Use of N-Benzoyl-L-tyrosine Thiobenzyl Ester as a Protease Substrate

HYDROLYSIS BY \( \alpha \)-CHYMOTRYPSIN AND SUBTILISIN BPN' *

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In the course of searching for specific chromogenic substrates which might be useful in screening for protease-deficient mutants of Bacillus subtilis, we have developed a method for the synthesis of N-benzoyl-L-tyrosine thiobenzyl ester (BzTyrSBzl) in good yield. Spontaneous base hydrolysis of this thiol ester is low, but several serine proteases hydrolyze it readily. Spectrophotometric measurement of the hydrolysis of the ester in the presence of 5,5'-dithiobis(2-nitrobenzoic acid) provides a continuous assay for chymotrypsin as sensitive as any assay reported in the literature. Serine proteases which hydrolyze this substrate may be detected in polyacrylamide disc gels by incubation in the presence of nitro blue tetrazolium.

Apparent \( K_m \) values of 0.02 and 7 mM and \( k_{cat} \) values of 37 s\(^{-1}\) and 126 s\(^{-1}\) were observed for the hydrolysis of BzTyrSBzl by \( \alpha \)-chymotrypsin and subtilisin BPN', respectively. Additionally, 5 mM indole was observed to behave as a strict competitive inhibitor of the \( \alpha \)-chymotrypsin-catalyzed hydrolysis of BzTyrSBzl but was observed to increase the maximal rate of hydrolysis of \( p \)-nitrophenyl acetate by \( \alpha \)-chymotrypsin by 30%, as previously described. These data, the published data of other workers, and results from studies with molecular models of trypsin and subtilisin BPN' are used as the basis for describing more fully a secondary hydrophobic binding pocket on \( \alpha \)-chymotrypsin. The pocket is immediately adjacent to the active site serine and is tentatively suggested to be composed of 4 aliphatic side chain residues and 2 glycine residues.

In studies on the intracellular proteolytic enzymes of Bacillus subtilis (trp'), we previously reported the use of partially purified N-benzoyl-L-tyrosine thiobenzyl ester to select for a protease-deficient mutant (1). Upon devising a method for the synthesis of the pure compound in good yield, herein reported, we undertook to measure the kinetics of hydrolysis of this thiol ester by \( \alpha \)-chymotrypsin and by subtilisin BPN'. The kinetic results obtained with \( \alpha \)-chymotrypsin suggested the presence of a secondary hydrophobic pocket, contiguous with the active site region. This idea was further supported by kinetic studies with the inhibitor indole and by observations made with molecular models of BzTyrSBzl and trypsin, a protease which has a polypeptide backbone structure closely similar to that of \( \alpha \)-chymotrypsin. Some of the kinetic data for \( \alpha \)-chymotrypsin reported in the literature are discussed in terms of this postulated secondary hydrophobic site.

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1. The abbreviations used are: BzTyrSBzl, N-benzoyl-L-tyrosine thiobenzyl ester; ZTyrONp, N-benzoyloxycarbonyl-L-tyrosine p-nitrophenyl ester; BzTyrOEt, N-benzoyl-L-tyrosine ethyl ester; AcTyrOEt, N-acetyl-L-tyrosine ethyl ester; AcTyrSEt, N-acetyl-L-tyrosine thioethyl ester; AcTyrSNp, N-acetyl-L-tyrosine p-nitrothiophenyl ester; AcTyrOEt, N-acetyl-L-tyrosine ethyl ester.

EXPERIMENTAL PROCEDURE

The \( \alpha \)-chymotrypsin (bovine pancreas; crystallized three times) and subtilisin BPN' (bacterial, type VII; lyophilized, crystallized) were purchased from Sigma Chemical Co. All other materials were reagent grade obtained from commercial sources.

