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Molecular Weight, Subunit Structure, and Amino Acid Composition of the Branched Chain Amino Acid Aminotransferase of *Salmonella typhimurium*†

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ABSTRACT: A molecular weight of 183,000 ± 5% and a sedimentation coefficient of 9.8 S ± 2% were obtained for the branched chain amino acid aminotransferase of *Salmonella typhimurium* by the use of ultracentrifugation techniques. Results of sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed a single monomeric species with a molecular weight of 31,500 ± 10%; threonine was identified as its N-terminal amino acid. It is concluded that the native form of the enzyme is a hexamer composed of seemingly identical subunits. The amino acid composition was also determined. Results of *p*-mercuribenzoate titration studies imply that two-thirds of the sulfhydryl groups (possibly two of the three cysteinyl residues calculated per subunit) are shielded in the native form and that the enzyme contains no disulfide bridges. However, it appears that a sulfhydryl group is not required for enzymatic activity.

The availability of highly purified branched chain amino acid aminotransferase (L-leucine:2-oxoglutarate aminotransferase, EC 2.6.1.6; trivial name: transaminase B) of *Salmonella typhimurium*, crystallized by Coleman and Armstrong (1971), made feasible for the first time a number of studies permitting the physical and chemical characterization of this enzyme. The following report is primarily concerned with the determination of the $s_{20,w}$ value at infinite dilution, the molecular weight, subunit structure, and the amino acid composition of this aminotransferase. Information regarding aggregation properties of the enzyme, identification of its N-terminal amino acid residue, and the effects of thiols and sulfhydryl reagents on the enzyme are also included.

Experimental Section

Enzyme Purification and Assay. The crystallization procedure and assay for transaminase B were as previously described by Coleman and Armstrong (1971). Enzyme preparations with specific activities of 3500–4000 μmol of isoleucine transaminated per hr per mg of protein were used in this study. Protein was determined by the method of Lowry *et al.* (1951).

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Materials. The following thiol compounds and sulfhydryl reagents were used: β-mercaptoethanol, reduced glutathione, *p*-mercuribenzoate, and *N*-ethylmaleimide (Sigma Chemical Co.); and 2-iodoacetamide (Eastman).

Proteins that served as standards in the sodium dodecyl sulfate polyacrylamide electrophoretic or Sepharose 6B chromatographic studies included: horse heart cytochrome *c*, bovine trypsin, bovine liver catalase, bovine fibrinogen, and rabbit muscle aldolase (Sigma Chemical Co.); bovine chymotrypsinogen A, rabbit muscle aldolase, and ovalbumin (Pharmacia Fine Chemicals, Inc.); horse heart myoglobin and bovine serum albumin (Schwarz/Mann); and yeast alcohol dehydrogenase (Miles-Seravac, Ltd.).

Other compounds and materials used included: sodium dodecyl sulfate (99% purity) and 2,4-dinitrophenyl (Dnp)[1] amino acid standards (Sigma Chemical Co.); guanidinium chloride (99% purity), methylenebisacrylamide and silica gel chromagram sheets (Eastman); Temed, ammonium persulfate, acrylamide, Bromophenol Blue, and α-ketoglutarate (Matheson Coleman and Bell); Coomassie Brilliant Blue (Mann Research Laboratories); thioglycolic acid (Fisher Scientific Co.); Sepharose 6B (Pharmacia Fine Chemicals, Inc.); analytical grade KCl (Mallinckrodt); and Baker-flex cellulose sheets (J. T. Baker Chemical Co.).

Sedimentation Velocity. The procedure described by Chervenka (1970) was employed. Enzyme was dissolved in 2 ml of 0.1 M potassium phosphate buffer, containing 0.1 M KCl and 2 mM potassium α-ketoglutarate (pH 6.5), and dialyzed overnight against 900 ml of the same solvent. Six boundary sedimentation experiments were performed with protein concentrations ranging from 5 to 0.8 mg/ml; dilutions were made using the dialysate. The centrifugations were conducted in a Beckman Model E ultracentrifuge with a calibrated An-D rotor at 52,640 rpm at 20.5°. Schlieren photographs were measured to the nearest 2 μ in a Gaertner microcomparator. The slope of log radius (*r*) *vs.* time, in minutes, for each centrifugation was obtained with a linear regression program. Straight line correlation coefficients for the individual experiments averaged 0.99999. Observed values of *s* were corrected to standard conditions, as described by Schachman (1957). For the determination of relative viscosity, flow times for dialysate and distilled, deionized water were measured in a capillary viscometer (Fisher Scientific Co.) in a 10-gallon water bath equipped with a Circu-Temp thermoregulator. Fluid densities were measured in 10-ml volumetric flasks calibrated with distilled, deionized water, employing the same controlled temperature bath to achieve thermal equilibration.

Molecular Weight Determination. The high speed method of equilibrium ultracentrifugation, as described by Yphantis (1964), was utilized. The study was conducted with a calibrated An-F rotor (four holes), which permitted simultaneous recording of equilibrium interference patterns for three initial protein concentrations (0.4, 0.8, and 1.0 mg/ml) at identical speed (12,590 or 15,220 rpm) and temperature (20.5°). Measurements of interference photographs were made to the nearest 2 μ in a Bausch and Lomb microcomparator. Replicate exposures were measured for the 15,220-rpm study. The transaminase B sample was dissolved in 0.01 M phosphate buffer (pH 7.8) containing 0.2 M KCl, and dialyzed overnight against two successive 500-ml volumes of the same solvent. The final dialysate was retained and used for dilutions and as reference solvent. Fluid densities were measured as described above. The partial specific volume of transaminase B was calculated from amino acid composition data.

