α-Dialkylamino Acid Transaminase from *Pseudomonas cepacia*. Purification, Crystallization, Physical, and Kinetic Properties*

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ABSTRACT: α-Dialkylamino acid transaminase was purified from a strain of *Pseudomonas cepacia*. The enzyme has been crystallized and subsequently characterized. By sedimentation equilibrium a molecular weight of 188,000 was determined for the native enzyme. A molecular weight of 47,000 (four subunits) was found when the enzyme was subjected to strong dissociating agents (7 M guanidine hydrochloride–8 M urea), whereas amino acid analysis combined with tryptic peptide maps showed eight subunits. Electrophoresis of the crystalline enzyme in polyacrylamide gels containing sodium dodecyl sulfate showed one band. A K_m value of 8.7×10^{-3} M for α-aminoisobutyric acid and one of 1.7×10^{-4} M for pyruvate were obtained from secondary plots of Lineweaver–Burk plots made at several concentrations of both substrates. Inhibition studies showed that 3×10^{-3} M D-cycloserine or 10^{-5} M L-cycloserine was necessary for 50% inhibition of the enzyme.

Decarboxylation-dependent transamination of α-dialkylamino acids was first demonstrated as a pyridoxal-catalyzed nonenzymatic reaction by Kalyankar and Snell (1962) in an extension of the pioneering studies of Herbst and Engel (1934). Later, an activity capable of catalyzing such a reaction was found in extracts of bacteria that had been isolated from enrichment cultures in which α-dialkylamino acids had served as the sole fixed nitrogen source (Aaslestad and Larson, 1964; Bailey and Dempsey, 1967; Dempsey, 1969). The activity has been purified to apparent homogeneity from one such organism, and several of the enzymatic properties of the enzyme have been studied (Bailey and Dempsey, 1967; Bailey *et al.*, 1970). The physical properties of such an enzyme have not been reported until now.

The enzyme catalyzes the overall conversion of AIB[1] and pyruvate to L-alanine, acetone, and carbon dioxide in two separate steps. In the first, AIB reacts with pyridoxal phosphate–enzyme to form pyridoxamine phosphate–enzyme, CO_2, and acetone. In the second, an exchange transamination occurs by which pyruvate is converted to L-alanine and pyridoxal phosphate–enzyme is regenerated. The enzyme is of interest, as has been pointed out earlier in greater detail (Bailey and Dempsey, 1967; Bailey *et al.*, 1970) because one active site cleaves two different types of groups attached to an α-carbon atom of an amino acid.

The original enzyme studied was isolated from a *Pseudomonas fluorescens* strain (Dempsey, 1969). In undertaking the study of the physical properties of this enzyme we found that the enzyme rapidly and irreversibly lost a large proportion of its activity during purification. After empirically determining that this loss was not preventible by any obvious method we sought another enzyme source. Having isolated and characterized a large number of strains containing this enzymatic activity (Dempsey, 1969) we looked among them for a more stable enzyme. The organisms were grown in identical media, harvested, disrupted by sonic oscillation, centrifuged, and the supernatant solutions fractionated with ammonium sulfate all in a manner identical from one organism to another. All of the active ammonium sulfate fractions were dialyzed in the same vessel for 1 week and portions of each fraction were assayed at several different intervals. The results of these assays showed that the enzyme from *Pseudomonas cepacia* retained 78% of its activity over a 1-week interval whereas that from *P. fluorescens* retained only 50%. In addition, *P. cepacia* provided an extract with a higher initial specific activity.

Experimental Section

Materials. AIB was obtained from Calbiochem and sodium pyruvate from Sigma Chemical Co. These substrates were recrystallized three times before use in the enzyme kinetic studies. Pyridoxal phosphate was purchased from Sigma Chemical Co. [1-^{14}C]AIB was obtained from New England Nuclear Corp. DEAE-cellulose type 40 was obtained from Schleicher & Schuell Inc. and was prepared as described by Peterson and Sober (1962). Hydroxylapatite was prepared by following precisely the procedure of Tiselius *et al.* (1956) as described by Sizer and Jenkins (1962).