Synthesis of N-Benzoyl-L-tyrosine Thiobenzyl Ester—Previously, other workers have reported methods for the synthesis of N-acetyl-phenylalanine thioethyl ester (2) and of N-acetyl-L-tryptophan thioethyl ester (3); however, in the present work a modification of the method of Weygand and Steglich (4) was used. Benzyl mercaptan (20 mmol) and dicyclohexylcarbodiimide (10 mmol) were dissolved in 30 ml of dry tetrahydrofuran; the mixture was cooled to -30°. To this stirred mixture was added, over a 20-min period, N-benzoyl-L-tyrosine (10 mmol) dissolved in 20 ml of chilled, dry tetrahydrofuran. Stirring was continued at -15° for 30 min, after which the mixture was allowed to warm to room temperature while stirring continuously for 2 hours. The dicyclohexyl urea was removed by vacuum filtration (recovery: 81%) and the tetrahydrofuran of the filtrate removed by means of a rotary evaporator. The residue was taken up in a minimal volume of ethyl acetate and any insoluble material remaining was removed by filtration. The ethyl acetate volume was reduced under vacuum and the resulting precipitate separated by filtration. The resulting white powder was washed extensively with diethyl ether until no mercaptan odor was detectable upon removal of the ether. The white amorphous powder melted with decomposition at 168-171°. The thiol ester was recrystallized from acetone-water. The over-all yield of BzTyrSBzl by this method was 76.2%. The elemental analysis, performed by Galbraith Laboratories, conformed closely to the theoretical values.
Upon storage at 0°C over an 8-month period, 2 to 3% of the ester was hydrolyzed. The accumulated free thiol could be eliminated by treating an acetone solution of the compound with 1% hydrogen peroxide, quenching with manganese dioxide, and filtering through a small pad of diatomaceous earth. The subsequent acetone solution remained thiol-free over a 2-month period at room temperature.

Similar procedures were employed in the syntheses of the ethane thiol and p-thiocresol esters of N-benzoyl-L-tyrosine. Preliminary assays indicated that these esters were less readily hydrolyzed than BzTyrSBzl, and thus they have not been studied further.

**Kinetics**—To measure the hydrolysis of BzTyrSBzl, cuvettes were prepared to contain 1.5 ml of 0.1 M Tris-CI, pH 7.8, amounts of acetone and BzTyrSBzl stock to give 0.75 ml of total acetone (25%, v/v), 0.02 ml of 5,5'-dithiobis(2-nitrobenzoic acid) stock, 0.05 ml of enzyme solution (1.36 x 10^-3 mg/ml of chymotrypsin, or 11.6 x 10^-4 mg/ml of subtilisin BPN'), and 0.70 ml of water. The order of addition of these solutions proved important to prevent precipitation at high BzTyrSBzl concentrations: mixing the Tris, water, acetone, and 5,5'-dithiobis(2-nitrobenzoic acid) together in the cuvettes and adding to this solution the appropriate amount of BzTyrSBzl stock was found to give clear solutions for the assay, provided the cuvettes were capped to minimize any evaporation of the acetone. Backgrounds were measured, the enzyme added, and a rate obtained by following the increase in absorbance at 412 nm as described for the assay of arylesterases (5) using thiopehyl acetate as a substrate.

**Stability of BzTyrSBzl toward Spontaneous Hydrolysis—**The data reported in Table I indicate that BzTyrSBzl undergoes very little spontaneous hydrolysis under the conditions studied. Even at a pH of 9.1, in the absence of any proteolytic enzyme, a rate of only about 1% per hour was observed. Practically, this means that no corrections for spontaneous rates of hydrolysis need be made for BzTyrSBzl when studying its enzymic hydrolysis under the conditions most usually employed.

**Hydrolysis of BzTyrSBzl by Proteolytic Enzymes—**Trypsin (EC 3.4.4.4), a-chymotrypsin (EC 3.4.4.5), and subtilisin BPN' (EC 3.4.4.16) were found to hydrolyze N-benzoyl-L-tyrosine thiobenzyl ester at significant rates. Fig. 1 shows the ranges for which the initial rate increased linearly with the concentration of a-chymotrypsin and subtilisin BPN'. As little as 2 ng/ml of a-chymotrypsin and 15 ng/ml of subtilisin BPN' can be measured at an initial substrate concentration of 0.32 mM. The former value is closely comparable to the most sensitive assay yet reported for chymotrypsin (10), that measuring the hydrolysis of N-benzoyloxybenzoyl-L-tyrosine p-nitrophenyl ester described by Martin et al. (11).

**RESULTS**

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Fig. 2. Plot of initial rate of hydrolysis of BzTyrSBzl (BTTBE) by α-chymotrypsin and subtilisin BPN' as a function of BzTyrSBzl concentration according to the equation of Hanes (12). The reaction mixtures contained in a total volume of 3 ml: 20 μmol of Tris-Cl, pH 8.0, 0.2 μmol of 5,5'-dithiobis(2-nitrobenzoic acid), amounts of BzTyrSBzl as indicated, 30% acetone (v/v), and either 2.6 × 10^{-11} mol of α-chymotrypsin or 2.2 × 10^{-11} mol of subtilisin BPN'. Enzyme concentrations were determined spectrophotometrically as indicated in Fig. 1, and other conditions of assay are as described in Fig. 1.