Sepharose 6B Chromatography. Transaminase B samples, containing 3–9 mg of protein in 1–2 ml of 2% sucrose, were applied to a Sepharose 6B column (2.5 × 40 cm) and eluted at a flow rate of 30 ml/hr. The effluent was monitored at 280 nm with the use of an ultraviolet monitor (Gilson Medical Instruments, Inc.) attached to a recorder (Texas Instruments, Inc.); 6-ml fractions were collected. The column was calibrated with fibrinogen (mol wt 340,000), catalase (230,000), and aldolase (158,000).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The electrophoretic procedure of Weber and Osborn (1969) was utilized to determine polypeptide chain weight; however, the concentrations of sodium phosphate buffer in the cathode and anode chambers were 0.025 and 0.05 M, respectively, rather than 0.1 M. Preliminary reductive denaturation (using protein concentrations of 0.2–0.3 mg/ml in a volume of 1–2 ml) involved: (1) incubation overnight at room temperature in 6 M guanidinium chloride with 0.1 or 1.0% β-mercaptoethanol (v/v) in 0.01 M sodium phosphate buffer (pH 7.1) (Reynolds and Tanford, 1970), or (2) warming for 2 hr at 37° in buffer of the same composition containing 1% sodium dodecyl sulfate (w/v) and 1% β-mercaptoethanol (Weber and Osborn, 1969). After such denaturation, the solutions were dialyzed overnight at room temperature against the same phosphate buffer, containing 0.15% sodium dodecyl sulfate and 0.15% β-mercaptoethanol. When guanidinium chloride was present, it was necessary to change the dialysate after several hours because of extensive precipitation of sodium dodecyl sulfate. Nonreductive denaturation experiments were performed as described above but without addition of β-mercaptoethanol. In the experiment in which 0.05 M iodoacetamide was present during denaturation in guanidinium chloride, the pH was adjusted to 8.3 and the denaturant concentration to 8 M; the pH was not adjusted in the corresponding experiment with 1% sodium dodecyl sulfate.

Gel monomer solution consisted of 0.1 M sodium phosphate (pH 7.1), 0.1% sodium dodecyl sulfate, 0.1% Temed, and 0.05% ammonium persulfate along with a selected concentration of total acrylamide. The electrophoretic apparatus was constructed in the laboratory. The method of Chrambach *et al.* (1967) was employed for precipitation and staining; however, exposure to 0.05% Coomassie Blue was performed overnight or longer, and the stained gels were allowed to stand 1–3 days in 10% trichloroacetic acid before photographing. The gels were photographed as described by Dunker and Rueckert (1969).

The following protein markers were used: cytochrome *c* (mol wt 11,700); myoglobin (17,200); trypsin (23,300); chymotrypsinogen A (25,700); yeast alcohol dehydrogenase (37,000); aldolase (40,000); ovalbumin (43,000); catalase (60,000); and serum albumin (68,000). Molecular weights are cited from Weber and Osborn (1969).

N-Terminal Amino Acid Determination. The N-terminal amino acid was determined as the Dnp derivative with the use of two separate enzyme preparations. The derivative was prepared by the method described by Naik and Horton (1971). In this study, however, the enzyme was denatured by treatment with sodium dodecyl sulfate or guanidinium chloride. For treatment with sodium dodecyl sulfate, 1 mg of enzyme preparation (1 ml), which had been dialyzed overnight against 5 mM potassium phosphate buffer (pH 8), was placed in an

[1] Abbreviations used are: Dnp, 2,4-dinitrophenyl; Temed, *N,N,N',N'*-tetramethylethylenediamine; N_2phF, 1-fluoro-2,4-dinitrobenzene.

ignition tube, made 0.5% with respect to sodium dodecyl sulfate and heated 5 min at 90–95°. $NaHCO_3$ (1.4 mmol) and water were then added to bring the final volume to 1.9 ml. N_2phF (21.5 μmol in 0.1 ml of ethanol) was added to the tube (wrapped in foil to shield the contents from light), and the tube was then incubated on a shaker for 24 hr at 37°. The reaction mixture was extracted twice with 2 ml of diethyl ether prior to precipitation of Dnp-protein by acidification to pH 1 with concentrated HCl. Dnp-protein was collected by centrifugation, washed successively with 0.05 or 0.1 N HCl, ethanol, and ether, and then hydrolyzed. For treatment with guanidinium chloride, dialyzed protein was gently evaporated to dryness and then dissolved in 1.9 ml of 5 M guanidinium chloride containing 5% $NaHCO_3$ (w/v), pH 8.5. The guanidinium chloride reaction mixture was treated as described above except that neither the extraction with ether nor acidification to pH 1 was performed because the Dnp-protein precipitated during the 24-hr incubation.

Two-dimensional thin-layer chromatography on 7.5-cm squares of Baker-flex cellulose (previously equilibrated with 0.1 M potassium phthalate (pH 6.0)) utilized *tert*-amyl alcohol saturated with the phthalate buffer (Naik and Horton, 1971) for the first direction and 1 M NaH_2PO_4 and 0.5 M Na_2HPO_4 (Pataki, 1966) for the second. With 7.5-cm squares of Eastman silica gel chromagram sheets, a benzene–pyridine–glacial acetic acid (80:20:2) solvent was used for the first direction, and a chloroform–benzyl alcohol–glacial acetic acid (70:30:3) solvent for the second. The latter two solvent systems are included by Pataki (1966) among those suitable for one-dimensional separation of ether-soluble Dnp-amino acids on silica gel. In this study, they also proved to be satisfactory in the two-dimensional technique. Water-soluble products of the hydrolysis were examined by the first of the two-dimensional systems described above and by one-dimensional development on silica gel strips (5 × 20 cm) with 1-propanol–NH_4OH (7:3), as described by Pataki (1966).

