Brock, C. J., Harris, J. I., & Sato, S. (1976) *J. Mol. Biol. 107*, 175–178.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.
Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1959) in *Data for Biochemical Research*, pp 226–231, Clarendon Press, Oxford.
Dougherty, H. W., Sadowski, S. J., & Baker, E. E. (1978) *J. Biol. Chem. 253*, 5220–5223.
Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.
Fridovich, I. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 41*, 35–97.
Fridovich, I. (1975) *Annu. Rev. Biochem. 44*, 147–159.
Gray, W. H. (1972) *Methods Enzymol. 25*, 333–344.
Harris, J. I., & Steinman, H. M. (1977) in *Superoxide and Superixode Dismutase* (Michelson, A. M., McCord, J. M., & Fridovich, I., Eds.) pp 225–230, Academic Press, London.
Harris, J. I., & Walker, J. E. (1977) in *Nucleotide-Dependent Dehydrogenases* (Sund, H., Ed.) pp 43–61, de Gruyter and Co., West Berlin.
Harris, J. I., Auffret, A. D., Northrop, F. D., & Walker, J. E. (1980a) *Eur. J. Biochem.* (in press).
Harris, J. I., Hocking, J. D., Runswick, M. J., Suzuki, K., & Walker, J. E. (1980b) *Eur. J. Biochem.* (in press).
Hayashi, R. (1977) *Methods Enzymol 47*, 84–93.
Hengartner, H., & Harris, J. I. (1975) *FEBS Lett. 55*, 282–285.
Henry, L. E. A., Halliwell, B., & Hall, D. O. (1976) *FEBS Lett. 66*, 303–306.
Klapper, D. G., Wilde, C. E., III, & Capra, J. D. (1978) *Anal. Biochem. 85*, 126–131.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Liu, T.-Y., & Chang, Y. H. (1971) *J. Biol. Chem. 246*, 2842–2848.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Martin, B., Svendsen, I., & Ottesen, M. (1977) *Carlsberg Res. Commun. 42*, 99–102.
McCord, J. M., & Fridovich, I. (1969) *J. Biol. Chem. 244*, 6049–6055.
McLachlan, A. D. (1977) *Int. J. Quantum Chem. 12*, 371–385.
McLachlan, A. D., & Walker, J. E. (1977) *J. Mol. Biol. 112*, 543–558.
Mendez, E., & Lai, C. Y. (1975) *Anal. Biochem. 68*, 47–53.
Milstein, C. (1966) *Biochem. J. 101*, 338–351.
Offord, R. E. (1966) *Nature (London) 211*, 591–593.
Richardson, J. S., Thomas, K. A., Byron, H. R., & Richardson, D. C. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1349–1353.
Rotilio, G., Bray, R. C., & Fielden, E. M. (1972) *Biochim. Biophys. Acta 268*, 605–609.
Smit, J. D. G., Pulver-Sladek, J., & Jansonius, J. N. (1977) *J. Mol. Biol. 112*, 491–494.
Steinman, H. M. (1978) *J. Biol. Chem. 253*, 8708–8720.
Tarr, G. E., Beecher, J. F., Bell, M., & McKean, D. J. (1978) *Anal. Biochem. 84*, 622–627.
Thompson, E. O. P., & Sanger, F. (1963) *Biochim. Biophys. Acta 71*, 488–491.
Walker, I. D., Harris, J. I., Runswick, M. J., & Hudson, P. (1976) *Eur. J. Biochem. 68*, 255–269.
Walker, J. E. (1979) *Proc. FEBS Meet., 1978 52*, 211–225.
Walker, J. E., Auffret, A. D., Brock, C. J., & Steinman, H. M. (1980a) in *Active Oxygen in Biological Species* (Bannister, J. V., Ed.) Pergamon Press, Oxford (in press).
Walker, J. E., Carne, A. F., Runswick, M. J., Bridgen, J., & Harris, J. I. (1980b) *Eur. J. Biochem.* (in press).
Walker, J. E., Wonacott, A. J., & Harris, J. I. (1980c) *Eur. J. Biochem.* (in press).
Woods, K. R., & Wang, K. T. (1967) *Biochim. Biophys. Acta 133*, 369–370.

# Purification and Properties of the Inducible Enzyme Cyanase†

Paul M. Anderson

ABSTRACT: Cyanase (cyanate hydrolase EC 3.5.5.3) has been purified 270-fold to a high state of purity from *Escherichia coli* B. The native enzyme has a molecular weight of ~150000 as estimated by sucrose density gradient centrifugation and gel-filtration chromatography on Bio-Gel P-300. The enzyme is an oligomer composed of apparently identical subunits which have a molecular weight of ~15000 as estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Amino acid analyses showed that the enzyme contains no tryptophan and a single histidine residue, based on a subunit molecular weight of 14661. Catalytic hydrolysis of cyanate was found to be dependent on the presence of bicarbonate and to be affected by ionic strength. The concentration of bicarbonate required to give half-maximal activity in the presence of 2 mM potassium cyanate was 0.1 mM. The apparent $K_m$ for cyanate in the presence of 3 mM bicarbonate is 0.6 mM. The initial product of the reaction is carbamate (or a related, unstable compound and/or carbamate precursor) which subsequently decomposes to ammonia and bicarbonate.

Studies in our laboratory and elsewhere have indicated that cyanate is a site-specific inhibitor of certain enzymes (Anderson & Carlson, 1975; Chollet & Anderson, 1978; Fan & Plaut, 1974; Shen & Colman, 1975; Sluyterman, 1967; Schroeder et al., 1969). As a result of these and current related studies and also because of recent interest in the potential use of cyanate as an antisickling agent (Brewer, 1976), we initiated an investigation of the properties of the enzyme cyanase (EC 3.5.5.3) reported to be present in rat and guinea pig tissues (Holtham & Schutz, 1948), bacteria (Taussig, 1960, 1965; Guilloton & Hargreaves, 1972), and plants (Lisanti, 1963; Lotti, 1963). This enzyme catalyzes hydrolysis of cyanate to ammonia and bicarbonate.