Culture of the Bacterium. The bacterium used in these studies was previously isolated and described as organism "i" (Dempsey, 1969). Classification of the organism by standard methods showed it to be a strain of *P. cepacia* (C. A. Lamartiniere and W. B. Dempsey, 1970, unpublished data). *P. cepacia* was grown at room temperature with vigorous aeration in 20-l. carboys containing 16 l. of 63 mM potassium phosphate (pH 7.0) which was 0.4 mM in $MgSO_4$ and 0.01 mM in $FeSO_4$. Glucose and AIB were autoclaved separately and added to a final concentration of 0.2%. Inoculation was with 1.6 l. of fully grown cells from shake cultures in the same medium. The bacteria were harvested in a refrigerated steam-driven Sharples centrifuge, lyophilized, and then stored at −80°.

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[1] Abbreviations used are: AIB, aminoisobutyric acid; SDS, sodium dodecyl sulfate; MSH, 2-mercaptoethanol; DTNB, 5,5′-dithiobis(2-nitrobenzoate); and α-DAT, α-dialkylamino acid transaminase.

Enzyme Assay. The release of CO_2 was measured by capturing in base the $[^{14}C]CO_2$ release from radioactive amino acid in the modification of the Snyder and Godfrey (1961) system previously described by Cuppy and Crevasse (1963). Acetone formation was measured as the dinitrophenylhydrazone (Bailey and Dempsey, 1967). All enzyme assays were run at 37°.

Purification. Lyophilized *P. cepacia* (30 g) were suspended in 300 ml of 20 mM potassium phosphate buffer (pH 7.5)–50 mM KCl and disrupted with a Branson Sonifier for 2 hr. The temperature was maintained below 8° throughout this and further purification steps. The sonicated mixture was centrifuged for 1 hr at 41,300*g*. Saturated ammonium sulfate at pH 7.5 was used to bring the supernatant fluid to 30% saturation and the mixture was allowed to stir overnight at 4°. The mixture was then centrifuged for 1 hr at 18,000*g* and the resulting supernatant fluid brought to 45% saturation in the same manner and allowed to stir at 4° for 4 hr. The mixture was centrifuged for 30 min at 18,000*g* and the precipitate was suspended in 20 mM potassium phosphate buffer (pH 8.5)–0.1 M in KCl and dialyzed overnight against two changes of 10 l. of this same buffer. The dialyzed material was applied to a 2.5 × 45 cm DEAE-cellulose column equilibrated with the same buffer. The column was washed until two protein peaks came off as measured by absorbancy at 280 nm and then a 2-l. linear gradient of 0.1–0.3 M KCl in 20 mM potassium phosphate buffer at pH 8.5 was applied. Fractions were assayed and the most active fractions were pooled and subsequently dialyzed overnight against two changes of 4 l. each of 20 mM potassium phosphate buffer (pH 7.0), 5×10^{-4} M in pyridoxal phosphate. The dialyzed material was then applied to a hydroxylapatite column equilibrated with the same buffer. Starting buffer was run through the column until no more protein came off as measured by absorbancy at 280 nm and then an 800-ml linear gradient of 0.02–0.3 M potassium phosphate (pH 7.0) containing 5×10^{-4} M pyridoxal phosphate was applied. The most active fractions were pooled and then brought to 70% saturation with solid ammonium sulfate, saturated ammonium sulfate at pH 8.0 being used to maintain the pH at 7.

The mixture was stirred overnight, the contents subsequently centrifuged and the precipitate was dissolved in 20 mM potassium phosphate buffer (pH 7.0) so that the protein solution was approximately 10–15 mg/ml. Crystallization was then initiated and performed at 4°. Finely ground ammonium sulfate was slowly added with stirring until a slight turbidity appeared. The solution was then stirred continuously while the turbidity increased. It was then stored in the refrigerator 4–7 days with occasional stirring during which we observed that silkiness could be seen upon swirling the suspension. Microscopic observation revealed crystal formation.

Molecular Weight Determination. High-speed sedimentation equilibrium studies of α-DAT were conducted in a Spinco Model E ultracentrifuge. Ultracentrifugation was performed with a 3.0-mm liquid column according to the method of Yphantis (1964) using a double-sector cell with sapphire windows. The protein concentration was between 0.05 and 0.3 mg per ml. Fringe displacement was measured with a Nikon profile projector Model 6C equipped with a digital micrometer. Ordinate displacements greater than 100 μ were used in calculations of molecular weight. A partial specific volume of 0.74 was assumed in molecular weight computation.