Amino Acid Composition. The method described by Spackman *et al.* (1958) was employed on two separate enzyme preparations. Analyses were performed with the use of a Beckman 120 C amino acid analyzer. Duplicate samples were hydrolyzed for 24, 48, 60, and 72 hr. The procedure described by Matsubara and Sasaki (1969) was used to determine tryptophan content; *viz.*, inclusion of 2% thioglycolic acid in one of each of the duplicate samples hydrolyzed. Cysteine and methionine values were determined as cysteic acid and methionine sulfone, respectively, in protein samples subjected to performate oxidation prior to acid hydrolysis (Hirs, 1967a).

p-Mercuribenzoate Titration. A spectrophotometric titration of transaminase B with *p*-mercuribenzoate was conducted in a manner suggested by Benesch and Benesch (1962) for proteins with relatively slow-reacting sulfhydryl groups. Six reaction mixtures were prepared; each contained dialyzed enzyme (20 μM with respect to subunit) and 0, 20, 40, 60, 80, or 160 μM *p*-mercuribenzoate, all in 0.067 M sodium phosphate (pH 6.7). Corresponding blanks were prepared without enzyme. The absorbance of each sample solution at 250 nm (Beckman DU spectrophotometer) was determined in comparison to that of the appropriate blank at intervals over a 1-week period. For the titration of the denatured enzyme, the above procedure was used with 8 μM subunit; and the reaction with *p*-mercuribenzoate was performed in 0.1 M sodium phosphate buffer (pH 6) in the presence of 4 M guanidinium chloride. The absorbance of each sample solution was determined after 20 min and 24 hr.

Results

Estimation of Sedimentation Coefficient. Schlieren patterns obtained for transaminase B showed the enzyme preparation to be free of any significant macromolecular contaminants. The corrected sedimentation coefficients obtained from six experiments exhibited an apparent slight negative concentration dependence over the range of 1–5 mg/ml, behavior which is typical of compact globular proteins (Van Holde, 1971). Least-squares analysis of the six values gave 9.78 S ± 0.25% as the value of $s_{20,w}$ at infinite dilution and a slope of −0.0735 S per unit concentration (mg/ml) ± 11%. Although the computed standard deviation of the intercept $s_{20,w}$ was minimal and the correlation coefficients for the plots of log radius (r) against time, in minutes, for finite concentrations were excellent (av = 0.99999), the observed values of s are presumed accurate to only ±2% (Chervenka, 1970). When compared with values for myogen A, myeloperoxidase, and catalase (Smith, 1968), the s value of 9.78 S represents a molecular weight in the range of 200,000, according to the formula of Martin and Ames (1961).

Molecular Weight Determination. The meniscus depletion type of sedimentation equilibrium experiment, developed by Yphantis (1964), was performed at 12,590 and 15,220 rpm using three different protein concentrations (0.4, 0.8, and 1.0 mg/ml). (Because of a gap in the 0.4 mg/ml pattern obtained at 15,220 rpm, due to blurring in the interference photographs, these particular data were not utilized in subsequent calculations.) Values of $\sigma_w(r)$, *i.e.*, d ln c/d(r^2/2), at each value of r, were obtained for each equilibrium interference pattern by a computer that fitted a least-squares straight line through successive sets of five adjacent data points to yield σ_w for the central point of each set. Only displacements greater than 100 μ were included. The value of σ for each pattern was obtained from a plot of $\sigma_w(r)$ in cm^{-2} *vs.* point concentration, measured in microns of fringe displacement, by extrapolation to zero concentration, where nonideal effects and contributions from component(s) heavier than the principal species should be absent. A summary of least-squares treatment of the plots is given in Table I, which also includes apparent molecular weights.

Since the slopes in Table I are all slightly positive, solute heterogeneity is indicated. The upward slope of σ_w with increasing displacement at 15,220 rpm is illustrated in Figure 1A. To ensure adequate clearance of solute from the meniscus region in high speed equilibrium experiments, Yphantis (1964) recommends a rotor speed that gives a value of $\sigma(\sigma_1)$ near 5 cm^{-2} in the typical run. This result was achieved at 15,220 rpm but not at 12,590 rpm. Consequently, the apparent molecular weights (av 183,200 ± 4500) obtained at the higher speed are considered to be more reliable. However, the average values of the apparent molecular weight at the two speeds differ by less than 5% and are thus within the expected error of the method.

An alternative method for evaluating σ_1, not requiring extrapolation, is available if only one heavier component is present with the ratio of its molecular weight to that of the principal species known, as would be the case for a monomer contaminated with dimer (Yphantis, 1964). Because column chromatography indicated the ability of transaminase B to dimerize (presented below), it was of interest to evaluate σ_1, assuming a monomer–dimer system, *i.e.*, $\sigma_1 = 2\sigma_n(r) - \sigma_w(r)$.[2] An average of 4.92 cm^{-2} ± 3.2% was obtained for

[2] The values of $\sigma_n(r)$, the effective reduced number-average molecular weight, were computed as described by Yphantis (1964).