† From the Department of Biochemistry, School of Medicine, University of Minnesota, Duluth, Duluth, Minnesota 55812. *Received December 26, 1979.* This work was supported in part by Grant GM-22434 from the National Institute of General Medical Sciences, U.S. Public Health Service.

0006-2960/80/0419-2882$01.00/0 

Our initial studies established that the activity which had been described in rat and guinea pig tissues was an artifact related to the assay conditions and that these tissues probably do not, in fact, posses a real cyanase activity (Collins & Anderson, 1977). We have, however, confirmed the presence of the inducible cyanase in *Escherichia coli* reported by Taussig (1965). The isolation of this enzyme and the properties of the highly purified enzyme are described in this report.

## Materials and Methods

*Materials.* Reagent-grade potassium cyanate was recrystallized before use. Sephadex G-25, DEAE[1]-Sephadex A-50, and phenyl-Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, Inc. Ammonium sulfate was ultrapure (enzyme grade) from Schwarz/Mann. Bio-Gel P-300 is a porous acrylamide gel obtained from Bio-Rad Laboratories. Nessler reagent was from Harleco. Ammonium carbamate was purchased from ICN Pharmaceuticals, Inc. Reagents used for electrophoresis, enzymes and marker proteins, and other biochemicals were purchased from Sigma Chemical Co.

*Measurement of Cyanase Activity.* The rate of hydrolysis of cyanate catalyzed by cyanase was determined by measuring ammonia formation with Nessler reagent (Koch & McMeekin, 1924; Lang, 1959). The standard assay mixture contained sodium bicarbonate (3 mM), potassium cyanate (2 mM), potassium phosphate buffer (50 mM, pH 7.6), and enzyme in a final volume of 2.0 mL. The reaction was terminated after 10 min at 37 °C by the addition of 0.9 mL of standard Nessler reagent which had been diluted 1:3 with water. The amount of ammonia formed (0.02–0.7 μmol) was determined by measuring the absorbance at 420 nm within 1–5 min after the addition of Nessler reagent. The absorbance was measured with a Beckman Model 24 spectrophotometer equipped with a sipper for rapid sample analysis.

*Electrophoresis.* Polyacrylamide gel electrophoresis under nondenaturing conditions was carried out at pH 8.9, according to the procedure of Davis & Ornstein (1964). Electrophoresis was conducted at 25 °C at 5 mA/column (5 × 80 mm) with a column electrophoresis cell (Model EC250) from E-C Apparatus Corp. Protein bands were fixed and stained by soaking the gels in methanol–acetic acid–water (5:1:5) containing 0.25% Coomassie blue. The gels were destained electrophoretically.

Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was carried out as described by Weber et al. (1972) with 10% gels. Protein samples were prepared for gel electrophoresis by heating at 100 °C for 5 min in a solution containing 0.01 M sodium phosphate, pH 7.0, 1% $NaDodSO_4$, and 1% mercaptoethanol. Protein bands were fixed and stained as indicated above after soaking in fixing solution to remove some of the $NaDodSO_4$.

*Estimation of Molecular Weight.* The subunit molecular weight was estimated by comparing the rate of migration of cyanase subunits with proteins of known subunit molecular weight during gel electrophoresis in the presence of $NaDodSO_4$ as described by Weber et al. (1972) and as outlined above. Electrophoresis was carried out at 26 °C using a vertical gel slab electrophoresis system (Model EC470) from E-C Apparatus Corp. Reference proteins used and their subunit molecular weights were phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 000), and α-lactalbumin (14 400). A linear relationship between the log of the subunit molecular weights of these reference proteins and their rates of migration was obtained.

The $s_{20,w}$ was determined by sucrose density gradient centrifugation as described by Martin & Ames (1961) and as more recently discussed by O'Brien et al. (1978). The linear sucrose gradients (5–20%) were prepared in 0.1 M potassium phosphate buffer, pH 7.6, in a total volume of 4.9 mL. The volume of the sample containing cyanase and/or protein standards added to the top of the gradient was 0.1 mL. Centrifugation was carried out in a Beckman L5-50 ultracentrifuge at 45 000 rpm with a SW 50.1 rotor for 12 h. Lactate dehydrogenase ($s_{20,w}$ = 7.3) and bovine serum albumin ($s_{20,w}$ = 4.6) were utilized as protein standards. After centrifugation the gradients were collected in fractions of 0.15 mL, and the location of the proteins was established by appropriate activity measurements (cyanase and lactate dehydrogenase) or by measurement of protein by the method of Lowry et al. (1951) (albumin). Since it was found that cyanase sedimented at nearly the same rate as lactate dehydrogenase, estimation of the $s_{20,w}$ for cyanase was made by the simple ratio method of Martin & Ames (1961) using lactate dehydrogenase as the reference protein.

The molecular size of native cyanase was estimated by gel-filtration chromatography as described by Andrews (1965) using Bio-Gel P-300 in a 2.5 × 50 cm column equilibrated with 0.1 M potassium phosphate, pH 7.6. Elution was carried out at a flow rate of 6 mL/h, and the eluate was collected in 3-mL fractions. The proteins utilized as references and their molecular weights were lactate dehydrogenase (140 000), human γ-globulin (150 000), glutamic-oxaloacetic transaminase (90 000), and ovalbumin (43 000). The location of the protein in the eluted fractions was determined by appropriate activity measurements (cyanase, lactate dehydrogenase, and glutamic-oxaloacetic transaminase) or by the measurement of protein by the method of Lowry et al. (1951) (γ-globulin and ovalbumin). A linear relationship between the elution volumes and the log of the molecular weights of the reference proteins was obtained.

*Amino Acid Analysis.* The amino acid composition of cyanase was determined by using a Beckman amino acid analyzer (Model 120B) modified to give increased sensitivity (Liao et al., 1973). The enzyme was hydrolyzed at 110 °C in 6 N HCl containing 0.02% phenol for 24, 48, and 72 h in triplicate (Moore & Stein, 1963). Total cysteine was determined as cysteic acid in hydrolyzed samples which had been oxidized with performic acid (Moore, 1963). Tryptophan was determined after hydrolysis of the enzyme in 4 N methanesulfonic acid (Simpson et al., 1976).

