, 380, Wiley, New York.
Taylor, B. L., Barden, R. E., & Utter, M. F. (1972) *J. Biol. Chem. 247*, 7383–7390.
Trotta, P. P., Estis, L. F., Meister, A., & Haschemeyer, R. H. (1974) *J. Biol. Chem. 249*, 482–491.
Vinograd, J., & Bruner, R. (1966a) *Biopolymers 4*, 131–156.
Vinogard J., & Bruner, R. (1966b) *Biopolymers 4*, 157–170.
Virden, R. (1972) *Biochem. J. 127*, 503–508.
Williams, C. H., Jr., Zanetti, G., Arscott, L. D., & McAllister, J. K. (1967) *J. Biol. Chem. 242*, 5226–5231.
Winzor, D. J., & Scheraga, H. A. (1963) *Biochemistry 2*, 1263–1267.

Reactivity of Human Leukocyte Elastase and Porcine Pancreatic Elastase toward Peptide 4-Nitroanilides Containing Model Desmosine Residues. Evidence That Human Leukocyte Elastase Is Selective for Cross-Linked Regions of Elastin†

Akira Yasutake and James C. Powers*

ABSTRACT: Elastin contains a number of cross-linking amino acid residues such as desmosine and isodesmosine which are primarily hydrophobic in character, but have a positively charged pyridinium ring. These cross-linking residues are formed by the action of lysyl oxidase upon Lys residues in tropoelastin, a precursor of elastin. A series of tetrapeptide 4-nitroanilides which contain Lys and a series of modified lysine residues were synthesized. The modified lysine residues [ϵ-carbobenzyloxy (Z), ϵ-benzoyl (Bz), ϵ-benzimidoyl (Bim), and ϵ-2-picolinoyl (Pic)] have various characteristics of desmosine and isodesmosine residues, such as a positive charge, a hydrophobic aromatic ring, or a pyridine ring. The reactivity of the tetrapeptide 4-nitroanilides containing the model desmosine residues at P_4, P_3, or P_2 with human leukocyte (HL) and porcine pancreatic (PP) elastase was measured at pH 7.5 and 25 °C. HL elastase exhibited high reactivity toward the substrates with P_4 or P_3 hydrophobic groups (Z, Bz, or Pic), and MeO-Suc-Lys(Pic)-Ala-Pro-Val-NA is 7 times more reactive than the previous best HL elastase substrate, MeO-Suc-Ala-Ala-Pro-Val-NA. The major change occurred in K_M values. The substrates containing Lys residues were either nonreactive or poor. Except for two substrates with P_2 hydrophobic residues (Bz and Pic), PP elastase was less reactive toward the substrates containing model desmosine residues than toward MeO-Suc-Ala-Ala-Pro-Val-NA. The data support the hypothesis that HL elastase cleaves elastin selectively near cross-linking residues. The results also indicate that HL elastase binds tightly to these regions and would be poorly effective toward regions of elastin or tropoelastin which contain Lys residues.

Elastin is a flexible, highly cross-linked protein found in high quantities in mammalian lung and arteries. The chemical structure of elastin is not yet known, but the fragmentary data available indicate that elastin has long sequences of small aliphatic amino acid residues such as Gly, Ala, Val, and Pro (Gray et al., 1973; Sandberg et al., 1977). Mature cross-linked elastin is formed by the action of lysyl oxidase on the soluble precursor protein tropoelastin. Lysyl residues of tropoelastin are oxidized to α-aminoadipic δ-semialdehyde residues which then condense with each other and with Lys residues to form

† From the School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332. *Received December 23, 1980.* This research was supported by a grant from the Council for Tobacco Research.

0006-2960/81/0420-3675$01.25/0