Molecular Weight and Subunit Structure of Pyridoxamine Pyruvate Transaminase*

Helmut Kolb, R. David Cole, and Esmond E. Snell

ABSTRACT: The sedimentation coefficient of pyridoxamine pyruvate transaminase ($s^0_{20,w}$ = 8.1 S) is almost unaffected by variations in pH between 5 and 9 or in temperature between 5 and 22°; it is also unaffected by addition of an excess of the substrate, pyridoxal, by reduction with $NaBH_4$ in the presence of pyridoxal, by dilution to 50 μg/ml, or by prolonged incubation in 4 M urea. From the sedimentation coefficient and the diffusion constant ($D^0_{20,w}$ = 5.2 × 10^{-7} sec cm^{-2}) a molecular weight of 148,000 was obtained; high- and low-speed sedimentation equilibrium runs gave values of 153,000 and 148,000, respectively. Previous studies (Ayling, J., and Snell, E. E. (1968a), *Biochemistry 7*, 1616) showed the presence of 2 combining sites for pyridoxal/150,000 daltons of enzyme. The Cotton effect at 233 mμ indicated that the conformation of the native enzyme was unchanged by the presence of pyridoxal and indeed was unchanged when pyridoxal was covalently linked to the enzyme by $NaBH_4$ reduction. In 8 M urea or 6 M guanidine hydrochloride, pyridoxamine pyruvate transaminase dissociates to yield a new species, $s_{20,w}$ about 2.0 S.

The carboxymethylated enzyme exhibits a similar sedimentation coefficient (1.6–1.9 S) in 8 M urea, 6 M guanidine hydrochloride, or 5% acetic acid. This species appears homogeneous on acrylamide gel electrophoresis and in sedimentation equilibrium runs; a molecular weight of 38,000 was obtained by the latter technique. Dissociation in urea or guanidine hydrochloride is accompanied by loss of activity, loss of the ability to bind pyridoxal, a very large increase in viscosity, and a profound reduction of the Cotton effect (near 233 mμ). Excess pyridoxal, but not excess pyridoxamine, partially protects the enzyme against urea denaturation. These data indicate that pyridoxamine pyruvate transaminase has an unusually stable structure which is further stabilized by interaction with pyridoxal. Each molecule contains two substrate binding sites and on the basis of physical properties consists of four apparently identical subunits.

A recent survey indicated that all pyridoxal phosphate dependent enzymes so far studied contained a number of subunits equal to or greater than the number of combining sites for pyridoxal phosphate (Boeker and Snell, 1968). Pyridoxamine pyruvate transaminase is unique among presently known transaminases in that it contains no pyridoxal phosphate; however, in this enzyme the substrates, pyridoxal and pyridoxamine, play the role ascribed to pyridoxal phosphate in the more usual transaminases (Wada and Snell, 1962; Ayling and Snell, 1968a,b). The stoichiometry of binding has been variously reported as 2 moles of pyridoxal/140,000 (Wada and Snell, 1962), 120,000 (Dempsey and Snell, 1963; Fujioka and Snell, 1965), or 150,000 g (Ayling and Snell, 1968a) of crystalline enzyme. The latter determination is least questionable on theoretical grounds, but all of the measurements are complicated by the difficulty in removing all of the bound vitamin B_6 from the enzyme. To assist in resolving this problem and because of the unique relationship of pyridoxamine pyruvate transaminase to pyridoxal phosphate dependent enzymes, it seemed worthwhile to compare its quaternary structure to that of other pyridoxal enzymes, particularly tryptophanase, whose quaternary structure is unusually sensitive to concentration, temperature, ionic environment, and pH (Morino and Snell, 1967a,b), and to other transaminases. With this in mind the work described in this paper was carried out.

Experimental Procedure

Materials and Methods. Pyridoxamine pyruvate transaminase was isolated as described previously (Ayling and Snell, 1968a) from a soil organism grown on pyridoxine as carbon and nitrogen source. For ultracentrifugation and kinetic measurements, the enzyme was crystallized three times from ammonium sulfate solutions and had a specific activity of 23 units/mg.

Pyridoxal, pyridoxamine, and sodium pyruvate were obtained from Nutritional Biochemicals Corp.; pyridoxine and other reagents were purchased from Merck & Co. Guanidinium chloride was purified by passing over a charcoal–Celite column before use (Satake *et al.*, 1960). Urea was deionized using a mixed-bed resin.

The transamination reaction was followed by the spectrophotometric determination at 410 mμ of pyridoxal formed from pyridoxamine and pyruvate, as described previously (Ayling and Snell, 1968a). The reaction mixture contained: 300 μl of 1.0 M Tris buffer (pH 8.52), 100 μl of 0.1 M pyridoxamine solution, 100 μl of 0.1

* From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received April 10, 1968.* Supported in part by Grants AM 1448, AI 1575, AM 2691, AM 6482, and AM 8845 from the National Institutes of Health, U. S. Public Health Service.

M sodium pyruvate, 50 μl of 0.2 M EDTA solution, and 2.45 ml of deionized H_2O, all contained in a 4-ml quartz cuvet with a 1-cm light path. The reaction was started by addition of 5–20 μg of enzyme and was followed at room temperature. The protein concentration was determined according to the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Pyridoxal combines stoichiometrically with pyridoxamine pyruvate transaminase (Dempsey and Snell, 1963); the azomethine linkage thus formed was reduced with sodium borohydride to give a stable, reduced enzyme–substrate complex by the following procedure. The enzyme was dialyzed for 5 days against four changes of buffer (0.02 M potassium phosphate, pH 7.0, or Tris, pH 8.5) to remove most of the bound pyridoxal. The resulting, nearly colorless, solution was titrated with 0.01 M pyridoxal until the absorbance at 410 mμ, read against a blank containing no enzyme, remained constant. A tenfold molar excess of sodium borohydride (0.025 M) was then added. After 10 min at room temperature, the solution was dialyzed against buffer appropriate for subsequent experiments.