Gel Electrophoresis. Analytical gel electrophoresis of the enzyme was carried out according to the method of Davis (1964). Electrophoresis of the native enzyme was conducted in 7.5% acylamide gel at pH 8.3. Enzyme dialyzed against 8 M urea, 0.1 M MSH, and 10 mM potassium phosphate buffer (pH 7.0) was subjected to gel electrophoresis in gels in which 8 M urea had been incorporated. Urea was also in the buffer system in this case. Both types of electrophoresis were carried out with a current of 2 mA/gel until the tracking dye reached the end of the gel. Gels were then stained with 5% Amido-Schwarz in 7% acetic acid.

SDS gel electrophoresis was carried out by a procedure of U. Laemmli and J. Maizel (personal communication from E. Ehrenfeld, 1970). Enzyme samples were heated for either 3 or 5 min at 70° in the presence of 2% SDS and 0.1 M MSH and then cooled and applied to SDS gels. A current of 3 mA/gel was applied for 2 hr at room temperature. The gel was subsequently washed with methanol–20% acetic acid (1:1, v/v) solution for 4 hr to remove the SDS and then stained with a 5% solution of Amido-Schwarz in 20% acetic acid–methanol (1:1, v/v). All gels were destained in a Canalco quick gel destainer in 7% acetic acid.

Dry Weight. The dry weight of α-DAT was determined with enzyme which had been recrystallized three times and then dialyzed against three changes of 2 l. each of 10 mM potassium phosphate buffer (pH 7.0.). The absorption spectrum was recorded on a Cary Model 15 spectrophotometer. Samples of approximately 2 mg of the above protein were first dried under reduced pressure in an oven at 120° and then held in a desiccator containing P_2O_5 until constant weight was obtained. The weight of the buffer in the samples was corrected for by drying portions of the final dialysate by the same procedures and then subtracting the buffer value.

Amino Acid Analysis. To ampoules containing 0.5 mg of α-DAT in 10 mM potassium phosphate buffer (pH 7.0) an equal volume of concentrated HCl was added. The mixture was then brought to 5.0 ml by the addition of constant-boiling HCl. The samples were frozen, evacuated, and then flushed with nitrogen while thawing. This procedure was repeated four times and then the ampoules were sealed. Triplicate samples were hydrolyzed at 110° for time periods of 24, 48, and 72 hr. After acid hydrolysis the HCl was removed by evaporation under reduced pressure and amino acid analysis was carried out on a Beckman 120C amino acid analyzer equipped with an integrator. Extrapolation to zero-time hydrolysis was employed for serine and threonine values. Cysteic acid and methionine sulfoxide were determined with the amino acid analyzer after performic acid oxidation and acid hydrolysis of the enzyme (Moore, 1963). Cysteinyl SH groups were determined by titration of the enzyme with Ellman's reagent (Ellman, 1959). Tryptophan was determined by the spectrophotometric method of Bencze and Schmid (1957).

Tryptic Peptide Mapping. The procedure for peptide mapping was as described by Chernoff and Liu (1961) with the following modifications. Enzyme (5 mg) dialyzed against 10 mM potassium phosphate buffer was boiled for 5–10 min and then made 50 mM in ammonium bicarbonate at pH 8.1. Trypsin was added to the protein at a ratio of 1:50 and digestion proceeded at 35° with shaking for 3 hr and then a similar addition of trypsin was made for 3 more hr for complete digestion. The mixture was then lyophilized and resuspended in a minimum amount of water. Approximately 2 mg of treated protein was then applied to Whatman No. 3 paper. Descending chromatography was accomplished with a solvent system of 1-butanol–pyridine–acetic acid–water (15:10:3:12, v/v) (Uyeda and Kurooka, 1970). Time of chromatography was 20 hr. The paper was allowed to dry and then moistened with pyridine–acetic acid–water (pH 6.5, 25:1:250, v/v) and subjected to electrophoresis (Uyeda and Kurooka, 1970) in a

TABLE I: Purification of α-Dialkylamino Acid Transaminase from *P. cepacia*.

