Extreme State of Ionization of Benzylsuccinate Bound by Carboxypeptidase A†

Allen R. Palmer, Paul D. Ellis, and Richard Wolfenden*

ABSTRACT: Benzylsuccinic acid, a powerful inhibitor of carboxypeptidase A, exhibits maximum inhibition at pH values where the inhibitor bears a single negative charge in solution. To establish the distribution of charges in the enzyme–inhibitor complex, this inhibitor was enriched with ^{13}C at each of the two carboxyl groups, alternatively. The observed chemical shifts suggest that *both* carboxyl groups are ionized in the bound inhibitor and that a hydrogen ion is taken up by the protein as the dianionic inhibitor is bound.

2-Benzylsuccinate is an exceptionally powerful reversible inhibitor of carboxypeptidase A, competitive against hydrolysis of peptide and ester substrates (Byers & Wolfenden, 1972, 1973). This inhibitor (Figure 1A) has served as a point of departure for the design of drugs antagonistic to angiotensin-converting enzyme (Cushman et al., 1977; Patchett et al., 1980) and as an affinity ligand for the isolation of carboxypeptidases (Peterson et al., 1976).

Benzylsuccinate bears no obvious structural resemblance to chemical intermediates in substrate hydrolysis, nor does its affinity for carboxypeptidase A change with changing pH in a way that resembles the influence of pH on V_{max} for normal substrates, as might be expected for an ideal transition-state analogue. Relieved of the energetic burden of gathering several substrate molecules from dilute solution, enzymes are generally expected to show affinities for "multisubstrate analogues" that match or surpass their combined affinities for the individual substrates (Wolfenden, 1972). The unusual affinity of benzylsuccinate for carboxypeptidase seemed understandable in terms of its resemblance to the collected product of peptide hydrolysis at the moment of their formation or as they combine with the enzyme in the reverse, peptide-forming reaction (Figure 1B) (Byers & Wolfenden, 1973). This feature is absent in 3-phenylpropionic acid (Elkins-Kaufman & Neurath, 1949), an inhibitor that is bound with an affinity similar to that of ordinary substrates (Figure 1C).

Information as to the exact structure of bound benzylsuccinate would be expected to provide some indication of the binding properties of the active site, useful in considering further improvements in inhibitor design. Positions of heavy atoms, in the less stable complex that is formed between benzylsuccinate and thermolysin, have been examined by X-ray diffraction, leading to reasonable inferences about the possibly analogous orientation of the inhibitor in the active site of carboxypeptidase A (Bolognesi & Matthews, 1979). Efforts to determine the structure of the inhibitory complex of benzylsuccinate with carboxypeptidase A directly, using X-ray diffraction methods in collaboration with Drs. F. Quiocho and W. N. Lipscomb, have been frustrated by disordering and solution of enzyme crystals soaked in solutions containing inhibitor. K_i values for reversible inhibition have, however, been found to vary with pH in a manner consistent with exclusive binding of a monoanionic species of benzylsuccinate (Byers & Wolfenden, 1973). This information does not, by itself, indicate the site of ionization in bound benzylsuccinate, nor does it establish that the inhibitor is actually bound as a monoanion. In an effort to establish the distribution of electrostatic charges in the bound inhibitor, we have enriched each of the critical carboxylic acid groups with ^{13}C and examined their chemical shifts by magnetic resonance spectroscopy at various pH values, in the presence and absence of the enzyme.

Experimental Procedures

Sample Preparation. Bovine pancreatic carboxypeptidase (Type II) was purchased from Sigma Chemical Co. and was maintained between 0 and 2 °C throughout. The crystalline enzyme, obtained by centrifugation, was washed twice with Tris-HCl buffer (0.05 M, pH 7.0) and then dissolved in the same buffer, containing NaCl (3 M) and D_2O (30%), for use as an internal lock in NMR experiments. The final concentration of carboxypeptidase used in these experiments was $(2.7–3.1) \times 10^{-3}$ M, determined spectrophotometrically by using an extinction coefficient $\epsilon_{280} = 6.42 \times 10^4$ M^{-1} cm^{-1} (Simpson et al., 1963).