Polyacrylamide gel electrophoresis was carried out as described by Davis (1964). The acrylamide concentration was 7.5%. Between 10 and 50 μg of protein were applied to the gel.

Analytical ultracentrifugation was carried out with a Beckman–Spinco Model E instrument, equipped with schlieren and Rayleigh interference optics. The temperature of the rotor during the run was held within ±0.02° of the indicated temperature. A standard 12-mm double-sector cell with sapphire windows was used with schlieren optics for the sedimentation velocity runs. The enzyme was dialyzed at 4° for several days against buffer solutions of appropriate pH and ionic strength. The enzyme was then diluted to the desired concentration with the last dialysate. The sedimentation coefficient was calculated as described by Schachman (1957).

The diffusion coefficient was determined in the ultracentrifuge using a capillary synthetic boundary cell and schlieren optics. The enzyme solution (0.15 ml) was placed in the bottom of the cell and the dialysate was used for the upper layer. The rotor speed was 6166 rpm. The apparent diffusion coefficient, D, was calculated from the area and height of the gradient as described by Gosting (1956).

For low-speed sedimentation equilibrium runs a rotor speed of 6995 rpm was used with interference optics. The initial protein concentration, c_0, of the sample was determined from the number of fringes observed in a synthetic boundary run as described by Richards and Schachman (1959). The molecular weight was calculated from a plot of log c *vs.* x^2 by using eq 1 where R is the gas constant, T is the absolute temperature, ω is the angular velocity of the rotor in radians per second, c the concentration of sample, x the distance from the center of rotation, ρ the density of the solution, and $\bar{v}$ the partial specific volume of the enzyme. The high-speed sedimentation equilibrium runs were carried out as described by Yphantis (1964), using interference optics. Three different protein concentrations were studied by placing 100-μl aliquots in a multichannel cell. The photographic plates were read using a Nikon microcomparator with a 50-fold magnification. From the plot of the log of the fringe displacement against x^2 the apparent molecular weight, M, at a given concentration was calculated using eq 1.

$$M = \frac{2RT\mathrm{d}\ln c}{\omega^2(1 - \bar{v}\rho)\mathrm{d}x^2} \qquad (1)$$

The partial specific volume, $\bar{v}$, calculated from the amino acid composition of the enzyme (Cohn and Edsall, 1943) was 0.742 ml/g.

The viscosity was measured in a capillary viscometer (Schachman, 1957) at 25 ± 0.05°. A solution (2 ml) containing 2 moles of pyridoxal/mole of enzyme in 0.02 M potassium phosphate (pH 7.0) was used at each concentration of native enzyme. It was not possible to determine the viscosity of pyridoxamine pyruvate transaminase in the absence of pyridoxal because the enzyme precipitated in the capillary. A similar procedure was followed with protein which had been inactivated in 6 M guanidinium chloride and then carboxymethylated according to Crestfield *et al.* (1963). The carboxymethylated sample was dialyzed against 6 M guanidinium chloride at 4°; the concentration of guanidinium chloride was checked by refractive index and density measurements by the formula of Kawahara and Tanford (1966). Five concentrations of enzyme were studied. Measurements of optical rotatory dispersion were made in cells of 0.1- or 0.5-mm light path in a Cary Model 60 spectropolarimeter. The temperature within the cell compartment was 25°.

The helical parameter, b_0, was evaluated from the Moffit equation (eq 2) as described by Fasman (1963); λ_0 was taken to be 212 mμ and the value used for the mean residue weight (MRW) was 120, calculated from the amino acid composition (Dempsey and Snell, 1962).

$$[\alpha]_\lambda = \frac{100}{MRW}\frac{(n^2 + 2)}{3}\left[\frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}\right] \qquad (2)$$

Results

Sedimentation Coefficient of Pyridoxamine Pyruvate Transaminase. Crystalline pyridoxamine pyruvate transaminase showed only a single protein band after acrylamide gel electrophoresis at pH 7.0 or 8.3 (Figure 1). The protein migrates only a short distance toward the anode at pH 7.0, which suggests that this pH is near the isoelectric point of this enzyme. Ultracentrifugation experiments with the crystallized enzyme using schlieren optics gave also a single symmetrical peak as shown in Figure 2. Sedimentation coefficients, determined at protein concentrations from 1.9 to 6.0 mg/ml, fell on a straight line which on extrapolation to zero concentration and correction for the density and viscosity of water at 20° yielded a value for $s^0_{20,w}$ of 8.1 S. The dependence of the sedimentation coefficient on the enzyme concentration is described by eq 3, where s_c is the observed sed-

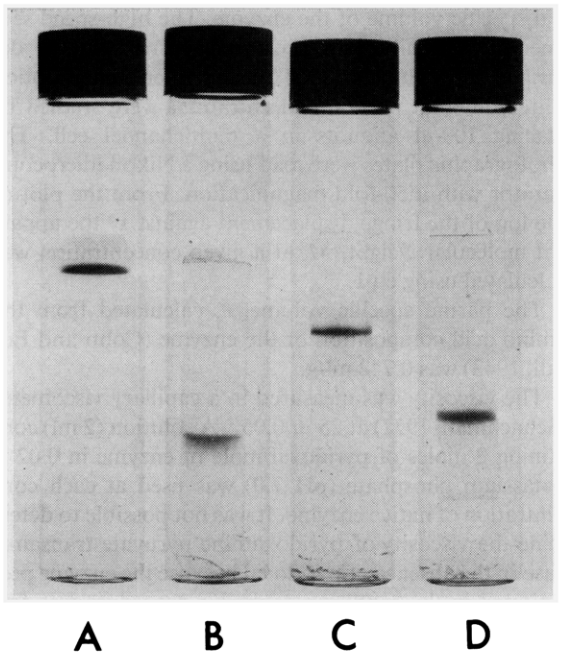

FIGURE 1: Electrophoresis of pyridoxamine pyruvate transaminase in polyacrylamide gel. (A and B) Native enzyme run at 4 mA/gel for 30 min in Tris buffer at pH 7.0 and 8.3, respectively. (C and D) Urea-denatured enzyme run at 4 mA/gel in 8 M urea–Tris buffer (pH 8.8) for 70 and 140 min, respectively. Migration took place from the top (−) to the bottom (+). The faint lower band in C and D represents the ion front and is not a second protein component.

$$s_c = 8.1(1 - 0.012c) \quad (3)$$

imentation coefficient at a given protein concentration, c (milligrams per milliliter).