Table II

| Enzyme and substrate | $K_a$ (μM) | $K_m$ (μM) | $k_{cat}$ (s^{-1}) |
|----------------------|------------|------------|-------------------|
| α-Chymotrypsin       |            |            |                   |
| BzTyrSBzl            | 0.02       | 37         |                   |
| BzTyrOEt             | 2.6        | 45         |                   |
| AcTyrOEt             | 0.0032     | 400        |                   |
| AcTyrEt              | 0.0065     | 0.99       |                   |
| AcTyrSNt             | 0.0084     | 0.085      |                   |
| AcTyrSnt             | 0.0045     | 0.032      |                   |
| Subtilisin BPN'      | 7.0        | 126        |                   |
| AcTyrOEt             | 10.0       | 658        |                   |

*Data of Folk and Schirmer (13): 0.04 M Tris-Cl, pH 7.8, 30% (v/v) MeOH, 25°C.
*Data of Martin et al. (11): 0.03 M Tris, pH 8.0, 12% (v/v) MeOH, 30°C.
*Data of Hirohara et al. (14): phosphate, pH 6.99, 0.34% (v/v) dimethylformamide and 1.97% (v/v) acetonitrile for first two and 6.2% (v/v) acetonitrile for the third one, 25°C (sic).
*Data of Glazer (15): 0.1 M KCl, pH 8.0, 8% (v/v) p-dioxane, 37°C.

primary intracellular serine protease of *B. subtilis*. The thiol esterase activity corresponds to azocasein hydrolyzing activity through all purification steps yet attempted, including two column chromatographic steps.

**Inhibitory Action of Indole on α-Chymotrypsin**—As the apparent $K_a$ of chymotrypsin for BzTyrSBzl was about 150-fold smaller than for BzTyrOEt (Table II), it was felt that this difference might represent a corresponding difference in the binding constants for these substrates. If this were true, it would imply the existence of a secondary hydrophobic site capable of binding the thiobenzyl group. To test this idea we have examined the effects of indole, an inhibitor thought to bind only in the recognition site or "tosyl hole" (18), on the saturation kinetics of BzTyrSBzl and of p-nitrophenyl acetate. The latter substrate was chosen as its kinetic properties are well known (for example, Ref. 19) and because it was thought that the nitrophenyl group might also bind in the secondary hydrophobic or benzyl site rather than in addition to binding in the tosyl hole. The date obtained from these studies are shown in Figs. 3 and 4, where they are plotted according to Lineweaver and Burk (20). Whereas indole behaved as a classical competitive inhibitor toward BzTyrSBzl (Fig. 3), it behaved as a mixed-type inhibitor (21) toward p-nitrophenyl acetate (Fig. 4). At a final concentration of 5 mM, indole increased the apparent $K_m$ of chymotrypsin for p-nitrophenyl acetate from 4.2 × 10^{-4} to 10.8 × 10^{-4} M, but indole also consistently increased the maximal velocity by 35%, from 9.6 to 12.8 nmol/min. This acceleration of the rate is consistent with the previous observation of Foster (22) that indole may cause up to a 1.6-fold increase in the deacylation step in the hydrolysis of p-nitrophenyl acetate by α-chymotrypsin. The increased velocity was shown not to be due to indole-catalyzed hydrolysis of p-nitrophenyl acetate by α-chymotrypsin. The increased velocity was shown not to be due to indole-catalyzed hydrolysis of p-nitrophenyl acetate by α-chymotrypsin. The increased velocity was shown not to be due to indole-catalyzed hydrolysis of p-nitrophenyl acetate by α-chymotrypsin.

**Observations Made with Molecular Models**—Using a molecular model of BzTyrSBzl, built to the same scale as Labquip models of subtilisin BPN' and trypsin, we have attempted to fit the substrate into the active site region of these enzymes. With the model of subtilisin BPN' and using the x-ray work on model peptides reported by Robertus et al. (23) we were able to fit the BzTyrSBzl substrate in a plausible way into the active site region. The benzyl group did not appear to occupy any obvious subsite on the enzyme.