NMR Measurements. ^{13}C spectra were obtained at 22 °C on a Bruker Model WH 400 spectrometer at the Regional NMR facility at the University of South Carolina. The resonance frequency for ^{13}C on this instrument is 100.62 MHz. Ten-millimeter diameter sample tubes were used, and the following acquisition parameters were employed: flip angle 40°; spectral window 20 000 Hz, recycle time 0.41 s; line broadening 10 Hz, broad-band proton decoupling 2.5 W. A total of 30 000 scans were recorded for each spectrum, over a period of approximately 3.5 h. A positive chemical shift was used to denote a resonance at lower shielding with respect to an external capillary containing Me_4Si.

Labeled Benzylsuccinic Acids. ^{13}C was introduced into benzylsuccinic acid, alternatively at positions 1 and 4, by appropriate modifications of the malonic ester syntheses described by Cohen & Milovanovič (1968).

For preparation of 2-benzylsuccinic acid-*1*-^{13}C, diethyl malonate-*1,3*-^{13}C (Merck Sharp & Dohme Canada, Ltd.; 10 mmol) was first converted to diethyl benzylmalonate by treatment for 10 min at room temperature with benzyl bromide (10 mmol) and sodium ethoxide (10 mmol) in ethanol (10 mL). After removal of the solvent by evaporation, the residue was dissolved in ether (20 mL) and extracted 3 times with water (20 mL). After the mixture was dried with magnesium sulfate, the ether layer was evaporated to dryness, yielding

† From the Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514 (R.W.), and the Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208 (A.P. and P.D.E.). *Received March 9, 1982.* This work was supported in part by Research Grants GM-18325 (to R.W.) and GM-26295 (to P.D.E.) from the National Institutes of Health and made use of the National Science Foundation Regional Nuclear Magnetic Resonance Facility at the University of South Carolina (CH78-18723).

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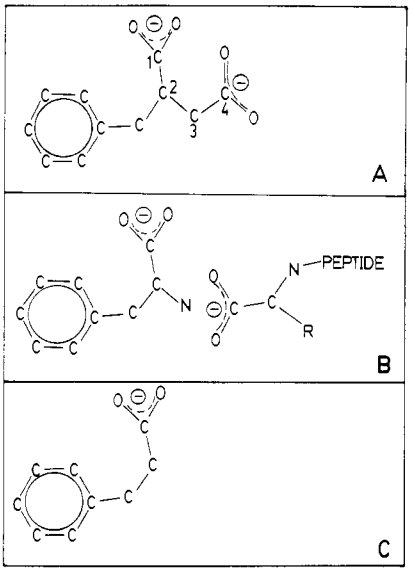

FIGURE 1: Structures of (A) 2-benzylsuccinate, (B) products of hydrolysis of a peptide with C-terminal phenylalanine, and (C) 3-phenylpropionate.

diethyl benzylmalonate-*1,3*-^{13}C as an oil (9.7 mmol). This procedure was repeated by using ethyl bromoacetate (10 mmol) in place of benzyl bromide. The resulting crude triethyl 1-phenylpropane-2,2,3-tricarboxylate (9.4 mmol) was boiled under reflux with potassium hydroxide (6 g) in ethanol (30 mL) for 8 h. Ethanol was removed by evaporation, water (50 mL) was added, and concentrated hydrochloric acid was added to adjust the pH to 1. After two extractions with ether (50 mL) the ether layers were dried with magnesium sulfate, and the ether was removed by vacuum. The residue was dried overnight under vacuum, yielding crude 1-phenylpropane-2,2,3-tricarboxylic acid-*2,2*-^{13}C (1.7 g, 6.7 mmol). This was heated at 170 °C for 1 h to yield crude 2-benzylsuccinic acid-*1*-^{13}C (1.24 g, 6 mmol), mp 160 °C after crystallization from water [lit. mp 160–161 °C (Weizman, 1943; Cohen & Milovanovič, 1968)].