The use of a scanning attachment at 280 mμ (Edelstein and Schachman, 1967) permitted direct measurement of the sedimentation coefficient at an enzyme contration of 50 μg/ml. A value of 8.3 S was obtained in good agreement with the extrapolated value, indicating that no dissociation occurs on dilution to this concentration. The sedimentation coefficient of pyridoxamine pyruvate transaminase also remains essentially constant in three different buffers, between pH 5 and 9 (Table I). The small differences observed are due to the variations in protein concentration. The sedimentation coefficient of a sample of enzyme largely freed of pyridoxal (0.2 mole remaining/150,000 g of enzyme) by dialysis for 1 week at 4° against 0.02 M potassium phosphate (pH 7.0) was unchanged by addition of excess pyridoxal (Table II). A change in temperature from 22 to 5° did not affect the sedimentation coefficient in either the presence or absence of pyridoxal (Table II). This constancy indicates a very stable molecule.

By treatment with sodium borohydride it is possible to bind pyridoxal covalently to the two substrate binding sites of the transaminase (Dempsey and Snell, 1963) with complete inactivation of the enzyme. The sedimentation coefficient of the reduced inactivated enzyme was the same as that of the native enzyme within experimental error and showed the same concentration dependence.

Diffusion Coefficient of Pyridoxamine Pyruvate Transaminase. The diffusion coefficient of native pyridox-

TABLE I: Sedimentation Coefficients of Pyridoxamine Pyruvate Transaminase in Different Buffers at Room Temperature.[a]

Buffer (0.02 M)	pH	Enzyme Concn (mg/ml)	$s_{20,w}$ (S)
Potassium phosphate	5.0	2.5	7.7
	6.2	2.5	7.6
	7.0[b]	2.5	7.6
	9.0	2.0	7.7
Sodium phosphate	7.0[b]	4.2	7.4
	8.0	2.8	7.6
Tris-HCl	8.5	3.0	7.5
	9.0	2.5	7.6

[a] The rotor speed was 59,780 rpm. [b] In the presence of 1.4×10^{-5} M mercaptoethanol.

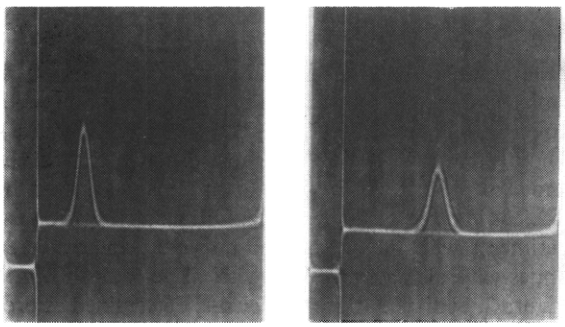

FIGURE 2: Sedimentation pattern of pyridoxamine pyruvate transaminase after 16 and 40 min at 59,780 rpm and 21°. The enzyme concentration was 4.5 mg/ml of 0.02 M potassium phosphate buffer (pH 7.0).

TABLE II: Sedimentation Coefficients of Pyridoxamine Pyruvate Transaminase as a Function of Temperature and Pyridoxal Concentration.

Moles of Pyridoxal/ Moles of Enzyme	Temp (°C)	Enzyme Concn (mg/ml)	$s_{20,w}$ (S)
20	22	2.5	7.6
2	21.2	3.0	7.5
0.2	22.4	2.0	7.7
3	4.8	3.5	7.4
0.2	6.2	2.6	7.6

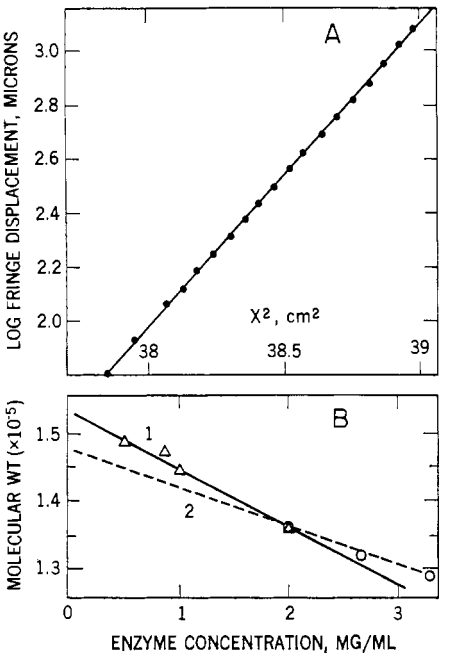

FIGURE 3: Determination of molecular weight of native pyridoxamine pyruvate transaminase. (A) Plot of log fringe displacement of the native enzyme (0.5 mg/ml) against x^2 after 18 hr at 17,250 rpm and 24.2°. (B) Dependence of the molecular weight of native pyridoxamine pyruvate transaminase upon enzyme concentration. Curve 1: results of sedimentation equilibrium runs at 17,250 rpm; curve 2: results of low-speed equilibrium runs at 6,995 rpm.