*Protein Measurement.* Except as indicated above, protein was routinely measured by the dye-binding procedure described by Bradford (1976) with reagents obtained from Bio-Rad Laboratories. Bovine serum albumin was used as the standard protein. The concentration of highly purified cyanase was determined by its absorbance at 280 nm. The $A^{1\%}_{1\text{ cm, }280\text{ nm}}$ was established by amino acid analysis of aliquots of cyanase of known $A_{280}$ diluted with norleucine of known concentration as an internal reference standard.

## Results

### *Enzyme Isolation*

*Growth of E. coli B and Induction of Cyanase Activity. E. coli* B was grown by custom fermentation by Grain Processing Corp. (Muscatine, IA) in a 1000-L aerated fermentation tank at 37 °C with a glucose–salts minimal medium. The minimal

[1] Abbreviations used: DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Caps, 3-(cyclohexylamino)propanesulfonic acid.

medium was inoculated with 40 L of stationary phase preculture. Potassium cyanate (80 g) freshly dissolved in 1 L of water was added to the medium when the cells had reached the half log phase of growth (absorbance at 550 nm of 0.6 in a 1-cm cuvette). After an additional 30 min the culture medium was cooled, and the cells were harvested by centrifugation. The resulting cell paste (2–3 kg from each fermentation batch) was stored frozen at −20 °C. The enzyme activity was stable for months when stored under these conditions.

The following purification procedure describes the isolation of cyanase from 100 g of cell paste. Unless indicated otherwise, all steps were carried out at 4 °C.

*Cell Extract.* The cell paste, suspended in 300 mL of 0.1 M potassium phosphate buffer, pH 7.6, was subjected to ultrasonic treatment for 20 min at full power (Branson Model W185). Cell debris was removed by centrifugation at 10000*g* for 20 min.

*Heat Denaturation.* The supernatant was placed in a 2-L Erlenmeyer flask, and the temperature was brought to 56 °C over a 5-min period of time by swirling in a water bath in which the temperature was 80 °C. The temperature was maintained at 56 °C for 5 min and then rapidly cooled in an ice bath to 25 °C or lower. The heat-denatured precipitate was removed by centrifugation at 10000*g* for 15 min.

*Ammonium Sulfate Fractionation.* Ammonium sulfate (30 g/100 mL of supernatant) was slowly added with stirring; slow stirring was continued for 10 min after all of the $(NH_4)_2SO_4$ had dissolved, and the insoluble protein was then removed by centrifugation at 10000*g* for 15 min. The enzyme, along with other protein, was precipitated from the resulting supernatant by repeating the above process with an additional 15 g of $(NH_4)_2SO_4$ for each 30 g of $(NH_4)_2SO_4$ used in the first fractionation. The precipitate obtained after centrifugation was dissolved in a minimal volume of 0.1 M potassium phosphate buffer, pH 7.6. The $(NH_4)_2SO_4$ was removed by gel filtration on a Sephadex G-25 column (5 × 30 cm) equilibrated with the same buffer used to dissolve the precipitate.

*DEAE-Sephadex Ion-Exchange Chromatography.* This step was carried out at room temperature. The solution containing enzyme was added to a column (5 × 20 cm) of DEAE-Sephadex A-50 equilibrated with 0.1 M potassium phosphate buffer, pH 7.6, containing 0.02% $NaN_3$. The enzyme and other protein were eluted from the column by increasing the phosphate-buffer concentration from 0.1 to 0.5 M; the mixing chamber and the reservoir of the linear gradient system each contained 1400 mL of 0.1 and 0.5 M potassium phosphate buffer, respectively; both solutions also contained 0.02% $NaN_3$. A relatively high concentration of buffer (approximately 0.4 M) was required before the enzyme was eluted, and a very large proportion of the total protein was eluted in several major protein peaks before the enzyme was eluted. Fractions containing the majority of the enzyme were pooled.

*Hydrophobic Column Chromatography.* This step was also carried out at room temperature. Sufficient solid potassium phosphate was added to the pooled fractions to bring the concentration to 0.6 M, pH 6.8. This solution was passed through a column (2.5 × 15 cm) of phenyl-Sepharose equilibrated with 0.5 M potassium phosphate, pH 6.8, containing 0.1 M NaCl and 0.02% $NaN_3$, and the column was washed with this same buffer (~300 mL) until protein was not being eluted from the column, i.e., until the $A_{280}$ had decreased to near zero. The enzyme was then eluted by a linear gradient of decreasing salt concentration. The mixing chamber contained 300 mL of the buffer used to equilibrate and wash the column, and the reservoir contained 300 mL of 0.01 M NaCl containing 0.02% $NaN_3$. Fractions of 15 mL were collected. The peak of enzyme activity was eluted after ~350 mL of eluant had passed through the column. The fractions containing most of the enzyme activity were pooled and concentrated by ultrafiltration. The concentrated solutions were stored at 4 °C in 0.1 M potassium phosphate buffer, pH 7.6.

Table I: Purification of Cyanase[a]

| purification step | vol (mL) | total protein (mg) | total act. (units) | sp act. (units/mg) | yield (%) |
|---|---|---|---|---|---|
| extract | 350 | 5040 | 6001 | 1.2 | 100 |
| heat denaturation | 285 | 1710 | 4970 | 2.9 | 83 |
| $(NH_4)_2SO_4$ fractionation | 51 | 900 | 3253 | 3.6 | 54 |
| DEAE-Sephadex chromatography | 300 | 39 | 3500 | 90 | 58 |
| hydrophobic chromatography | 5.8 | 9.9 | 3200 | 323 | 53 |

[a] Procedures are described in the text. A unit is defined as that amount of activity which catalyzes the hydrolysis of 1 μmol of cyanate in 1 min at 37 °C.