Purification Step	Vol (ml)	Total Protein (mg)	Sp Act. (μmoles of CO_2/ min per mg)	Yield (%)
Sonicate	293	9099	0.40	100
30–45% $(NH_4)_2SO_4$	197	3640	0.79	79
DEAE	238	501	4.67	64
Hydroxylapatite	250	224	7.80	48
First crystallization	10	156	8.60	37
Second crystallization	10	122	9.0	30
Third crystallization	10	103	8.8	25

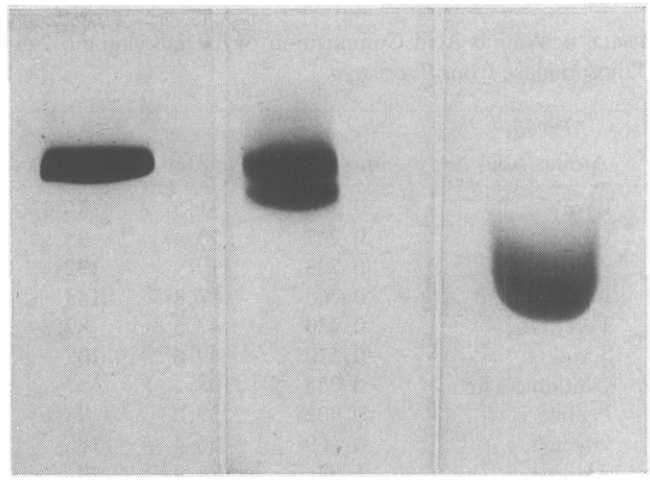

FIGURE 1: Analytical gel electrophoresis of α-dialkylamino acid transaminase from *P. cepacia*. Acrylamide concentration in all gels was 7.5%. Left: 40 μg of native enzyme. Center: 60 μg of enzyme dialyzed *vs.* 8 M urea–0.1 MSH and electrophoresed on gel containing 8 M urea. Right: 32 μg of enzyme heated for 5 min at 70° in 2% SDS and electrophoresed for 2 hr with 3 mA of current. In each case the sample is applied to the top of the gel as the picture is now oriented.

Savant high-voltage electrophoresis apparatus by applying a potential of 2000 V for 85 min. The paper was dried and sprayed with ninhydrin reagent. After the spots were marked, the paper was treated with Sakaguchi reagent to detect arginine-containing peptides.

Immunological Studies. Three-times-recrystallized α-DAT (4 mg) was mixed with Freund's complete Adjuvant (Difco) to form a stable emulsion and injected into the thigh of a young adult rabbit. After 10 days 2 mg of α-DAT was injected again to obtain a secondary response. Three weeks after the initial injection a high titer of precipitating antibody was detected.

Microbiological Assay of Vitamin B_6. Pure α-DAT which had first been dialyzed against 20 mM AIB was hydrolyzed for 5 hr in 0.055 N H_2SO_4 at 121°. Total vitamin B_6 content was assayed with *Saccharomyces carlsbergensis* by the method previously reported (Dempsey, 1965).

Results

Enzyme Purification. Table I summarizes the purification procedure used to isolate α-DAT from *P. cepacia*. This method was used repeatedly with the same results indicated here. A homogeneous protein with a 25% recovery was obtained as the final product after the 22-fold purification. Slender needle-like crystals were observed upon microscopic examination of crystallized enzyme. Homogeneity was suggested by our finding of constant high specific activity in the most active fractions of the hydroxylapatite chromatography, by a straight line when fringe displacement was plotted against the distance from the center of rotation on sedimentation equilibrium experiments, by a single band upon polyacrylamide gel electrophoresis at pH 8.3, and a single well-defined line on double-immunodiffusion plates. The enzyme was relatively stable during storage at 5° for 2–3 days, however after several weeks at 5°, the catalytic activity gradually dropped from a specific activity of 8 to one of 2. This activity loss was not reversible by further recrystallization. Sulfhydryl compounds, pyridoxal phosphate, and substrates were ineffective in preventing this slow loss of activity.

Molecular Weight. α-Dialkylamino acid transaminase from *P. cepacia* was centrifuged to equilibrium at 17,980 rpm for 24 hr at 12°. An average molecular weight of 188,000 was obtained for the native enzyme. Ultracentrifugal analysis of the enzyme at 31,410 rpm and 20° in 8 M urea, 0.1 M MSH yielded data from which a molecular weight of 48,000 was calculated. Similarly, a molecular weight of 46,000 was obtained when the enzyme was centrifuged in 7 M guanidine·HCl–0.1 M MSH at 35,600 rpm and 20°.