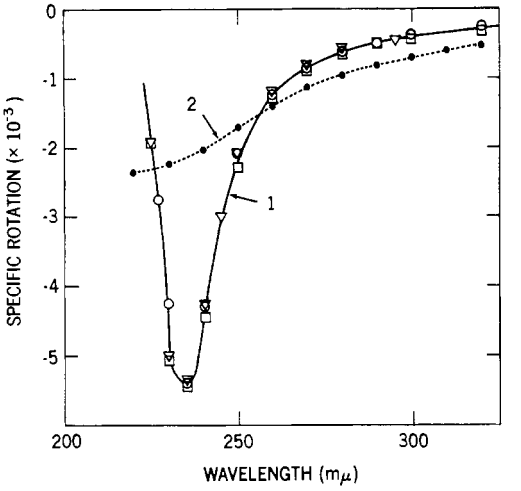

FIGURE 4: Ultraviolet rotatory dispersion spectrum of pyridoxamine pyruvate transaminase. Curve 1: —▽—, native enzyme with 0.4 mole of pyridoxal bound/mole of enzyme; —□—, native enzyme with 2 moles of pyridoxal bound/mole of enzyme; —○—, $NaBH_4$-reduced, inactive enzyme. Curve 2: —●—, denatured enzyme. The denatured enzyme was in 6 M guanidinium chloride; other samples were in 0.02 M sodium phosphate buffer (pH 7.0).

amine pyruvate transaminase was measured with the ultracentrifuge at three different enzyme concentrations in 0.02 M sodium phosphate buffer (pH 7.0). Since the apparent diffusion constants were concentration dependent, the three values were extrapolated to zero protein concentration to obtain a value of $D^0_{20,w}$ of 5.2×10^{-7} sec/cm².

The frictional coefficient, calculated from the diffusion coefficient and the molecular weight (150,000; see following section) as described by Tanford (1967), was 1.18, a value which is consistent with a reasonably globular shape of the native enzyme.

Molecular Weight of Pyridoxamine Pyruvate Transaminase. A. FROM SEDIMENTATION AND DIFFUSION COEFFICIENTS. The molecular weight M was calculated according to eq 4 given by Svedberg and Pederson (1940),

$$M_{s,D} = \frac{s_0 RT}{D_0(1 - \bar{v}\rho)} \qquad (4)$$

where s_0 and D_0 are values of the sedimentation and diffusion coefficients, respectively, both extrapolated to zero protein concentration, and other symbols are as given in eq 1. This method yielded a molecular weight of 148,000.

B. FROM SEDIMENTATION EQUILIBRIUM. High-speed sedimentation equilibrium experiments at low protein concentrations (Yphantis, 1964) yielded a straight line when the logarithm of the fringe displacement was plotted against the square of the distance from the center of rotation, thus confirming the homogeneity of the enzyme (Figure 3A). The molecular weight, calculated at several protein concentrations and extrapolated to zero concentration (Figure 3B), was 153,000.

The molecular weight was also determined at several higher protein concentrations with lower rotor speeds (Schachman, 1957; see Methods) and extrapolated to zero concentration (Figure 3B). The value obtained, 148,000, is in excellent agreement with that obtained by the two other procedures used. The validity of the average value obtained by these measurements is confirmed by the finding that exactly two combining sites for pyridoxal are present per 150,000 daltons of enzyme (Ayling and Snell, 1968a).

Optical Rotatory Dispersion Spectra of Pyridoxamine Pyruvate Transaminase. With ultracentrifugation experiments no difference in conformation between native and reduced pyridoxamine pyruvate transaminase could be detected. Since optical rotatory dispersion is a more sensitive tool to examine conformational changes, experiments were done with native enzyme in the presence and in the absence[1] of pyridoxal and with reduced enzyme. As shown in Figure 4 all three experiments gave the same optical rotatory spectrum in the range of 220–300 mμ. The conformation parameter, b_0, calculated from a Moffit plot (see Methods), was −190.

Effect of Denaturing Agents on Activity and Sedimentation Behavior of Pyridoxamine Pyruvate Transaminase. Pyridoxamine pyruvate transaminase was inactivated by 6 or 8 M urea at a rate dependent on the concentration of pyridoxal (Figure 5). In contrast to pyridoxal, pyridoxamine at a concentration ten times that nec-

[1] We have not been able to reduce the bound pyridoxal below 0.2–0.4 mole/mole of enzyme (*cf.* Ayling and Snell, 1968a); the pyridoxal-free enzyme referred to here contained 0.4 mole/mole.

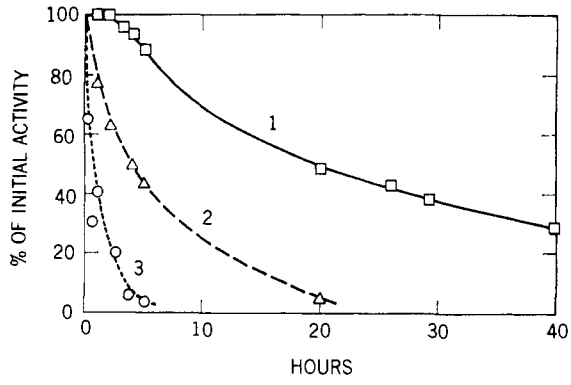

FIGURE 5: The effect of pyridoxal on the rate of inactivation of pyridoxamine pyruvate transaminase in 8 M urea. The enzyme was titrated with pyridoxal as described under Methods. In curve 1 the solution contained ten times the amount of pyridoxal necessary to saturate the enzyme. In curve 2 the enzyme was titrated to the saturation point (2 moles of pyridoxal bound/mole of enzyme); in curve 3 the enzyme was half-saturated with pyridoxal (1 mole of bound pyridoxal/mole of enzyme).