TABLE I: Molecular Weight of Transaminase B as Determined by the High Speed Method of Equilibrium Ultracentrifugation.[a]

Initial Protein Concn (mg/ml)	Rpm	Slope (cm^{-2}/μ)	Intercept (σ_1) (cm^{-2})	Apparent Molecular Weight	Standard Deviation
1.0	12,590	$+1.1 \times 10^{-4}$	3.60	193,500	±6500
0.8	12,590	$+1.6 \times 10^{-4}$	3.49	187,600	±3800
0.4	12,590	$+0.8 \times 10^{-4}$	3.56	191,400	±4800
		Av for 12,590 rpm values	3.55	190,800	±5000
1.0	15,220	$+3.9 \times 10^{-4}$	4.89	179,900	±4800
1.0	15,220	$+5.6 \times 10^{-4}$	4.92	181,000	±1800
0.8	15,220	$+3.1 \times 10^{-4}$	5.00	183,900	±4800
0.8	15,220	$+3.9 \times 10^{-4}$	5.11	188,000	±6600
		Av for 15,220 rpm values	4.98	183,200	±4500

[a] Linear regressions of $\sigma_w(r)$, in cm^{-2}, on concentration, in μ, were obtained by computer analysis of the data over ~180–1000 μ displacement. σ_1 refers to the estimated effective reduced molecular weight of the enzyme at infinite dilution. The following values were used in the calculation of molecular weights: $\bar{v} = 0.732\ cm^3/g$ and $p = 1.0091\ g/cm^3$. Values from replicate exposures at 15,220 rpm are presented. Standard deviations are equivalent to the standard deviations of σ_1 provided by the least-squares computer program.

the four 15,220 rpm patterns, which is in good agreement with the average value of 4.98 cm^{-2} presented in Table I. Figure 1B contains plots of σ_w, σ_n, and $(2\sigma_n - \sigma_w)$ *vs.* displacement for one equilibrium pattern.

For calculation of the molecular weights presented in Table I, a partial specific volume ($\bar{v}$) of 0.732 cm^3/g, evaluated from amino acid composition data (Table IV), was used. Results of sedimentation equilibrium in H_2O and D_2O solvents (Edelstein and Schachman, 1967) furnished a $\bar{v}$ value of 0.739 cm^3/g. However, the calculation of this experimental value did not include data obtained at concentrations below 300–350 μ because of steep slopes in the ln c *vs.* r^2 plots in this region (attributed to convective disturbances). Substitution of 0.739 cm^3/g for $\bar{v}$ yields an average molecular weight of 188,200 (15,220 rpm); thus, the use of either $\bar{v}$ value results in molecular weight values which agree within 3%.

Sepharose 6B Chromatography. Use was made of a calibrated Sepharose 6B column to explore the effects of several buffers used in the ultracentrifuge experiments. As seen in Table II, the state of aggregation of transaminase B is affected by the eluting buffer, seemingly by its ionic strength. In buffer systems of ionic strength 0.2–0.3, the apparent molecular weight is consistently in the range of 200,000. Furthermore, no detectable peaks with higher or lower mobilities were eluted in this range of ionic strength and at these protein concentrations. Thus, the enzyme preparations examined in the ultracentrifuge were in solvent systems in which a single molecular species appears to predominate.

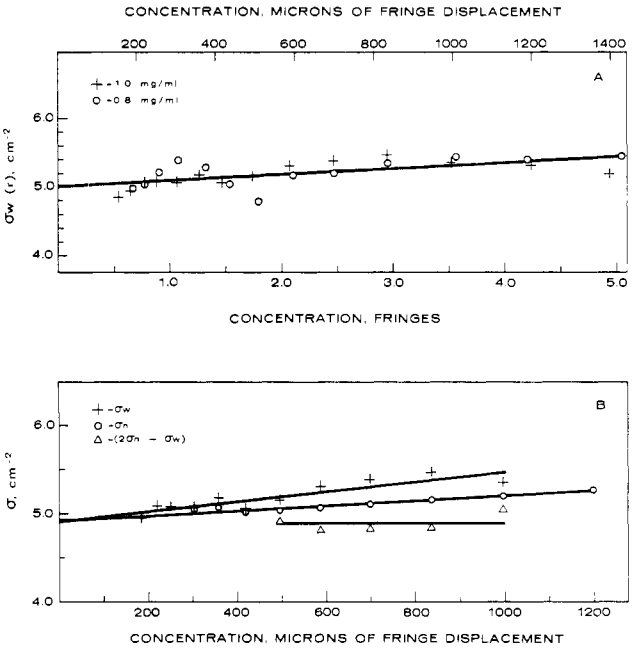

FIGURE 1: Determination of σ_1 from effective reduced weight and number average molecular weights. (A) The point values for σ_w for two initial concentrations are extrapolated to infinite dilution. An average of two linear regression lines is shown. (B) $(2\sigma_n - \sigma_w)$ is obtained at point concentrations from 500 to 1000 μ in a single cell, and the average of these values should approximate σ, if dealing with a monomer–dimer system.

TABLE II: Chromatography of Transaminase B on Sepharose 6-B Column.