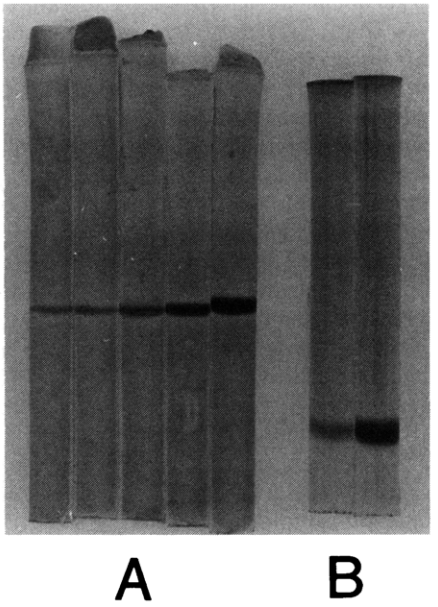



FIGURE 1: Acrylamide gel electrophoresis of purified cyanase. (A) Nondenatured, from left to right: 0.3, 1, 3, 10, and 30 μg of protein. (B) Electrophoresis in $NaDodSO_4$, from left to right: 3 and 30 μg of protein. Electrophoretic procedures are described under Materials and Methods.

The results of a typical enzyme isolation are summarized in Table I. The last step yields enzyme of high purity as judged by acrylamide gel electrophoresis in the absence or presence of $NaDodSO_4$ (Figure 1). The purified enzyme is stable for months at 4 °C (or room temperature, if precautions are taken to prevent microbial growth) in dilute or concentrated neutral or slightly alkaline salt solutions but loses activity when dialyzed against water.

### *Properties of the Purified Enzyme*

*Molecular Weight.* Sucrose density gradient centrifugation studies using lactate dehydrogenase ($s_{20,w}$ = 7.0) as the major marker protein gave a value of $s_{20,w}$ of 7.3 for cyanase. This value corresponds to an approximate molecular weight of 149 000 for a spherical molecule (Martin & Ames, 1961). The $s_{20,w}$ measured by this method was not significantly affected

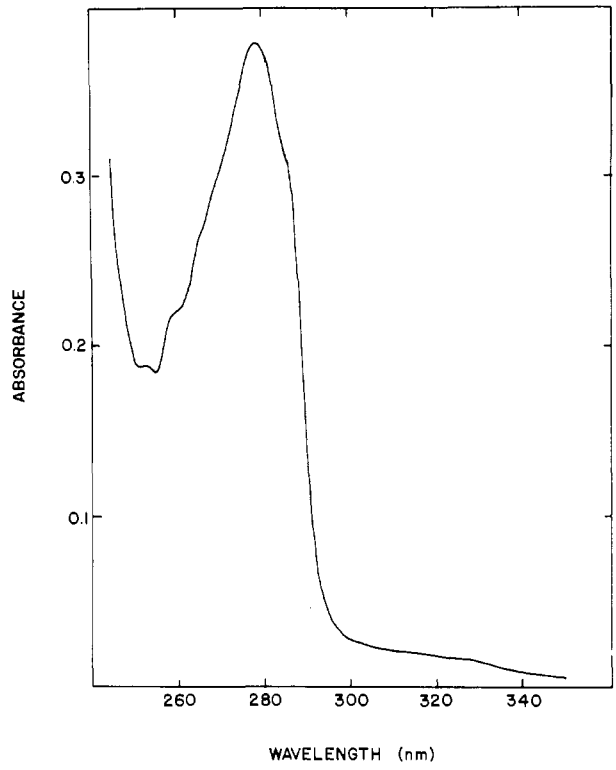



FIGURE 2: Ultraviolet spectrum of cyanase. The enzyme (0.91 mg/mL) was in 0.025 M potassium phosphate buffer, pH 7.5.

by the concentration of cyanase (0.05–1.0 mg/mL in the sample added to the gradient) or by temperature (4 or 22 °C). The molecular weight of the native enzyme estimated by gel filtration on Bio-Gel P-300 is approximately 145 000 at 22 °C and 153 000 at 4 °C. Cyanase eluted in nearly the same volumes as lactate dehydrogenase at 22 °C and γ-globulin at 4 °C. The elution profiles for cyanase activity were symmetrical and sharp even when the concentration of cyanase was quite high. The elution profile was not affected by the presence of 3 mM $NaHCO_3$ or 0.1 M NaCl.

Gel electrophoresis of the purified cyanase in the presence of $NaDodSO_4$ gave a single migrating band (Figure 1) which corresponded to a molecular weight of ~15 200; the cyanase subunit migrated at nearly the same rate as α-lactalbumin in all experiments. This value is in close agreement with the minimum molecular weight of 14 661 calculated from the amino acid composition (Table II).

*Amino Acid Composition.* The amino acid composition of cyanase is shown in Table II. The enzyme contains no tryptophan and a single histidine residue (based on a minimum molecular weight of 14 661). A total of two half-cystine residues was estimated to be present in each subunit on the basis of the number of cysteic acid residues present after performic acid oxidation. Free sulfhydryl groups could not be detected by titration with 5,5′-dithiobis(2-nitrobenzoate) before or after treatment with $NaDodSO_4$ as described by Habeeb (1972) or in the presence of 6 M guanidine hydrochloride or 8 M urea. The absorptivity ($A^{1\%}_{1\,cm,280\,nm}$), determined by measuring the protein concentration in a solution of known $A_{280}$ by quantitative amino acid analysis, was found to be 4.1. This value is somewhat higher than the value of 3.3 calculated from the amino acid composition, as described by Edelhoch (1967). The ultraviolet spectrum of the highly purified cyanase shown in Figure 2 is consistent with the amino acid composition, i.e., the presence of tyrosine but no tryptophan residues (Edelhoch, 1967); the spectrum is similar to the spectrum of the protein kinase inhibitor reported by Demaille et al. (1977) which has one tyrosine but no tryptophan or cystine residues.