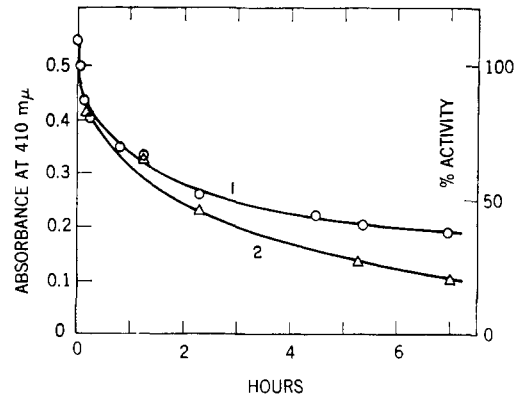

FIGURE 6: Kinetics of inactivation of pyridoxamine pyruvate transaminase in 8 M urea at 20° as measured by loss of bound pyridoxal (curve 1) or by enzymatic assay (curve 2). At time zero, 1.44 g of urea and a few drops of water were added to 1.8 ml of a solution containing 4.3 mg of enzyme/ml which was 75% saturated with pyridoxal (approximately 1.5 moles of bound pyridoxal/mole of enzyme) bringing the final volume of the solution to 3 ml and the final urea concentration to 8 M. The solution was placed in a 4-ml cuvet with a 1-cm light path. A small aliquot (10 μl) was removed at the times indicated and assayed for activity by the standard procedure.

essary for saturation did not protect the enzyme against inactivation by urea, even though both substrates have similar affinities for the enzyme (Ayling and Snell,1968a)

One interpretation of such results would be that the enzyme–pyridoxal complex assumes a physical structure resistant to denaturation, but that once partially denatured the enzyme loses its ability to bind this substrate at the active site. It was possible to test this interpretation by correlating enzymic inactivation with loss of absorbance at 410 mμ, which results from binding of pyridoxal. In fact, loss of absorbance does accompany inactivation (Figure 6), and addition of excess pyridoxal to urea-inactivated enzyme resulted in neither absorption at 410 mμ nor reactivation of the enzyme. The failure of the urea-treated transaminase to bind pyridoxal seems, therefore, to result from a structural change in the enzyme, which could be an unfolding or dissociation into subunits, or a combination of these processes.

That part of the structural change was a dissociation of the enzyme into subunits was revealed by sedimentation velocity experiments. After partial inactivation in 8 M urea, two peaks of very different sedimentation velocities appeared (Figure 7); after complete inactivation, only the more slowly moving peak ($s_{20,w}$ = 2.4 S) was present. A peak of similar sedimentation coefficient (2.0 S) was seen in 6 M guanidine hydrochloride, which inactivated the enzyme completely within 15 min even in the presence of a 20-fold excess of pyridoxal. No peaks were detected between those of the subunit and the native structure at any concentration of urea or guanidine hydrochloride studied.

The extent of inactivation by urea correlates very well with the amount of slowly sedimenting material in the cell (Figure 7). It can be concluded that the dissociated subunit is inactive and that the activity of the urea-treated pyridoxamine pyruvate transaminase results from undissociated enzyme, present even after an incubation time of several hours.

Small variations in the sedimentation coefficient of the subunit obtained in different denaturing solvents (Table III) suggest that different degrees of unfolding of the subunit may occur during denaturation. A few experiments indicated that disulfide cross-linking occurs slowly in solutions of denatured enzyme; however, when this reaction was prevented by inactivation and carboxymethylation in guanidine hydrochloride, the sedimentation coefficient (1.9 S) was about the same as that of the slowly moving peak seen in urea or guanidine hydrochloride solutions. That the enzyme molecule is extensively unfolded during denaturation was revealed by measurements of intrinsic viscosity (Figure 8). The intrinsic viscosity of the native enzyme saturated with pyridoxal was 4.5 ml/g, while that of the carboxymethylated enzyme in 6 M guanidinium chloride was 32.0 ml/g. An extensive conformational change was also revealed by studies of optical rotatory dispersion, which showed that the deep absorption trough at 233 mμ observed with the native enzyme was largely eliminated by dissolving in 6 M guanidinium chloride (Figure 4). It therefore seems reasonable that the variation of sedimentation coefficients in Table III could result from relatively small quantitative differences in the extent of unfolding. Such variations also would be observed if minor changes in $\bar{v}$ occur as a result of differential binding of solvent or solutes in the various denaturing solvents.

Molecular Weight of Subunits of Pyridoxamine Pyruvate Transaminase. To determine the molecular weight of the subunits formed on denaturation, sedimentation equilibrium runs (Yphantis, 1964) were conducted on a sample of carboxymethylated enzyme following dialysis against 8 M urea or 6 M guanidinium chloride for several days at 4°. Plots of the data obtained (Figure 9) were linear in both cases, indicating that the dissociated enzyme was homogeneous in molecular weight. The cal-

TABLE III: Effect of Denaturing Agents on the Activity and Sedimentation Coefficients of Pyridoxamine Pyruvate Transaminase at Room Temperature.

Forms of Enzyme and Dissociating Agent	Act. (%)	Protein Concn (mg/ml)	$s_{20,w}$ (S)
Native enzyme			
2 M urea	100	4.0	7.4
4 M urea	100	5.0	7.2
8 M urea[a]	60	4.5	7.0, 2.7
8 M urea[b]	0	3.5	2.4
6 M guanidine hydrochloride	0	3.2	2.0
5% acetic acid	0	3.5	1.8
5% sodium dodecyl sulfate	0	3.8	2.2
Carboxymethylated			
8 M urea	0	3.0	1.9
6 M guanidine hydrochloride	0	5.0	1.8
5% acetic acid	0	3.5	1.6

[a] Incubated in urea 2 hr before ultracentrifugation.
[b] Incubated in urea 10 hr before ultracentrifugation.

culated molecular weights (Table IV) in the two solvents agreed, suggesting that the calculations are not seriously distorted by interactions between the solvent and the protein subunits. As a further check, however, enzyme which had been inactivated and carboxymethylated in

TABLE IV: Molecular Weights of Pyridoxamine Pyruvate Transaminase in Denaturing Solutions.