Column Buffer System	Ionic Strength	Approx Mol Wt
1. 0.01 M potassium phosphate–0.2 M KCl (pH 7.8)	~0.2	200,000
2. 0.1 M potassium phosphate–2 mM α-ketoglutarate (pH 6.5)	~0.2	200,000
3. 0.1 M potassium phosphate–0.1 M KCl–2 mM α-ketoglutarate (pH 6.5)	~0.3	200,000
4. 0.01 M potassium phosphate–2 mM α-ketoglutarate (pH 7.0)	~0.03	300,000
5. 0.01 M Tris-chloride (pH 7.5)	~0.01	400,000

The elution behavior of transaminase B in buffers of low ionic strength (Table II) provides insight into the association proclivities of the molecule. The apparent molecular weight of 400,000 at an ionic strength below 0.01 implies dimerization of the 200,000 molecular weight species, whereas the value of 300,000 observed in an ionic strength near 0.03 could represent a mixture of "200,000" and "400,000" species.[3] It

[3] In the case of small zone elution, continuous dilution would occur during migration through the column so that any dependence of state of aggregation on protein concentration would result in a continuous variation in apparent mobility, thereby obscuring detection of individual 200,000 and 400,000 molecular weight species.

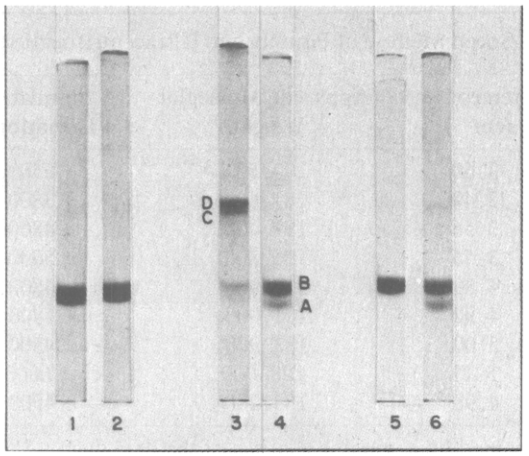

FIGURE 2: Electrophoretic patterns on gels containing 5% total acrylamide with acrylamide–methylenebisacrylamide at 38:1. The direction of migration was downward. Gels 1 and 2 are transaminase B patterns obtained after denaturation in guanidinium chloride–β-mercaptoethanol and sodium dodecyl sulfate–β-mercaptoethanol, respectively. Gels 3 and 4 are the patterns after denaturation without β-mercaptoethanol in guanidinium chloride and sodium dodecyl sulfate, respectivly, whereas gels 5 and 6 are patterns resulting from these respective treatments in the presence of iodoacetamide. The designations A, B, C, and D are explained in the text.

is realized that these observed values for molecular weights are, at best, rough approximations since gel filtration mobilities of proteins are related to their effective hydrodynamic radii rather than to their molecular weights *per se*.

Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis. Analyses by sodium dodecyl sulfate polyacrylamide gel electrophoresis of enzyme samples that were reduced (β-mercaptoethanol) and denatured (sodium dodecyl sulfate or guanidinium chloride) reveal that a single band is produced (Figure 2, gels 1 and 2). Table III is a summary of the results obtained from a series of experiments that utilized a number of protein markers to determine the average molecular weight of the subunit. A value of 31,500 ± 2.2% was obtained. Although the standard deviation derived for the results of this series is low, a value of ±10% is a more realistic esimate of reliability of molecular weights obtained by this technique (Weber and Osborn, 1969).

TABLE III: Sodium Dodecyl Sulfate Polyacrylamide[a] Gel Electrophoresis of Transaminase B (Reduced).[b]

Denaturing Agent	% Total Acrylamide	Subunit Weight (Front as Reference)
1% SDS, 1% β-mercaptoethanol	5.2	30,422
1% SDS, 1% β-mercaptoethanol	7.2	31,722
1% SDS, 1% β-mercaptoethanol[c]	10.0	31,420
1% SDS, 1% β-mercaptoethanol	10.0	32,268
6 M GdmCl, 1% β-mercaptoethanol	5.2	31,052
6 M GdmCl, 1% β-mercaptoethanol	5.2	30,833
6 M GdmCl, 0.1% β-mercaptoethanol	7.2	32,189
6 M GdmCl, 0.1% β-mercaptoethanol	10.3	32,087
Av subunit weight		31,499
Standard deviation		±684 (±2.2%)

[a] Abbreviations used: SDS, sodium dodecyl sulfate; GdmCl, guanidinium chloride. [b] Acrylamide–methylenebisacrylamide = 38:1 except as noted in *c*. [c] Acrylamide–methylenebisacrylamide = 49:1.

As seen in Figure 2 (gels 3 and 4), denaturation in the absence of reducing agent results in different electrophoretic patterns. Two bands (A and B), with apparent molecular weights of ~27,000 and ~30,000, respectively, are obtained with either sodium dodecyl sulfate or guanidinium chloride treatment. With guanidinium chloride denaturation, however, a second set of bands (C and D, with apparent molecular weights of ~57,000 and ~61,000, respectively) is found. From visual comparison of band intensities and widths, it is inferred that the relative amounts of the components are D > C and B > A. Apparent relative amounts of C and B vary with different experiments, but band D is always the strongest and band A is always present in only trace amounts. The reduced complex is presumed to be closely related to band B because of the similarity of their mobilities.