In making the same types of observations using the trypsin model, as an approximation to α-chymotrypsin (24), we were immediately struck by the fact that when the tyrosine was positioned in the tosyl hole such that the tyrosine amide nitrogen could hydrogen bond to the carbonyl of Ser-214, the...
thiobenzyl group fit very neatly into what appeared, in our model, to be a shallow hydrophobic pocket. Assuming that a similar steric arrangement exists in α-chymotrypsin, the pocket in α-chymotrypsin would be lined by Ala-55, Gly-196, Ileu-212, Gly-216, Ileu-99, and Val-213. The latter 2 residues appear to comprise the entrance to this hydrophobic cavity. A schematic representation of the subsites of α-chymotrypsin following the suggestion of Cohen and Lo (25) and including the newly postulated hydrophobic site (hp) is shown in Fig. 5.

Qualitative Detection of Benzyl Mercaptan—Four tetrazolium dyes (2,3,5-triphenyl-2H-tetrazolium, p-nitro blue tetrazolium, neotetrazolium, and MTT tetrazolium) were screened for capacity to react with benzyl mercaptan, which would be released during enzymic hydrolysis of BzTyrSBzl. Of these dyes, p-nitro blue tetrazolium proved to be most sensitive and was examined further. Table III shows that the minimum detectable concentration of benzyl mercaptan is 5 × 10⁻⁴ M. We have successfully used nitro blue tetrazolium to detect thiol esterase activity in polyacrylamide disc gels but have not been able to use it in screening for mutants because colonies of B. subtilis on agar plates were found to reduce this dye in the absence of substrate; however, benzyl mercaptan causes a detectable precipitate with a 2% solution (w/v) of AgNO₃ (pH 7.0) at concentrations down to 20 μM.

**DISCUSSION**

Prior to the present work, Frankfater and Kédya (26) had reported studies on the hydrolysis of p-nitrophenyl thiolacetate by α-chymotrypsin and Goldenberg et al. (2) and Polgar (27) had reported the synthesis and studies on the hydrolysis of the more specific substrate N-acetyl-L-phenylalanyl thiophenil ester, respectively. Very recently during the course of the present work, Hirohara et al. (14) described some detailed kinetic studies on the hydrolysis by α-chymotrypsin of two specific substrates, the thioeithanol and p-nitrothiophenyl esters of N-acetyl-l-tryptophan. Each of the studies cited measured the hydrolysis of the thiol esters by following changes in the ultraviolet or the visible absorption of the p-nitrophenyl thiolate ion.

The use of Ellman’s reagent 5,5'-dithiobis(2-nitrobenzoic acid) to assay the thiol released during the hydrolysis of BzTyrSBzl by α-chymotrypsin or subtilisin has provided a convenient continuous assay at visible wavelengths which is comparable in sensitivity for α-chymotrypsin to the most sensitive assay yet reported (11). In addition the thiobenzyl ester has the advantage of being very much more stable than the p-nitrophenyl esters, and presumably also the p-nitrothiophenyl esters, toward spontaneous hydrolysis at alkaline pH values (11). If one wished to assay for hydrolysis of the thiol ester at pH values below neutrality, which is not possible with Ellman’s reagent, presumably one of the pyridyl disulfides described by Grassetti and Murray (28) could be used as the chromogenic agent.