Form of Enzyme and Dissociating Agent	Temp (°C)	Protein Concn (mg/ml)	Mol Wt (g)
Native enzyme			
8 M urea	23	1.25	32,000
8 M urea	23	0.62	35,000
8 M urea	23	0.50	36,000
8 M urea	23	0[a]	38,000
Carboxymethylated enzyme			
6 M guanidine hydrochloride	21	1.25	34,000
6 M guanidine hydrochloride	21	0.62	37,000
5% acetic acid	22	0.50	39,000

[a] Extrapolated.

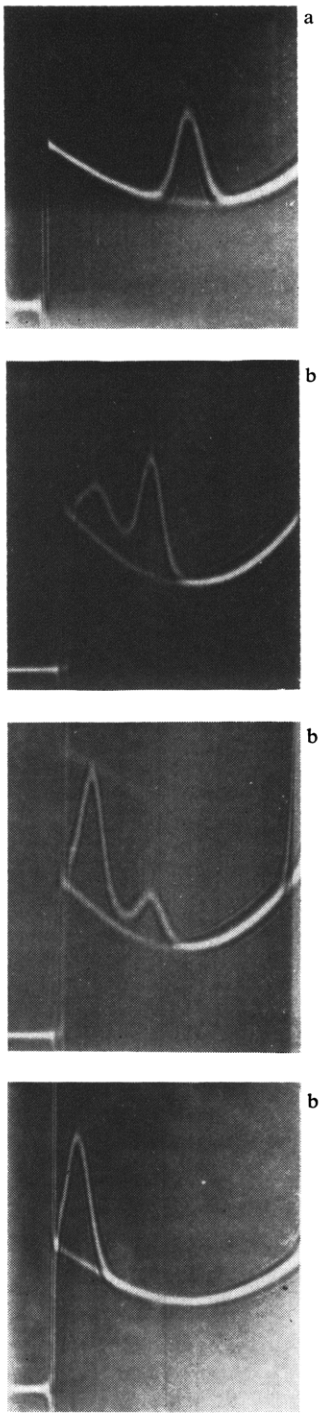

FIGURE 7: Sedimentation pattern of pyridoxamine pyruvate transaminase at different incubation times and pyridoxal concentrations in 8 M urea. The enzyme concentration was 8 mg/ml. Each picture was taken approximately 80 min after reaching 59,780 rpm. The temperature was 20°. Concentrations were calculated by integration of the area under the gradient curve. The activity was determined under standard conditions from a parallel experiment. In the top schlieren pattern (marked a), the pyridoxal concentration was ten times that necessary to saturate the enzyme; in the remaining patterns (marked b), the enzyme was saturated with pyridoxal as determined by spectrophotometric titration.

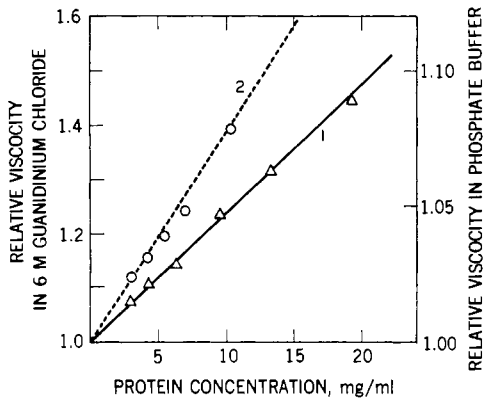

FIGURE 8: Relative viscosity of solutions of native (curve 1) and denatured (curve 2) pyridoxamine pyruvate transaminase at 25.2°. Solutions of the denatured enzyme were in 6 M guanidinium chloride; those of native enzyme were in 0.02 M potassium phosphate buffer (pH 7.0) containing 10 moles of pyridoxal/mole of enzyme.

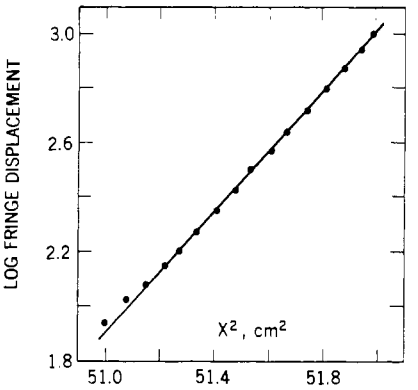

FIGURE 9: Molecular weight determination of dissociated pyridoxamine pyruvate transaminase. The graph shows a plot of log fringe displacement of the denatured protein in 8 M urea against x^2. The protein concentration was 0.5 mg/ml, the temperature 23°, and the rotor speed 44,770 rpm.

8 M urea was dialyzed for 1 week against four changes of 5% acetic acid (pH 2.45). The protein was soluble at that point and sedimented as a single peak with an $s_{20,w}$ of 1.6 S. After dilution with 5% acetic acid to three different concentrations (0.02, 0.025, and 0.05 mg/ml) and dialysis against three changes of 5% acetic acid for 5 days, the samples were subjected to a high-speed equilibrium run at 23,150 rpm. Linear plots of log c *vs.* x^2 were obtained from which a molecular weight of 39,000 was calculated (Table IV).

These molecular weight determinations indicate that pyridoxal pyruvate transaminase dissociates in 8 M urea or 6 M guanidine hydrochloride into four subunits of equal size. Electrophoresis in polyacrylamide gel containing 8 M urea (Figure 1C,D) failed to resolve these subunits, indicating that they also are identical by this criterion.

Several attempts to reconstitute active enzyme after exposure to urea or guanidine were uniformly unsuccessful despite precautions to remove the denaturants slowly, to prevent oxidation of sulfhydryl groups, and to operate within a reasonable pH range of 5–9. Instead the protein seems to aggregate (mol wt $\sim 10^6$) or precipitate. Pyridoxal was not bound to the dissociated protein and did not promote reactivation under these conditions.