The results reveal that on denaturation in the absence of β-mercaptoethanol, the transaminase B monomer exists in two forms. With regard to the heavier species noted with guanidinium chloride treatment, it is plausible that at least one sulfhydryl group on each subunit is exposed upon extension of the molecule and that interchain disulfide bridge formation produces heavier species, potentially of three types. The appearance of only two bands instead of three may be a mass action phenomenon; *viz.*, an excess of one form (band B) compared to the other (band A) would produce little or no AA, a major percentage of BB (band D), and moderate amounts of AB (band C). In the sodium dodecyl sulfate treatment, on the other hand, the sulfhydryl groups remain protected (Fish *et al.*, 1970) with the result that only the two monomeric forms are observed. It is also possible that band A could result from formation of intrachain disulfide bridges in a small fraction of the denatured monomers. If so, it may be reasoned that although interchain disulfide bridges occur only rarely in the sodium dodecyl sulfate treated samples (lack of bands C and D), intrachain disulfide bond formation (band A) can occur during formation of the sodium dodecyl sulfate complex. If these assumptions are valid, alkylation of the sulfhydryl groups as they are exposed to the denaturants should prevent formation of intra- and interchain bridges, in which case bands A, C, and D should not appear on electrophoresis. To test this hypothesis, a 5000-fold molar excess of iodoacetamide was employed as the alkylating agent. As seen in Figure 2, gel 5, only band B was present in the guanidinium chloride–iodoacetamide treated sample. By contrast, no significant change in band patterns was noted when iodoacetamide was included during sodium dodecyl sulfate denaturation (Figure 2, gel 6). In the latter case, the reagent may not have reacted at all, either because sodium dodecyl sulfate shielded the sulfhydryl groups or because the pH was kept at 7.1 instead of 8.3 where carboxymethylation is facilitated.

N-Terminal Amino Acid Determination. The only Dnp-amino acid identified by thin-layer chromatography of the ether extractable matter from the Dnp-protein hydrolysate was Dnp-threonine. The ether phase also contained dinitrophenol and dinitroaniline, by-products of the reaction. ε-Dnp-lysine and a small amount of dinitroaniline were extracted from the aqueous phase with *sec*-butyl alcohol–ethyl acetate (1:1). The lysine derivative was distinguished from Dnp-arginine following chromatography in that it produced

a brown color on treatment with ninhydrin instead of remaining yellow (Pataki, 1966). Thus, on the basis of accumulated evidence, threonine is identified as the reactive N-terminal amino acid.

Amino Acid Composition. Table IV provides a summary of the results obtained from an analysis of the amino acid composition of the enzyme. All composition data are based on the relationship of total weight recovered from the analyzer to the subunit weight of 31,500. The estimated total number of residues per subunit is 288 (mol wt 31,800). The number of proline residues is estimated to be 11, which is lower than the 12.52 residues derived from experimental data. As noted in Table IV, the four analyses used for the proline calculations had a standard deviation of ±12.5%, which is an accurate reflection of the variability noted among the results. Because the observed variation may be due to variable contributions by cysteine which cochromatographs, in part, with proline (Moore and Stein, 1963), a conservative estimate of 11 residues (lowest value obtained) is considered the most reasonable number. Data from samples hydrolyzed in the presence of 2% thioglycolic acid were not used for the proline determinations because of the high levels of cysteine obtained by this procedure (Matsubara and Sasaki, 1969). In the case of tryptophan, an estimate of 85% recovery (Matsubara and Sasaki, 1969) was made; a similar estimate was made for cysteine–cystine recovery as cysteic acid (Hirs, 1967b).

A $\bar{v}$ value of 0.732 cm³/g was calculated from the amino acid composition data (Schachman, 1957). The value of $\bar{v}$ could be slightly higher (up to 0.734 cm³/g) if the number of amide residues, currently identified as aspartic acid and glutamic acid, was known.

TABLE IV: Amino Acid Composition of Transaminase B.[a]

Amino Acid Residue	μmoles/100 μmoles of Amino Acids Recovered	No. of Residues per 31,500 daltons	Estimated Nearest Integer/ 31,500
Lysine	4.02 ± 0.09	11.55	12
Histidine	2.48 ± 0.19	6.98	7
Arginine	6.18 ± 0.12	17.81	18
Aspartic acid	9.14 ± 0.35	26.15	26
Threonine	4.91 ± 0.18	14.01	14
Serine		18	18
Glutamic acid	9.84 ± 0.17	28.93	29
Proline	4.16 ± 0.52	12.52	11
Glycine	10.23 ± 0.25	29.21	29
Alanine	9.08 ± 0.26	25.92	26
Valine	8.20 ± 0.27	23.40	23
Isoleucine	5.83 ± 0.19	16.69	17
Leucine	6.94 ± 0.16	19.65	20
Tyrosine	3.45 ± 0.12	9.75	10
Phenylalanine	4.23 ± 0.18	12.01	12
Tryptophan	1.91 ± 0.07	5.35	6
Cysteine		2.46	3
Methionine		6.73	7

[a] Duplicate samples, one containing 2% thioglycolic acid, were hydrolyzed for 24, 48, 60, and 72 hr. Proline values represent the average obtained in hydrolysates without thioglycolic acid; tryptophan values are the average for the 24-, 48-, and 60-hr hydrolysates with thioglycolic acid; and isoleucine values are the average of the 60- and 72-hr hydrolysates. The 60-hr thioglycolic acid value was not used in the calculations for threonine. Serine values were obtained by extrapolation to zero time. Cysteine and methionine were determined as cysteic acid and methionine sulfone, respectively.