In discussing the data presented in Table II several points may be noted with respect to α-chymotrypsin. As shown above, Folk and Schirmer (13) found that the ⁴₃₄₃₄₄ for BzTyrOEt was 43 s⁻¹, which is very similar to what we report for BzTyrSBzl. On the other hand the apparent ⁴₃₄₄ for BzTyrSBzl is 150-fold lower for BzTyrSBzl than for BzTyrOEt. A similar relationship was observed by Hirohara et al. (14) when they compared the steady state kinetics of AcTrpOEt with those of AcTrpSNp, although in this case the ⁴₄ for the thiol ester was only 14-fold lower than that of the oxygen ester. Do these apparent ⁴₄ differences reflect increased affinity of α-chymotrypsin for these substrates which have an aromatic residue esterified to the carboxyl group? The leaving ability of benzyl mercaptan would be expected to lie between that of ethyl mercaptan and p-nitrophenyl mercaptan. The difference in the ⁴₄ and the apparent ⁴₄ for AcTrpSNp reported by Hirohara et al. (14) (Table II) is about 13.5. Multiplying the apparent ⁴₄ for BzTyrSBzl by this factor gives 0.27 μM which is an estimate of the ⁴₄ for BzTyrSBzl. Gutfreund and Hammond have found that the value of the “true” K₄ (that is, K₁ + K₄/K₃) of BzTyrOEt for α-chymotrypsin is not more than about 3 times larger than the apparent ⁴₄ at a pH of 7.2 in 20% isopropryl alcohol (29). Thus, rough estimates for the values of ⁴₄ are 0.3 μM and 8 μM for BzTyrSBzl and BzTyrOEt, respectively. As the amount and nature of the organic solvents used by various workers can make significant differences in the ⁴₄ values observed, these values must be taken as only approximations. Insofar as the approximations are valid, we infer that there is a relatively important hydrophobic binding site on the carboxyl side of the aromatic amino acid-binding pocket of α-chymotrypsin. Indeed, Hirohara et al. (14) have interpreted the lower ⁴₄ values of the sulfur esters compared to the oxygen esters in terms of the greater hydrophobicity of the sulfur relative to oxygen.

In the case of subtilisin BPN’ it should be noted that the ⁴₄ values for BzTyrSBzl and AcTyrOEt are quite similar, but that the ⁴₄ for AcTyrOEt is significantly higher; this is

**TABLE III**

Detection of benzyl mercaptan through its production of purplish-blue insoluble formazan

| Benzyl mercaptan concentration | Precipitate observed |
|-------------------------------|---------------------|
| 10                           | ++++                |
| 5                            | ++++                |
| 1                            | ++                  |
| 0.5                          | +                   |
| 0.1                          | −                   |

*The rate constants refer to those in the standard formulation:

\[ E + S \stackrel{k_{-1}}{\rightleftharpoons} E'S \stackrel{k_{1}}{\rightarrow} E'P \]

where \( E' \) represents the acyl-enzyme covalent intermediate.
consistent with the studies of the molecular models which suggested there was no site on subtilisin BPN' which could specifically accommodate the benzyl group.

More than 10 years ago Jones and Niemann (30) suggested that hydrophobicity of the carbalkoxy group of amino acid esters may contribute significantly to the ability of the ester to bind α-chymotrypsin. More recently a number of workers have presented evidence which may be interpreted in terms of a secondary hydrophobic site on α-chymotrypsin; their observations are briefly summarized. Zerner and Bender (31) found the $K_m$ for $N$-acetyl-L-tyrosine ethyl ester was 96 mM whereas that for the corresponding $p$-nitrophenyl ester was 2.2 mM. Similarly, Fastrez and Fersht (32) have reported that the apparent $K_m$ of α-chymotrypsin for $N$-acetyl-$L$-lysine arginyl methyl ester per mol of $α$-chymotrypsin, but of only 1 mol of the substrate binding pocket.

East and Trowbridge (34) have provided calorimetric evidence for the binding of 2 mol of $N$-phenyl-$p$-toluenesulfonyl-$L$-arginine methyl ester per mol of $α$-chymotrypsin, but of only 1 mol of the substrate per mol of the zymogen. Finally, Berliner and Wong (35) have deduced three binding modes for spin-label substituted phenylsulfonyl fluorides, one of which is outside the substrate binding pocket.

Examination of the molecular models of $BzTyrSBzl$ and trypsin made plausible the suggestion of Hirohara et al. (14) that it was the greater hydrophobicity of sulfur which accounted for the increased binding of the sulfur analogue of $N$-acetyltryptophan ethyl ester. We observed that the LY-methyl ester per mol of $α$-chymotrypsin, but of only 1 mol of $α$-chymotrypsin residues Ileu-99 and Val-213 would be in close contact with the substrate binding pocket. Alternatively, we suggest that the enzyme may be in the secondary hydrophobic pocket, thereby causing the increase observed in $V_{max}$. Obviously, other interpretations are not excluded at present. The data strongly imply that indole and $p$-nitrophenyl acetate can bind simultaneously to one enzyme molecule. These observations on α-chymotrypsin may be related to the substrate activations which have been noted for some time with trypsin (see for example, Ref. 38).