Discussion

The stability of pyridoxamine pyruvate transaminase over a wide range of conditions contrasts markedly with that of two bacterial pyridoxal phosphate dependent enzymes, tryptophanase (Morino and Snell, 1967a,b) and arginine decarboxylase (Boeker and Snell, 1968), studied previously in this laboratory. For example, the tetrameric apotryptophanase reversibly dissociates to dimers (in the presence of Na^+ or K^+) merely by lowering the temperature and protein concentration, by moderately increasing the pH, or by treating with low concentrations of sodium dodecyl sulfate, whereas the tetrameric pyridoxamine pyruvate transaminase even in the absence of its substrate is totally unaffected by similar treatments. Both of these enzymes are irreversibly dissociated to monomers when treated with high concentrations of denaturants (Table III), but at pH 9 in the absence of denaturants the transaminase retains its tetrameric form while apotryptophanase dissociates. Stability more like that of the transaminase is acquired by tryptophanase as it binds pyridoxal phosphate in the presence of Na^+ or K^+. This binding, which also produces a profound conformational change in the enzyme, renders tryptophanase so stable that only high concentrations of sodium dodecyl sulfate will dissociate the tetramer. When this dissociation does occur it goes irreversibly all the way to monomers, resembling the dissociation of pyridoxamine pyruvate transaminase in 8 M urea or 6 M guanidinium chloride. While pyridoxal binding does stabilize the tetrameric form of the transaminase, and therefore must product local changes in conformation near the active site, neither the binding of pyridoxal nor the reduction of the azomethine bond between pyridoxal and the transaminase led to conformational alterations sufficient to affect the hydrodynamic parameters or the value of b_0. The latter value, calculated from optical rotatory dispersion data, indicates a significant extent of ordered structure in the native transaminase, particularly when viewed in the light of its profound change in the presence of guanidinium chloride. Similar situations have been observed by Fasella and Hammes (1965) when pyridoxal phosphate was coupled to aspartate aminotransferase by reduction, and by Wilson and Meister (1966) who coupled pyridoxal phosphate to aspartate β-decarboxylase. Apparently, then, a conformation of considerable stability preexists in pyridoxamine pyruvate transaminase and is simply reinforced by the binding of pyridoxal, whereas such a conformation is acquired by tryptophanase only as a result of binding pyridoxal phosphate. Stabilization of both enzymes through binding of pyridoxal or of pyridoxal phosphate is also evidenced by the fact that both the pyridoxal–transaminase complex and holotryptophanase are stable to the shear stress involved in viscometric measurements, whereas the pyridoxal-free transaminase

and apotryptophanase are readily denatured under such conditions.

The fact that pyridoxamine binds as well as pyridoxal to the transaminase (Ayling and Snell, 1968a) but does not reinforce the tetrameric structure implies that interaction with pyridoxal may produce cross-linking as a result of bridging between a portion of a peptide chain that binds the pyridine nucleus of the coenzyme and another portion of the same or a separate peptide chain that supplies the amino group involved in azomethine formation. If a separate peptide chain were involved, reduction of the azomethine linkage between the pyridoxal and the enzyme might strengthen such cross-linking sufficiently so that dimers might be found in urea solutions. This was not the case; no dimers were detected in 4 or 8 M urea, but only tetramers and monomers. Indeed the aggregation–dissociation properties were like those of the native enzyme in the presence of pyridoxal.

When treated vigorously enough with urea, guanidinium chloride, or acetic acid, pyridoxal pyruvate transaminase dissociates into four subunits even in the presence of excess pyridoxal. As judged by their homogeneity in molecular weight and electrophoretic properties the subunits appear to be identical or nearly so. Chemical tests of this supposition, including the determination of amino- and carboxyl-terminal residues and the distribution of peptides following treatment with CNBr or trypsin, are in progress. If the subunits are in fact identical, the organization of the molecule must be such that two subunits provide only a single combining site for pyridoxal. A somewhat similar situation has been observed in tryptophanase, where two identical peptide chains provide a single combining site for pyridoxal phosphate (Morino and Snell, 1967b), but in this case the two chains are combined through a disulfide linkage. The subunit structure of pyridoxamine pyruvate transaminase appears to differ even more from that of glutamate oxaloacetate transaminase (mol wt 80,000–110,000), which is reported to contain two identical subunits (Polyanovsky and Shpikiter, 1965; Polyanovsky and Vorotnitskaya, 1965; Scardi, 1968) and to provide two combining sites for pyridoxal phosphate (Wada and Morino, 1964; Scardi, 1968; Banks *et al.*, 1968). The latter enzyme also is reported (Polyanovsky and Ivanov, 1964) to dissociate into single subunits at assay concentrations (for a contrary view, see Banks *et al.*, 1968). Direct measurement of the sedimentation coefficient of pyridoxamine pyruvate transaminase at protein concentrations approaching those used for assay provides no evidence for dissociation of this enzyme under assay conditions.

The molecular weight of the associated form of pyridoxamine pyruvate transaminase, established here as 150,000, represents an upward revision from the value of 120,000 found in previous investigations by titration with pyridoxal or its derivatives (Dempsey and Snell, 1963; Fujioka and Snell, 1965). The latter value was not corrected for dissociation of pyridoxal from the enzyme at the equivalent point, and would be expected on this basis to be somewhat low. When such correction is made, a value of 150,000 also is obtained by titration with substrate on the assumption of two combining sites for pyridoxal per mole of enzyme (Ayling and Snell, 1968a). The revised value and the presence of four subunits make it likely that eight rather than the six SH groups previously reported (Fujioka and Snell, 1965) are present per mole of enzyme; this problem is being currently reexamined.

Acknowledgments

We are grateful to Miss Doris Midgarden for assistance with several of the ultracentrifugal experiments.

References

Ayling, J., and Snell, E. E. (1968a), *Biochemistry 7*, 1616.

Ayling, J., and Snell, E. E. (1968b), *Biochemistry 7*, 1626.

Banks, B. E. C., Doonan, S., and Vernon, C. A. (1968), *in* Pyridoxal Catalysis: Enzymes and Model Systems, Snell, E. E., Braunstein, A. E., Severin, E. S., and Torchinsky, Yu. M., Ed., New York, N. Y., Interscience, p 305 ff.