Titration with p-Mercuribenzoate. To obtain additional information about the sulfhydryl residues in the native enzyme, a series of *p*-mercuribenzoate-titration experiments was conducted. The results of the first experiment revealed that in the presence of excess reagent approximately 1 equiv of *p*-mercuribenzoate had reacted per enzyme subunit within 40 min; however, further reaction was not extensive within 24 hr of incubation (Figure 3A). It was necessary, therefore, to incubate the enzyme with *p*-mercuribenzoate for prolonged periods of time to observe significant additional reaction of the enzyme with the sulfhydryl reagent. One of the curves in Figure 3A depicts results obtained after 5–7 days of incubation and, as indicated, 2.75 sulfhydryl groups were titrated per subunit, a value that is in accord with the total of three cysteine residues obtained from the amino acid composition data. The results also imply that the enzyme does not possess disulfide bridges. Because the enzyme precipitates at pH 5, the recommended pH of 4.6 (Benesch and Benesch, 1962) could not be utilized in these experiments. Also, because of the ability of denatured enzyme to form disulfide bridges (presented above), no attempt was made to prepare denatured enzyme by treatment with guanidinium chloride for subsequent *p*-mercuribenzoate titration. However, titration of the enzyme with *p*-mercuribenzoate in the presence of 4 M guanidinium chloride was attempted, and the results obtained after an incubation period of 20 min are presented in Figure 3B. A value of 3.35 sulfhydryl groups was calculated per subunit. The absorbance values of the *p*-mercuribenzoate–guanidinium chloride samples after 24 hr of incubation were erratic; *i.e.*, significantly lower than those presented in Figure 3B and, therefore, could not be used to acquire additional information. Nevertheless, the values obtained by both of the *p*-mercuribenzoate titration procedures are in agreement with the amino acid composition data.

Thiol and Sulfhydryl Reagents. Ten-minute incubation of the enzyme at 37° with 100 mM β-mercaptoethanol prior to the assay or addition of 1, 10, or 100 mM β-mercaptoethanol to the assay mixture does not increase the transaminase activity. Likewise, reduced glutathione (100 mM), when added to the assay, has no stimulatory effect on enzymatic activity. The effects of sulfhydryl reagents on the activity are also minimal. Preincubation of the enzyme for 15 min at 37° with 1 or 10 mM iodoacetamide, 1 mM *N*-ethylmaleimide, or 1 mM *p*-mercuribenzoate does not significantly inhibit the observed reaction rate, nor does addition of 1 mM $HgCl_2$ to the assay medium. Thus, to date, there is no evidence that the presence of a sulfhydryl group is required for activity.

Discussion

The results of this study on the branched chain amino acid aminotransferase of *S. typhimurium* reveal that the enzyme has an apparent molecular weight of 183,000 ± 5% and a sedimentation coefficient of 9.8 S ± 2%. The single monomeric constituent of the enzyme has a molecular weight of 31,500 ± 10%; thus, it is concluded that the active enzyme is a hexamer. Too, identification of threonine as the only N-terminal amino acid implies that the subunits of the hexamer may be identical.

The association of subunits to form the quaternary structure of the native protein is noncovalent in character; however, each subunit appears to contain at least one sulfhydryl

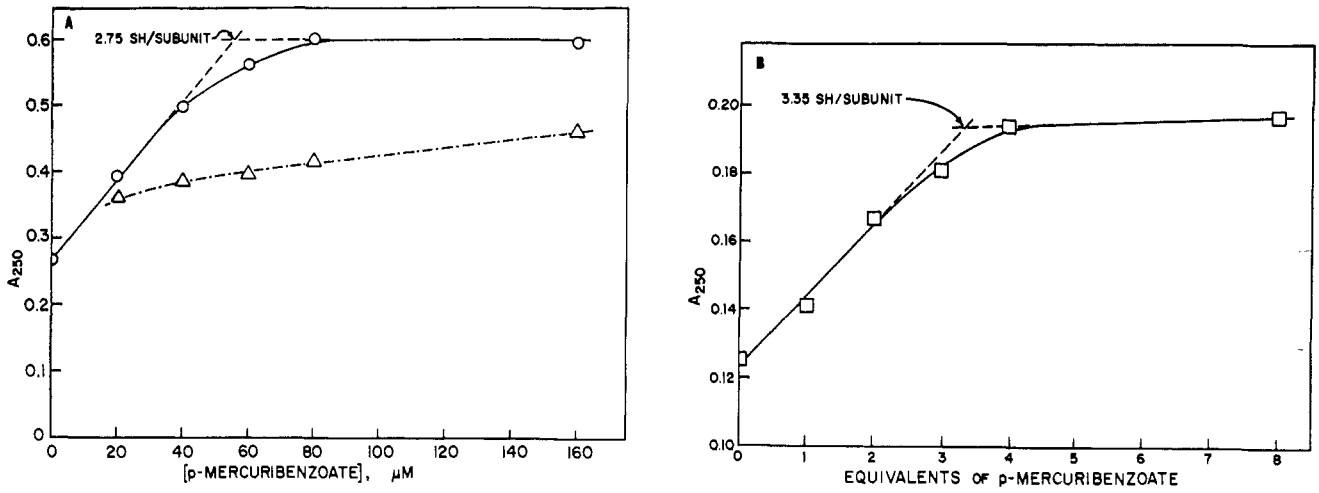

FIGURE 3: (A) Titration of transaminase B with *p*-mercuribenzoate. The (△) and (○) curves represent absorbance values obtained after 24-hr and 5–7-days incubation, respectively. (B) Titration with *p*-mercuribenzoate in presence of 4 M guanidinium chloride. Absorbance values were obtained after 20-min incubation.

group which is shielded from oxidation in the hexamer but which becomes available for interchain disulfide bridge formation on denaturation in 6 M guanidinium chloride (though not in 1% sodium dodecyl sulfate). Titration with *p*-mercuribenzoate also provides evidence for protected sulfhydryl groups, since approximately two-thirds of the titratable cysteine residues require prolonged incubation with the reagent before appreciable mercaptide formation occurs. Thus, it is possible that two of the three sulfhydryl groups in each of the subunits of the hexamer are shielded.