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REFERENCES

1. Hageman, J. H., and Carlton, B. C. (1973) J. Bacteriol. 114, 612-617.
2. Goldenberg, V., Goldenberg, H., and McLaren, A. D. (1950) J. Am. Chem. Soc. 72, 5477.
3. Ingles, D. W., and Knowles, J. R. (1966) Biochem. J. 99, 275-282.
4. Weyzand, F., and Steitz, W. (1960) Biochim. Biophys. Acta 29, 20-33.
5. Augustinsson, K.-B., and Axenfors, B. (1972) Anal. Biochem. 48, 428-436.
6. Bllman, G. L. (1959) Arch. Biochem. Biophys. 82, 70-77.
7. Alden, R. A., Birktoft, J. J., Kraut, J., Robertus, J. D., and Wright, C. S. (1971) Biochim. Biophys. Res. Commun. 45, 303-307.
8. Dreyer, W. J., Wade, R. D., and Neurath, H. (1955) Arch. Biochem. Biophys. 59, 145-156.
9. Matsubara, H., Kasper, C. B., Brown, I. M., and Smith, E. L. (1963) J. Biol. Chem. 240, 1125-1130.
10. Walsh, K. A., and Wilcox, P. E. (1970) Methods Enzymol. 19, 31-38.
11. Martin, C. J., Golubow, J., and Axelrod, A. E. (1959) J. Biol. Chem. 234, 294-298.
12. Hanes, C. S. (1932) Biochem. J. 26, 1406.
13. Folk, J. E., and Schirmer, E. W. (1966) J. Biol. Chem. 241, 181-192.
14. Hirohara, H., Bender, M. L., and Stark, R. H. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 1643-1647.
15. Glazer, A. N. (1967) J. Biol. Chem. 242, 433-436.
16. Inagami, T., and Sturtevant, J. M. (1965) J. Biol. Chem. 235, 1019-1023.
17. Martin, C. J., Golubow, J., and Axelrod, A. E. (1958) Biochim. Biophys. Acta 27, 430-431.
18. Blow, D. M. (1971) in The Enzymes (Boyer, P. D., ed) 3rd ed, Vol. III, pp. 185-212, Academic Press, New York.
19. Bender, M. L., Kédzv. F. J., and, and Fediger, F. C. (1967) J. Chem. Ed. 44, 58-65.
20. Lineaweaver, H., and Burke, D. (1934) J. Am. Chem. Soc. 56, 658-662.
21. Dixon, M., and Webb, E. C. (1964) Enzymes, 2nd ed, p. 324, Academic Press, New York.
22. Foster, R. J. (1961) J. Biol. Chem. 236, 2461-2465.
23. Robertus, J. D., Alden, R. A., Birktoft, J. J., Kraut, J., Powers, J. C., and Wilcox, P. (1971) Biochemistry 11, 2439-2449.
24. Stroud, R. M., Kay, L. M., and Dickerson, R. E. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 125-140.
25. Cohen, S. G., and Lo, L. W. (1970) J. Biol. Chem. 245, 5718-5727.
26. Frankfater, A., and Kédzv, F. J. (1971) J. Am. Chem. Soc. 93, 4039-4043.
27. Polger, I. (1972) Acta Biochim. Biofis. Acad. Sci. Hung. 7, 319-334.
28. Grassetti, D. R., and Murray, J. F., Jr. (1967) Arch. Biochem. Biophys. 119, 41-49.
29. Gutfreund, H., and Hammond, B. R. (1959) Biochem. J. 73, 530-536.
30. Jones, J. B., and Niemann, C. (1962) Biochemistry 1, 1003-1006.
31. Zerner, B., and Bender, M. L. (1964) J. Am. Chem. Soc. 86, 3669-3674.
32. Fastrez, J., and Fersht, A. R. (1973) *Biochemistry* **12**, 1067–1074
33. Steitz, T. A., Henderson, R., and Blow, D. M. (1969) *J. Mol. Biol.* **46**, 337–348
34. East, E. J., and Trowbridge, C. G. (1973) *J. Biol. Chem.* **248**, 7552–7557
35. Berliner, L. J., and Wong, S. S. (1974) *J. Biol. Chem.* **249**, 1668–1677
36. Inagami, T., York, S. S., and Patchornik, A. (1965) *J. Am. Chem. Soc.* **87**, 120–127
37. Davis, L., and Hess, G. P. (1974) *J. Mol. Biol.* **82**, 27–33
38. Kallen-Trummer, V., Hoffmann, W., and Rottenberg, M. (1970) *Biochemistry* **18**, 3090–3094
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