Boeker, E. A., and Snell, E. E. (1968), *J. Biol. Chem. 243*, 1678.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, p 370.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.

Davis, G. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Dempsey, W. B., and Snell, E. E. (1963), *Biochemistry 2*, 1414.

Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242*, 306.

Fasella, P., and Hammes, G. G. (1965), *Biochemistry 4*, 801.

Fasman, G. D. (1963), *Methods Enzymol. 6*, 928.

Fujioka, M., and Snell, E. E. (1965), *J. Biol. Chem. 240*, 3044.

Gosting, L. F. (1956), *Advan. Protein Chem. 11*, 429.

Kawahara, K., and Tanford, C. (1966), *J. Biol. Chem. 241*, 3228.

Lowry, O. H., Rosebrough, N. F., Farr, A. L., and Randall, R. F. (1951), *J. Biol. Chem. 193*, 265.

Morino, Y., and Snell, E. E. (1967a), *J. Biol. Chem. 242*, 5591.

Morino, Y., and Snell, E. E. (1967b), *J. Biol. Chem. 242*, 5602.

Polyanovsky, O. L., and Ivanov, V. I. (1964), *Biokhimiya 29*, 623.

Polyanovsky, O. L., and Shpikiter, V. O. (1965), *Dokl. Akad. Nauk USSR 163*, 1011.

Polyanovsky, O. L., and Vorotnitskaya, I. E. (1965), *Biokhimiya 30*, 619.

Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.

Satake, K., Rasmussen, P. S., and Luck, J. M. (1960), *J. Biol. Chem. 235*, 2801.

Scardi, V. (1968), *in* Pyridoxal Catalysis: Enzymes and Model Systems, Snell, E. E., Braunstein, A. E.,

Severin, E. S., and Torchinsky, Yu. M., Ed., New York, N. Y., Interscience, p 179 ff.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Svedberg, T., and Pederson, K. O. (1940), The Ultracentrifuge, Oxford, Clarendon.
Tanford, C. (1967), Physical Chemistry of Macromolecules, New York, N. Y., Wiley.
Wada, H., and Morino, Y. (1964), *Vitamins Hormones 22*, 411.
Wada, H., and Snell, E. E. (1962), *J. Biol. Chem. 237*, 133.
Wilson, E. M., and Meister, A. (1966), *Biochemistry 5*, 1166.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Physicochemical Basis of the Recognition Process in Nucleic Acid Interactions. II. Interactions of Polyuridylic Acid and Polycytidylic Acid with Nucleoside Mono- and Triphosphates*

Paul O. P. Ts'o and Wai Mun Huang†

ABSTRACT: Insoluble complexes of adenine nucleotides (AMP, dAMP, ATP, and dATP) and polyuridylic acid as well as guanine nucleotides (GMP, dGMP, and GTP) and polycytidylic acid are formed reversibly when the concentrations of the polynucleotides and especially of the nucleotides are sufficiently high and the temperature is sufficiently low. At moderate concentration of the interactants (0.01–0.02 M), Mg^{2+} ion is usually required for the interaction. These insoluble complexes have a definite stoichiometry, generally 2U to 1A or 1C to 1G and, in one case, 2C to 1G was found. The base-pairing specificity is the same as that of the polynucleotide interactions. On the other hand, a soluble complex between the mononucleotides and their complementary polynucleotides cannot be detected by optical rotatory dispersion, sedimentation, and viscosity, techniques which were employed successfully before to characterize the adenosine–$(U)_n$ system. These data indicate that interaction in these mixtures only takes place when accompanied by a phase transition, such as precipitation or gelation. The following mechanism is proposed. When the conditions are sufficiently favorable, the monomer–polymer interaction occurs through hydrogen bonding between the base pairs and through the cooperative stacking of the mononucleotides along side the complementary polynucleotides. In the complex, mononucleotides become polymerlike. Thus, a phase transition occurs, since the polymer–polymer complexes are insoluble under these conditions. This transition of physical state provides the additional driving force needed for the interaction to proceed by removing the complex into another phase of the system.

For the past decade our laboratory has been actively engaged in studies concerning the physicochemical basis of nucleic acid conformation and interaction. A considerable amount of work has been done on the problem of monomer–monomer interaction of the bases, nucleosides, and nucleotides in aqueous solution, from our laboratory (Ts'o *et al.*, 1963; Ts'o and Chan, 1964; Chan *et al.*, 1964; Schweizer *et al.*, 1965, 1968; Broom *et al.*, 1967) and from others (see Discussion). The first paper of this series concerns the problem of nucleosides' interaction with polynucleotides (Huang and Ts'o, 1966a). This problem was also investigated independently with different emphasis in laboratories of National Institutes of Health (Howard *et al.*, 1966; Maxwell *et al.*, 1966).

The interaction of nucleoside monophosphates with the polynucleotides was first investigated by Howard *et al.* (1964) using the technique of infrared spectroscopy. Definitive information about the interaction between guanosine mononucleotides and $(C)_n$ was obtained. These authors then extended their investigation to the problem of guanosine triphosphate interaction with $(C)_n$ and adenosine triphosphate interaction with $(U)_n$ (Miles *et al.*, 1966). In our laboratory, the research on the interaction of nucleotides and nucleoside triphosphates

* From the Department of Radiological Sciences, The Johns Hopkins University, Baltimore, Maryland. *Received April 12, 1968*. Presented in part at the 10th Annual Biophysical Society Meeting, Boston, Mass., 1966, Abstracts, p 16. This material was also submitted by W. M. H. as part of a Ph.D. thesis to Johns Hopkins University, Feb 1967. This work was supported in part by grants from the National Science Foundation (GB-5483 and GB-767) and by a program project grant, National Institutes of Health (GM-10802-04, 05).

† Present address: Department of Biochemistry, Albert Einstein College of Medicine, Bronx, N. Y. 10461.