Gel Electrophoresis. The results of gel electrophoresis are shown in Figure 1. Electrophoresis of the native enzyme showed one band in 7.5% acrylamide gels (left). Enzyme dialyzed 18 hr against 10 mM potassium phosphate buffer (pH 7.0) and 8 M urea–0.1 M MSH and then applied to 7.5% acrylamide gel containing 8 M urea revealed two discrete bands after electrophoresis (center tube). Using a Gilford linear transport Model 240 spectrophotometer we found a ratio of 1 to 4 for the amount of the light band relative to the amount of the dark band in this case.

Enzyme heated for either 3 or 5 min in 2% SDS and then subjected to electrophoresis in the polyacrylamide gel of Laemmli and Maizel showed one diffused band by optical analysis or after staining (right tube).

Amino Acid Analysis. Results of the amino acid analysis of α-DAT is shown in Table II; 100% of the dry weight of the crystalline enzyme was accounted for by amino acids. Titration of the native enzyme with Ellman's reagent demonstrated no exposed SH groups, however, after incubation of the enzyme with 4 M urea, eight SH groups were detected. Reduction of the enzyme with $NaBH_4$ and treatment with 4 M urea before titration failed to demonstrate additional SH groups.

Tryptic Peptide Mapping. Figure 2 shows a tracing of the ninhydrin-positive spots seen after two-dimensional chromatography of the tryptic digest of α-DAT. A total of 23 spots was seen.

Kinetics. The overall reaction catalyzed by this enzyme is the sum of a decarboxylation-dependent transamination and an exchange transamination (Bailey and Dempsey, 1967). During our studies we found that high concentrations of pyruvate inhibited the reaction (Figure 3) while no substrate inhibition was observed with AIB. True K_m values for this reaction have not been previously determined in any system and were obtained for pyruvate and AIB by using the second-

TABLE II: Amino Acid Composition of α-Dialkylamino Acid Transaminase from *P. cepacia*.

Amino Acid	μmoles/mg	μg/mg	Residues/ Mole
Lysine	0.246	31.5	46.2
Histidine	0.207	28.4	38.9
Arginine	0.703	110	132
Aspartic acid	0.763	87.8	143
Threonine	0.470	47.5	89.3
Serine	0.570	49.6	107
Glutamic acid	0.835	108	157
Proline	0.402	39.0	75.6
Glycine	0.489	56.4	186
Alanine	1.07	75.8	201
Valine	0.476	47.2	89.5
Isoleucine	0.441	49.9	82.9
Leucine	1.12	126	210
Tyrosine	0.213	34.8	40.0
Phenylalanine	0.316	46.5	59.4
Tryptophan	0.071	13.2	13.4
Cysteine	0.099	11.0	18.6
Methionine	0.270	35.4	50.8

ary plot system of Dalziel (1957). This method involved determining the V_{max} values for AIB and pyruvate in several separate experiments at different concentrations of the unvaried substrate. These values were 1.7×10^{-4} M for pyruvate and 8.7×10^{-3} M for AIB. Pyridoxal phosphate was found necessary for enzyme catalysis since CO_2 release from AIB by pure dialyzed enzyme in the standard assay was increased 200-fold in the presence of pyridoxal phosphate.

Using varying concentrations of D- or L-cycloserine (an alanine analog) we attempted to demonstrate stereospecificity of the enzyme. It was found that 3×10^{-3} M D-cycloserine was necessary for 50% inhibition while 10^{-5} M L-cycloserine gave the same effect (Figures 4 and 5). A similar comparison with similar results has been reported for the *P. fluorescens* enzyme (Bailey *et al.*, 1970), and for other pyridoxal phosphate containing enzymes, *e.g.*, Martinez-Carrion and Jenkins (1965). In each case the possibility that the inhibition seen with D-cycloserine was due to contamination with the L isomer was not ruled out.