*Kinetic Properties.* The presence of bicarbonate is required for catalytic activity, as shown in Figure 3. An apparent binding constant (or $K_m$) for this effect of bicarbonate is not easily established, since bicarbonate is formed during the course of the reaction. However, this contribution to the total bicarbonate concentration (as well as the contribution by endogenous bicarbonate) becomes less significant for added bicarbonate concentrations >0.05 mM. The double reciprocal plot for the data points of Figure 3A from 0.05 to 2.5 mM is linear as shown in the inset. A value of approximately 0.1

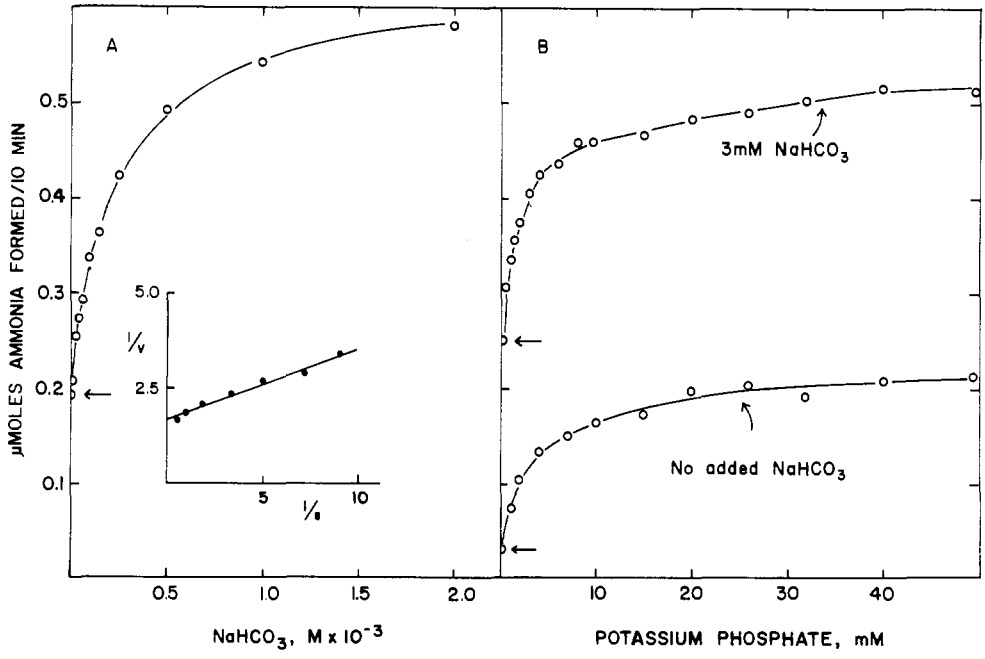



FIGURE 3: Effect of sodium bicarbonate and potassium phosphate on cyanase activity. Enzyme activity was measured as described for the standard assay procedure under Materials and Methods, except that the concentrations of sodium bicarbonate or potassium phosphate were varied as indicated. The assay mixtures contained 0.15 μg of cyanase. The arrows indicate data points obtained without addition of sodium bicarbonate (A) or potassium phosphate (B).

Table II: Amino Acid Composition of Cyanase

| amino acid | no. of residues found[a] | no. of residues nearest integer | amino acid | no. of residues found[a] | no. of residues nearest integer |
|---|---|---|---|---|---|
| histidine | 1.0 | 1 | glycine | 10.1 | 10 |
| lysine | 8.7 | 9 | alanine | 15.9 | 16 |
| arginine | 5.7 | 6 | valine[d] | 6.3 | 6 |
| tryptophan[b] | 0.0 | 0 | methionine | 3.0 | 3 |
| aspartic acid | 15.7 | 16 | isoleucine[d] | 9.8 | 10 |
| threonine[c] | 7.3 | 7 | leucine | 17.8 | 18 |
| serine[c] | 5.2 | 5 | tyrosine | 3.2 | 3 |
| glutamic acid | 12.0 | 12 | phenylalanine | 4.9 | 5 |
| proline | 5.7 | 6 | half-cystine[e] | 2.1 | 2 |

[a] Assuming a single histidine residue and a molecular weight of 14 661. [b] Determined after hydrolysis in 4 N methanesulfonic acid. [c] Extrapolated to zero time of hydrolysis. [d] Extrapolated to infinite time of hydrolysis. [e] Determined as cysteic acid after performic acid oxidation.

mM was determined from these data for the concentration of bicarbonate required for half-maximal activity. The effect of bicarbonate is not due to added sodium cation, since the same effect is observed with the potassium salt.

Catalytic activity also appears to be affected by ionic strength as illustrated in Figure 3B. These results, together with those in Figure 3A, indicate that the effect of buffer concentration is not due to contaminating bicarbonate and also that the effect of bicarbonate is not due to an ionic strength effect, although this may be an additional contribution of bicarbonate in the absence of added buffers (Figure 3B). Phosphate is not specifically required for catalytic activity (e.g., see Figure 5); a similar dependence on ionic strength is observed with other buffers such as Hepes.

The choice of a suitable buffer for kinetic studies is complicated for several reasons: many anions act as inhibitors, probably by competing with the cyanate anion for binding at the active site (Taussig, 1965); the enzyme activity is dependent upon ionic strength; and many buffers interfere with the Nessler reagent used in these studies for measurement of ammonia. Phosphate buffer is the only buffer we have found which is not subject to these problems at higher ionic strength.

The pH optimum for the catalytic activity is 7.4 in phosphate buffer (Figure 4). The apparent $K_m$ value for cyanate in 0.06 M potassium phosphate buffer, pH 7.6, in the presence of 3 mM added bicarbonate is 0.6 mM.