With regard to the sulfhydryl moieties of the enzyme, the lack of inhibition of enzymatic activity by several sulfhydryl blocking reagents and the lack of detectable activation by either β-mercaptoethanol or glutathione imply that a sulfhydryl group is not required for transaminase activity. An alternative possibility is that the "shielded" sulfhydryl groups identified in this study are also protected from interaction with any of the inhibitors, at least during the exposure intervals tested. However, in the case of the analogous cytoplasmic enzyme from pig heart, Taylor and Jenkins (1966b) observed severe inhibition with *N*-ethylmaleimide, *p*-mercuribenzoate, and $HgCl_2$ using virtually identical procedures. Thus, it is clear that the *Salmonella* and the cytoplasmic pig heart aminotransferases display distinctly different responses with respect to sulfhydryl reagents.

The subunit structures of other branched chain amino acid aminotransferases have not been examined; however, a number of molecular weights have been reported. The single microbial enzyme that has been investigated is that of *Pseudomonas aeruginosa*, for which a molecular weight of 130,000 (Sephadex G-200 chromatography) was reported by Norton and Sokatch (1970). With respect to mammalian systems, the molecular weight of the cytoplasmic pig heart aminotransferase was reported to be 75,000 (low speed sedimentation equilibrium) by Taylor and Jenkins (1966a) and that of the mitochondrial form, studied by Aki *et al.* (1967), is very similar (Sephadex G-100 chromatography and *s* value). The supernatant form of the enzyme from hog brain and that of rat ascites hepatoma (two forms with very similar properties) possess molecular weights of 39,000 (equation of Martin and Ames (1961)) and 42,600 (Sephadex G-100 chromatography), respectively (Aki *et al.*, 1968; Ogawa *et al.*, 1970). Thus, the reported data suggest that the mammalian enzymes possess fewer subunits in their quaternary structures than do the microbial forms. Although comparisons among the published data are admittedly of limited value, it may be significant that the weight of the *Pseudonomad* enzyme is very close to a multiple of 31,500 and that the weights of the mammalian forms cluster around 40,000 and multiples thereof. These resemblances among the aminotransferases suggest that future studies could reveal sequence homologies among bacterial forms and also among mammalian types.

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Nitrogen Isotope Effects on the Papain-Catalyzed Hydrolysis of *N*-Benzoyl-L-argininamide†

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ABSTRACT: The papain-catalyzed hydrolysis of *N*-benzoyl-L-argininamide shows nitrogen isotope effects (k^{14}/k^{15}) at 25° of 1.021 at pH 8.0, 1.024 at pH 6.0, and 1.023 at pH 4.0. These isotope effects are much larger than those observed in the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide (O'Leary, M. H., and Kluetz, M. D. (1972), *J. Amer. Chem. Soc. 94*, 3585) and are near the upper limit of nitrogen isotope effects which are observed in reactions in which a bond is broken to an isotopic nitrogen atom. The isotope effects are interpreted in terms of the partitioning of a tetrahedral intermediate. In the case of chymotrypsin, the tetrahedral intermediate partitions rather equally between return to starting materials and loss of ammonia, whereas in the case of papain, the intermediate returns to starting material much more frequently than it decomposes to acyl-enzyme, and carbon–nitrogen bond breaking is entirely rate determining. This interpretation is consistent with the expected properties of the oxygen and sulfur nucleophiles present at the active sites of the two enzymes.

The sulfhydryl-dependent plant protease papain has been studied extensively (Lowe, 1970; Glazer and Smith, 1971). Both the amino acid sequence (Glazer and Smith, 1971) and the X-ray structure of the enzyme (Drenth *et al.*, 1971) have been reported. The minimal mechanism of action is the three-step sequence

$$E + S \rightleftharpoons ES \longrightarrow \underset{+\ P_1}{\text{acyl-E}} \xrightarrow{H_2O} E + P_2 \qquad (1)$$

where ES is the Michaelis complex and acyl-E is the covalent acyl-enzyme intermediate. This acyl-enzyme is formed by the reaction of the substrate with the sulfhydryl group of a cysteine residue at the active site—a reaction which is assisted by the participation of a nearby histidine residue (Husain and Lowe, 1968).

Papain is often compared with chymotrypsin, and it is clear that these two enzymes are similar in many respects. Strong evidence has been presented for the existence of a tetrahedral intermediate in acyl-enzyme formation in the case of chymotrypsin (Fersht and Requena, 1971; Lucas *et al.*, 1973). The presence of such intermediates in nonenzymatic reactions of esters and amides is well documented in many cases (Bender, 1960; Shain and Kirsch, 1968), and it seems natural that such intermediates should also occur in enzymatic reactions. No evidence bearing on the existence of such an intermediate in papain-catalyzed hydrolysis has been presented.

Heavy-atom isotope effects have been used extensively in studies of mechanisms of organic reactions (Fry, 1970), but until recently little use has been made of such isotope effects in studies of enzyme-catalyzed reactions. Heavy atom isotope effects are potentially of great use in studies of enzyme mechanisms because the information they provide is complementary to that provided by rapid-kinetic studies. Whereas the latter provide information about rapid steps in the reaction prior to the rate-determining step, the former provide information about the rate-determining step and any moderately slow steps prior to it.

The pH dependence of nitrogen isotope effects on the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan-

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