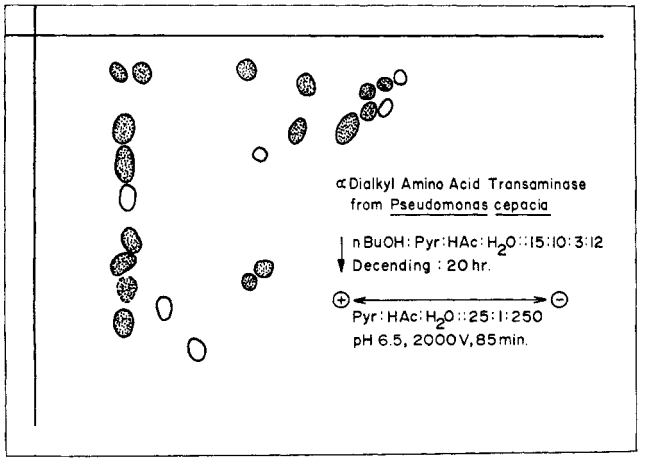

FIGURE 2: Tracing of peptide map of tryptic digest of α-dialkylamino acid transaminase from *P. cepacia*. Closed circles are from ninhydrin stain. Dark areas represent Sakaguchi stain.

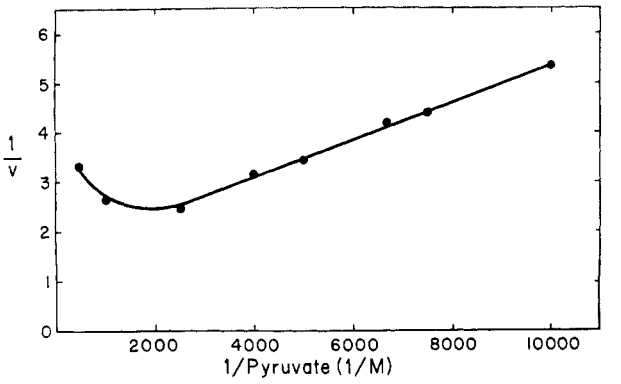

FIGURE 3: A plot of the reciprocal of initial velocity *vs.* the reciprocal of pyruvate concentration. AIB concentration was 20 mM.

Spectral Properties. The spectrum of a dilute solution of the pure enzyme dialyzed against 10 mM potassium phosphate (pH 7.0) showed a maximum absorbance at 278 nm with an extinction coefficient of 0.662 for a 1-mg/ml solution in 1-cm cells. Other maxima were not seen. Solutions of the enzyme at 1.5 mg/ml showed a very small maximum at 320 nm after exhaustive dialysis against pH 7.0 phosphate buffer and 20 mM AIB. By addition of pyridoxal phosphate to such an enzyme preparation, we were able to calculate from the resulting spectra an approximate dissociation constant of 1.4×10^{-5} M for an enzyme–pyridoxal phosphate complex (one binding site per 188,000 mol wt enzyme was assumed). It is obvious from this value that the amount of spectral change seen during this spectral titration was very small, therefore the error in this figure may be significant. The interesting thing we found was that the only new absorbance maximum which appeared was at 320 nm, all other maxima observed to change with each addition of pyridoxal phosphate were indentical with those of free pyridoxal phosphate. Upon inspection, the spectrum of the α-DAT preparation after dialysis against AIB but before pyridoxal phosphate addition also showed a small absorbance maximum at 320 nm. Analysis of total vitamin B_6 content of this preparation by a microbiological assay showed 0.25 mole of pyridoxal phosphate was bound per 188,000 g of enzyme. From this figure a molar absorptivity at 320 nm of about 10,000 was calculated by assuming all the unique absorbance at this wavelength derived from bound pyridoxal phosphate.

Discussion

Sedimentation equilibrium analysis of the native enzyme showed an average molecular weight value of 188,000 for the enzyme in buffer. An average value of 47,000 was found when sedimentation equilibrium analysis was performed in either 7 M guanidine·HCl or 8 M urea. These data suggested that the native enzyme was composed of four subunits. When the results of tryptic peptide analysis were combined with those of the amino acid analysis, however, we found that the native enzyme was apparently composed of eight subunits. That is, we found 23 spots on the chromatogram, 17 were arginine-containing and 6 were not. The amino acid analysis showed 132 arginine and 46 lysines. This would predict that approx-