*Ammonia Is Not the Initial Product.* Evidence that the *initial* product of the reaction catalyzed by cyanase may actually be carbamate (or a related compound) rather than ammonia (and bicarbonate) was obtained by measuring ammonia formation with time in the presence of high concentrations of cyanase under conditions in which the transient existence of carbamate could be detected. Carbamate is stable in alkaline solutions such as Nessler reagent and does not give a color reaction with this reagent (Gorin, 1959). Thus, ammonia can be selectively measured by Nessler reagent in the presence of carbamate. Since carbamate decomposes almost instantaneously to carbon dioxide and ammonia in neutral solutions and the rate of decomposition is accelerated by phosphate buffer (Jespersen, 1975), a time lag in ammonia formation, which would be expected if carbamate was the initial product, would not be observed under the normal assay conditions. At a higher pH in Hepes–bicarbonate buffer, however, the rate of decomposition of carbamate is slow enough to measure by manual techniques. This was established by measuring ammonia formation with time when ammonium carbamate was dissolved in buffer under these conditions. Under these conditions, when sufficient cyanase was present to give nearly complete hydrolysis of all cyanate present in ~4 min, a time lag in ammonia formation was observed (Figure 5A, open circles). This lag in ammonia formation could be eliminated if the sample being analyzed was first added to monobasic potassium phosphate for 2 s before ad-

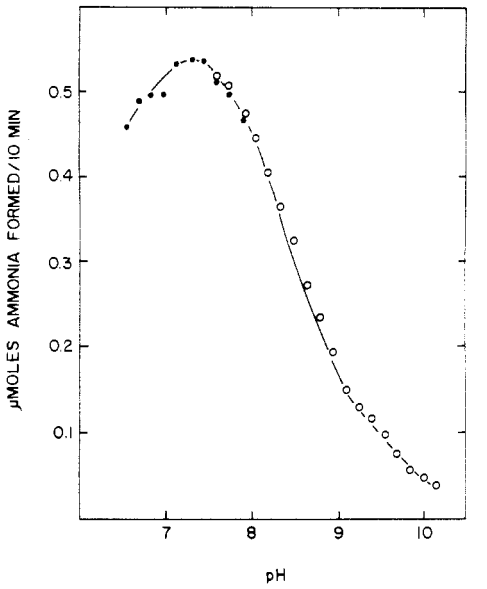



FIGURE 4: Effect of pH on cyanase activity. Enzyme activity was measured as described under Materials and Methods. The 2-mL reaction mixtures contained cyanase (0.15 μg), potassium cyanate (4 mM), sodium bicarbonate (3 mM), and either 30 mM potassium phosphate (●) or 30 mM potassium phosphate plus 2 mM sodium pyrophosphate and 2 mM Caps (○) at the indicated pH values.

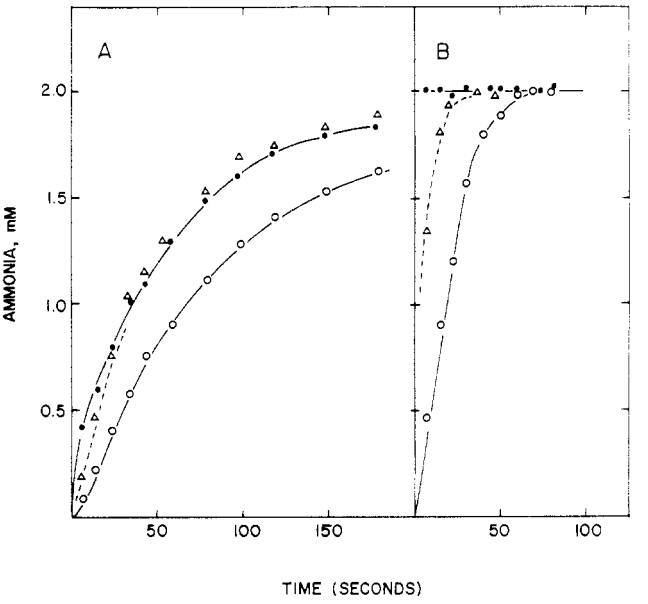



FIGURE 5: Ammonia formation with time. The reaction was carried out at 23 °C. (A) The reaction mixtures contained cyanase (0.06 mg), potassium cyanate (2 mM), sodium bicarbonate (3 mM), and Hepes buffer (20 mM, pH 9.1) in a final volume of 2.0 mL. At the indicated times a 0.1-mL aliquot was quickly removed and added to either 1.0 mL of Nessler reagent previously mixed with 50 μL of 1 M monobasic potassium phosphate (○ and △) or to 50 μL of 1 M monobasic potassium phosphate, followed by immediate (2-s) addition of 1 mL of Nessler reagent (●). The open triangles (△) represent results obtained when carbonic anhydrase (3 mg/mL) was also present in the reaction mixture. (B) is the same as (A) except that the reaction mixtures contained a higher concentration of cyanase (0.6 mg in a final volume of 1 mL).

dition to Nessler reagent or if carbonic anhydrase was present in the reaction mixture (Figure 5A, closed circles and open triangles, respectively); carbamate is quantitatively converted to ammonia during the 2-s exposure to acidic phosphate, and carbonic anhydrase catalyzes decomposition of carbamate (Jones & Lipmann, 1960). The 2-s exposure to acidic phosphate does not result in significant hydrolysis of cyanate. Since cyanate is a potent competitive inhibitor of carbonic anhydrase (Thorsland & Lindskog, 1967), the initial lag in ammonia formation observed in Figure 5A (open triangles) before the cyanate concentration is appreciably reduced would be expected.

The time course of ammonia formation under these same conditions when the concentration of cyanase present in the reaction mixture was sufficiently high to give nearly complete hydrolysis of all cyanate in a few seconds is shown in Figure 5B. Although all the cyanate present had been converted within 7 s to a product which immediately yielded stoichiometric amounts of ammonia when added to acidic phosphate (closed circles), the production of ammonia was much slower (open circles). The presence of carbonic anhydrase greatly reduced the time required for conversion of cyanate to ammonia (open triangles).

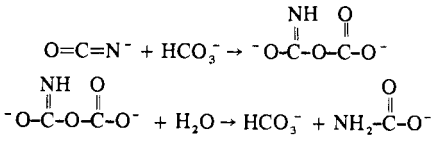
The results of several control experiments demonstrated that the formation of ammonia from carbamate, added with rapid stirring to buffer as solid ammonium carbamate, under the same conditions as described in Figure 5, was qualitatively very similar to the results described in Figure 5B; decomposition was approximately 90% complete after 50 min, the presence of carbonic anhydrase accelerated the rate of ammonia formation, and the 2-s exposure to acidic phosphate resulted in complete decomposition to ammonia.