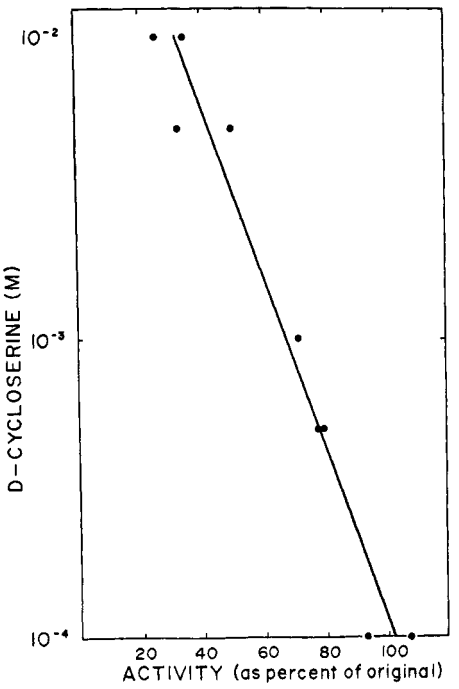

FIGURE 4: The effect of varying concentrations of D-cycloserine on α-dialkylamino acid transaminase from *P. cepacia*. The points were fitted by method of least squares.

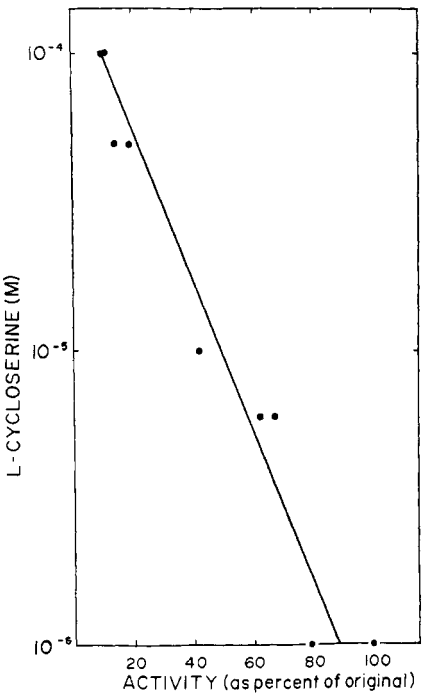

FIGURE 5: The effect of varying concentrations of L-cycloserine on α-dialkylamino acid transaminase. The points were fitted by method of least squares.

imately 23 spots would be present for an 8-subunit protein and 45 for a 4-subunit protein regardless of whether the calculation was based upon total lysine plus arginine or upon the content of either amino acid separately.

The reason for the discrepancy between the data from 7 M guanidine·HCl and those from the tryptic map is not clear at present. Obviously it may be that neither 7 M guanidine·HCl nor 8 M urea is able to separate the subunits completely. In support of this view is our finding that the subunits apparently fail to separate completely during polyacrylamide gel electrophoresis in 8 M urea (Figure 1). Similarly, there may be errors in interpreting the peptide map which at this writing are not obvious. It should be pointed out here, however, that the crystalline α-DAT from an *Arthrobacter* species has the same molecular weight as the *P. cepacia* enzyme and does show eight subunits upon treatment with 7 M guanidine·HCl (H. Itoh, C. A. Lamartiniere, and W. B. Dempsey, 1971, unpublished data).

Amino acid analysis of α-DAT from *P. cepacia* did not demonstrate any obvious peculiar amino acids. Determination of SH groups by the amino acid analyzer and by titration with DTNB yielded different results. Only 8 SH residues were found upon titration with DTNB and yet 19 were calculated from the amino acid analysis. While the reason for this discrepancy is also not known, it may be that both SH analyses and ultracentrifugal analyses give unexplainable data because the enzyme resists dissociation, a property very possibly selected for by our choice of the most stable enzyme of the several available to us.

We found by the secondary plotting techniques of Dalziel (1957) that the K_m for AIB was 8.7×10^{-3} M or the same as the apparent K_m reported for the *P. fluorescens* enzyme (Bailey and Dempsey, 1967) whereas the K_m for pyruvate of 1.7×10^{-4} M is tenfold lower than the apparent K_m reported for the *P. fluorescens* enzyme. This latter difference no doubt comes from the fact that the normal concentration of pyruvate (0.02 M) in the assay system is inhibitory as can be estimated from Figure 3.

Our finding that the enzyme is more susceptible to inhibition by low concentrations of L-cycloserine than it is to D-cycloserine is also in keeping with the findings made by Bailey *et al.* (1970) on the *P. fluorescens* enzyme. In explaining this effect with the *P. fluorescens* enzyme they suggested as a result of other careful studies with model compounds that L-cycloserine, after forming an aldimine with the coenzyme pyridoxal phosphate, lost its α hydrogen to form a stable L-cycloserine–pyridoxal phosphate enzyme complex because the spatial relationship between the methylene of the L-cycloserine and the enzyme active site directed that α hydrogen to the appropriate orientation for cleavage. The methylene of D-cycloserine, on the other hand, directed the *carbonyl* of D-cycloserine into that orientation and as a result, no such stable complex of inhibitor and enzyme could arise (Bailey *et al.*, 1970).

Acknowledgments

The authors thank K. Sims for technical assistance and G. Brooks for assistance in amino acid analysis. We gratefully acknowledge the assistance in performing and interpreting the ultracentrifuge runs which was rendered by K. Uyeda and E. G. Richards. We also thank P. A. Srere for advice and encouragement during this project.

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Inelastic Light-Scattering Study of the Size Distribution of Bovine Milk Casein Micelles*

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ABSTRACT: We have used the technique of inelastic laser light scattering to measure the diffusion coefficients, and thereby the hydrodynamic radii, of fractionated casein micelles from bovine milk The micelles were fractionated by rate-zone centrifugation in sucrose density gradients. Electron microscopic examination of the fractionated material, and the good fit of the experimental light-scattering spectrum to that expected for a monodisperse suspension of spheres, indicated that the fractions were effectively monodisperse. From measurements on various fractions a cumulative distribution curve of the weight fraction of micelles with radii less than or equal to R was constructed. This distribution had about 80% of the casein in micelles with radii between 500 to 1000 Å, and 95% between 400 and 2200 Å, with a most probable radius of about 800 Å. These radii, determined for micelles in solution, are two to three times larger than those found by other workers using electron microscopy on apparently comparable micellar preparations.

The shape and size distribution of bovine milk casein micelles have been studied by a number of workers with the electron microscope (Nitschmann, 1949; Hostettler and Imhof, 1952; Knoop and Wortmann, 1960; Saito and Hashimoto, 1964; Rose and Colvin, 1966; Carroll *et al.*, 1968). From these studies it can be concluded that casein micelles are strongly polydispersed, roughly spherical in shape, and that their diameters range from 400 to 3000 Å with an average size of about 800 Å. In all these investigations, different techniques for preparation and fixation of the micelles and the shadowing of the specimen were used. In interpreting electron micrographs the possibility is ever present of being misled by artifacts produced by a particular preparative method. Moreover, it is extremely difficult to count small micelles and discriminate them from the background materials on the grid (Carroll *et al.*, 1968). In addition, the limitation of studying only dehydrated micelles under the electron microscope may give a size distribution which no longer represents that in the native milk. Size measurements of casein micelles dispersed in simulated milk ultrafiltrate (SMUF)[1] (Jenness and Koops, 1962), as in the present study, should more correctly reflect their actual size distribution in milk.

Morr *et al.* (1971) described a rate-zone ultracentrifugation technique for fractionating skim milk casein micelles in sucrose gradients. Casein micelles were separated from soluble caseins and the micellar caseins were further separated into a number of rather uniform size fractions. The reduced polydispersity of these fractions has made it possible to determine the average size of the micelles in each fraction in a solvent system similar to milk, thereby permitting a reconstruction of the size distribution curve.

In recent years, the technique of inelastic light scattering has been widely used to measure the diffusion constants and hydrodynamic radii of biological macromolecules (Dubin *et al.*, 1967, 1970; Cummins *et al.*, 1969; Rimai *et al.*, 1970; Ford *et al.*, 1969). This method measures the spectrum of laser light inelastically scattered from a solution of macromolecules undergoing brownian motion. It is ideally suited for the study of dilute solution of micelles, whose scattered light spectrum has high intensity and spectral width conveniently in the audiofrequency range.

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[1] SMUF is a salt solution (pH 6.6) containing calcium, magnesium, potassium, sodium, phosphate, citrate, carbonate, sulfate, and chloride, which simulates milk ultrafiltrate.