## Discussion

The relatively simple procedure established for isolating highly purified cyanase from *E. coli* in good yield was made possible in part because the enzyme is quite stable, and the two chromatography steps can be carried out at room temperature. Although present in small amounts, several minor contaminating proteins are present in the final product when these steps are carried out at 4 °C.

The estimates of molecular size indicate that the enzyme is an oligomer composed of 8–12 polypeptide chains which have a molecular weight of ~15 000. Preliminary sequence studies indicate that the 8–12 subunits are probably identical. Although the two different methods used to estimate the molecular weight of the oligomer are potentially subject to considerable error, the relatively close agreement in the estimated molecular weight determined by these two different methods suggests that the shape of the oligomer is probably not highly asymmetric and that the molecular weight estimations are reasonable approximations.

The requirement for bicarbonate for catalytic activity has not been previously reported for cyanase from *E. coli*, although it has been shown to be required for the induced cyanase activity present in a species of *Flavobacterium* (Guilloton & Hargreaves, 1972). The mechanism of bicarbonate activation is unknown. One possibility currently under investigation is that bicarbonate participates as a recycling substrate, perhaps analogous, e.g., to the reaction of carboxylic acids with carbodiimides:



Regardless of the actual mechanism, the studies reported above indicate that the initial product of the reaction is not ammonia and bicarbonate but, instead, is an unstable intermediate (presumably carbamate) which then decomposes nonenzymatically to give ammonia and bicarbonate. Carbamate has also been shown to be an initial product in the hydrolysis of urea catalyzed by urease (Jesperson, 1975; Blakeley et al., 1969) and in the phosphorylation of adenosine 5′-diphosphate (ADP) by carbamoyl phosphate catalyzed by carbamate kinase (Jones & Lipmann, 1960).

The physiological function of this novel enzyme has not been established. Cyanate has been shown to be a natural constituent of some tissues, and it has been suggested that it arises from slow decomposition of urea (Birch & Schutz, 1946); however, it seems more likely that the cyanate measured in these studies actually originated from carbamoyl phosphate, which is known to break down to cyanate in neutral or alkaline solution (Allen & Jones, 1964). Thus, the role of cyanase may be to detoxify cyanate originating from carbamoyl phosphate, which might be produced in excess under certain conditions in some species.

## Acknowledgments

I thank Barbara Wilson and Edward Snyder for their technical assistance and Dr. John Saari for performing the amino acid analyses.

## References

Allen, C. M., & Jones, M. E. (1964) *Biochemistry 3*, 1238.
Anderson, P. M., & Carlson, J. D. (1975) *Biochemistry 14*, 3688.
Andrews, P. (1965) *Biochem. J. 96*, 595.
Birch, K. M., & Schutz, F. (1946) *Br. J. Pharmacol. 1*, 186.
Blakeley, R. L., Hinds, J. A., Kunze, H. E., Webb, E. C., & Zerner, B. (1969) *Biochemistry 8*, 1991.
Bradford, M. (1976) *Anal. Biochem. 72*, 248.
Brewer, G. J. (1976) *Am. J. Hematol. 1*, 121.
Chollet, R., & Anderson, L. L. (1978) *Biochim. Biophys. Acta 525*, 455.
Collins, C. A., & Anderson, P. M. (1977) *Biochem. Biophys. Res. Commun. 79*, 1255.
Davis, B. J., & Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321, 404.
Demaille, J. G., Peters, K. A., & Fischer, E. H. (1977) *Biochemistry 16*, 3080.
Edelhoch, H. (1967) *Biochemistry 6*, 1948.
Fan, C. C., & Plaut, G. W. E. (1974) *Biochemistry 13*, 45.
Gorin, G. (1959) *Biochim. Biophys. Acta 34*, 268.
Guilloton, M., & Hargreaves, A. B. (1972) *C. R. Hebd. Seances Acad. Sci., Ser. D 275*, 1827.
Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 457.
Holtham, S. B., & Schutz, F. (1948) *Experientia 4*, 298.
Jespersen, N. D. (1975) *J. Am. Chem. Soc. 97*, 1662.
Jones, M. E., & Lipmann, F. (1960) *Proc. Natl. Acad. Sci. U.S.A. 46*, 1194.
Koch, F. C., & McMeekin, T. C. (1924) *J. Am. Chem. Soc. 46*, 2066.
Lang, C. A. (1959) *Anal. Chem. 30*, 1692.
Liao, T. H., Robinson, G. W., & Salnikow, J. (1973) *Anal. Chem. 45*, 2286.
Lisanti, L. E. (1963) *Ann. Sper. Agar. 17*, 45.
Lotti, G. (1963) *Chim. Ind. (Milan) 45*, 561.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372.

Moore, S. (1963) *J. Biol. Chem. 238*, 235.
Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819.
O'Brien, R. D., Timpone, C. A., & Gibson, R. E. (1978) *Anal. Biochem. 86*, 602.
Schroeder, D. D., Allison, N. J., & Buchanan, J. M. (1969) *J. Biol. Chem. 244*, 5856.
Shen, W.-C., & Colman, R. E. (1975) *J. Biol. Chem. 250*, 2973.
Simpson, R. J., Neuberger, M. R., & Lin, T.-Y. (1976) *J. Biol. Chem. 251*, 1936.
Sluyterman, L. A. E. (1967) *Biochim. Biophys. Acta 139*, 439.
Taussig, A. (1960) *Biochim. Biophys. Acta 44*, 510.
Taussig, A. (1965) *Can. J. Biochem. 43*, 1063.
Thorsland, A., & Lindskog, S. (1967) *Eur. J. Biochem. 3*, 117.
Weber, K., Pringle, J. R., & Osborn, M. (1972) *Methods Enzymol. 26*, 3.

# Interaction of Dansylated Peptidyl Chloromethanes with Trypsin, Chymotrypsin, Elastase, and Thrombin†

Glenn S. Penny and Douglas F. Dyckes*

ABSTRACT: A series of $N^{\alpha}$-1-(dimethylamino)-5-naphthalenesulfonyl (dansyl) derivatives of peptidyl chloromethanes (chloromethyl ketones) were synthesized and employed to introduce the fluorescent dansyl moiety specifically into the active sites of proteinases via affinity labeling. Dansylalanyllysylchloromethane (DALCM) was utilized to inactivate and fluorescently label trypsin and the trypsin-like enzyme thrombin. Dansylleucylphenylalanylchloromethane (DLPCM) was synthesized and selectively employed as an inhibitor of chymotrypsin. The di-, tri-, and tetrapeptides—dansylprolylalanylchloromethane (DPACM), dansylalanylprolylalanylchloromethane (DAPACM), and dansylprolylalanylprolylalanylchloromethane (DPAPACM)—were synthesized and their interaction with elastase was evaluated. The compounds DALCM, DLPCM, and DAPACM all proved to be effective, fast-acting proteinase inhibitors. Studies of energy transfer in the enzyme–inhibitor conjugates led to results entirely consistent with the proposed conformational homology of thrombin with the other serine proteinases studied. The fluorescent affinity labels are believed to possess enormous potential for the localization, isolation, and characterization of enzymes.

Site-specific affinity labeling of serine proteinases with amino acid and peptidyl chloromethanes has been a valuable tool in the study of the structure and function of proteolytic enzymes. For example, by incorporating radioactive labels into the amino acid chloromethanes Tos-Lys-$CH_2Cl$ (TLCK)[1] and Tos-Phe-$CH_2Cl$ (TPCK), it was first possible to identify the histidine residues at the active sites of trypsin (Ong et al., 1964), chymotrypsin (Shaw & Springhorn, 1967), and thrombin (Glover & Shaw, 1971). Similar affinity labels have become probes of the microenviroment of enzyme active sites by replacing the tosyl group with a spin-label (Kosman, 1972) or a fluorescent molecule (Schoellmann, 1972; Vaz & Schoellmann, 1976). Peptidyl chloromethanes have also been found useful in mapping the three-dimensional structure of substrate binding sites (Segal et al., 1971; Shotton et al., 1972).

Many biological effects of amino acid chloromethanes have been documented. TLCK is thought to interact with acrosin and thus prevent fertilization (Zaneveld et al., 1970). It further irreversibly inhibits the translation of mRNA into protein in rabbit reticulocyte (Freedman et al., 1973) and *Escherichia coli* (Rossman et al., 1974), and into polio-virus specific proteins in infected HeLa cells (Summers et al., 1972). Proteolytic activity associated with the rampant growth of tumor and cancer cells has been curbed by the action of TLCK and TPCK, and their application has resulted in the inhibition of the adhesion of Ehrlich ascites tumor cells to plastic (Whur et al., 1974; Powers, 1977). In each of these cited instances, the effective use of TLCK and TPCK as inhibitors is thought to be evidence for the involvement of proteolytic enzymes in the indicated biological processes. In most cases, however, the isolation of the enzymes responsible has not been accomplished.

The dansylated peptidyl chloromethanes described in this work were designed to combine the advantages for visualization and isolation of a fluorescent label and the enhancement of reactivity afforded by peptidyl chloromethanes. Peptide derivatives of phenylalanyl-, lysyl-, and alanylchloromethanes have been found to be as much as 150 times more effective than their tosyl derivatives in the inhibition of chymotrypsin (Powers & Wilcox, 1970; Morihara & Oka, 1970; Kurachi et al., 1973), trypsin (Coggins et al., 1974; Kettner et al., 1978), and elastase (Powers & Tuhy, 1973; Thompson & Blout, 1973; Thompson & Dennis, 1973). The inclusion of a dansyl moiety permits ready visualization of these affinity

† From the Department of Chemistry, University of Houston, Houston, Texas 77004. *Received August 8, 1979.* This research was supported by the U.S. Public Health Service (Grant No. HL 19514).

[1] Abbreviations used: Ac, acyl; BAPA, $N^{\alpha}$-benzoyl-L-arginine-*p*-nitroanilide; Boc, tBoc, *tert*-butyloxycarbonylurethane protecting group; BPTI, basic pancreatic trypsin inhibitor; dansyl, Dns, 1-(dimethylamino)-5-naphthalenesulfonyl; DALCM, $N^{\alpha}$-dansyl-L-alanyl-L-lysylchloromethane; DALM-thrombin, Dns-Ala-Lys-$CH_2$-thrombin, $N^{\alpha}$-dansyl-L-lysylthrobino(His-43)methane; DALM-trypsin, Dns-Ala-Lys-$CH_2$-trypsin, $N^{\alpha}$-dansyl-L-alanyl-L-lysyltrypsino(His-46)methane; DAPACM, $N^{\alpha}$-dansyl-L-prolyl-L-alanylchloromethane; DAPAM-elastase, Dns-Ala-Pro-Ala-$CH_2$-elastase, $N^{\alpha}$-dansyl-L-alanyl-L-prolyl-L-alanylelastino(His-45)methane; Diazald, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide; DLPCM, $N^{\alpha}$-dansyl-L-leucyl-L-phenylalanylchloromethane; DLPM-chymotrypsin, Dns-Leu-Phe-$CH_2$-chymotrypsin, $N^{\alpha}$-dansyl-L-lysyl-L-phenylalanylchymotrypsino(His-57)methane; DMF, dimethylformamide; $Me_2SO$, dimethyl sulfoxide; DPACM, $N^{\alpha}$-dansyl-L-prolyl-L-alanylchloromethane; $Et_3N$, triethylamine; M.A., mixed anhydride; NMR, nuclear magnetic resonance; PNGB, *p*-nitrophenyl-*p*-guanidinobenzoate; THF, tetrahydrofuran; TLC, thin-layer chromatography; TLCK, $N^{\alpha}$-tosyl-L-lysylchloromethane; tosyl, Tos, *p*-toluenesulfonyl; TPCK, Tos-Phe-$CH_2Cl$, $N^{\alpha}$-tosyl-L-phenylalanylchloromethane or L-1-tosylamido-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; Z, (benzyloxycarbonyl)urethane protecting group.

0006-2960/80/0419-2888$01.00/0 