For preparation of 2-benzylsuccinic acid-*4*-^{13}C, acetic acid-*1*-^{13}C (Merck Sharp & Dohme Canada, Ltd.) was first distilled from tetraacetyl diborate (Eichelberger & La Mer, 1933) to remove traces of moisture. The dry acid (26 mmol) was treated with red phosphorus (Ward, 1922) (31 mg), and bromine (4.5 g) was added dropwise. The mixture was heated for 1 h at 100 °C, and the resulting bromoacetic acid (16 mmol) was obtained by distillation at 203 °C. This was converted, by refluxing with ethanol in benzene in the presence of concentrated sulfuric acid (Natelson & Gottfried, 1955), to ethyl bromoacetate-*1*-^{13}C (10 mmol), obtained by distillation at 155 °C. This compound was then introduced into the synthetic procedure described in the preceding paragraph, along with unlabeled diethyl benzylmalonate (Aldrich Chemical Co.), to prepare 2-benzylsuccinic acid-*4*-^{13}C (1.2 g, 6 mmol), mp 160 °C after crystallization from water.

Results

Assignment of Resonances from Bound DL-*2-Benzylsuccinate-4-*^{13}C. The K_i for the L isomer of 2-benzylsuccinate (4.5×10^{-7} M) is substantially lower than the K_i for the D isomer (3.0×10^{-6} M) (Byers & Wolfenden, 1973). Spectra recorded during titration of carboxypeptidase A (3×10^{-3} M) with DL-2-benzylsuccinate-*4*-^{13}C are shown in Figure 2. After addition of 0.5 equiv of inhibitor (Figure 2B), two new resonances were observed at 224.2 and 226.0 ppm. At this inhibitor concentration, both isomers should be tightly bound,

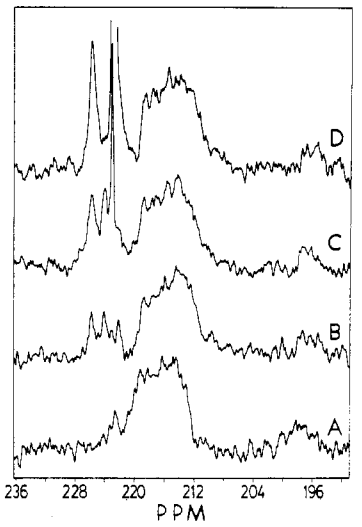

FIGURE 2: Spectra of carboxypeptidase A during titration with 2-benzylsucinic acid-*4*-^{13}C: (A) no inhibitor; (B) 0.5 equiv of inhibitor; (C) 1.5 equiv of inhibitor; (D) 6 equiv of inhibitor. Conditions are described in the text.

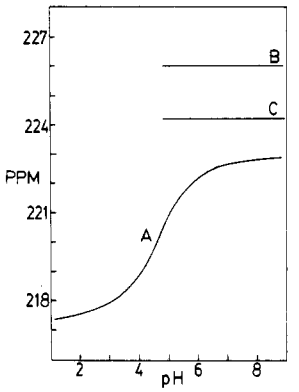

FIGURE 3: ^{13}C chemical shift of 2-benzylsuccinic acid-*4*-^{13}C, plotted as a function of pH: (A) free inhibitor; (B) L isomer bound to the protein; (C) D isomer bound to the protein.

so that these resonances were assigned to the D and L isomers of the inhibitor bound to the protein. After addition of 1.5 equiv of inhibitor (Figure 2C), a third, sharper, resonance appeared at 223.0 ppm. This was attributed to unbound inhibitor that was in excess of sites available on the enzyme. After addition of 6 equiv of inhibitor (Figure 2D), the resonance at 223.0 ppm had increased very much, as expected for the unbound inhibitor. The resonance at 224.2 ppm had vanished, and the resonance at 226 ppm had increased. The resonance at 224.2 ppm was therefore assigned to the enzyme-bound D isomer, eventually displaced by the tighter binding L isomer that was assigned the resonance at 226 ppm. This displacement approached completion only as the L isomer approached concentrations equivalent to the concentration of enzyme.

pH Effects on Chemical Shifts of 2-Benzylsuccinate-4-^{13}C. So that these results could be used to determine the state of ionization of bound inhibitors, the chemical shift of benzylsuccinate-*4*-^{13}C was determined at varying degrees of ionization in the absence of enzyme (Figure 3). At low pH, the chemical shift was 217.2 ppm. As the carboxylic acid group became ionized, its chemical shift changed to 222.8 ppm, matching the chemical shift of unbound inhibitor in the experiments with enzyme at pH 7 described above. Apparent pH values were recorded in 30% D_2O, with a glass electrode calibrated against aqueous buffers. True pH values would accordingly be

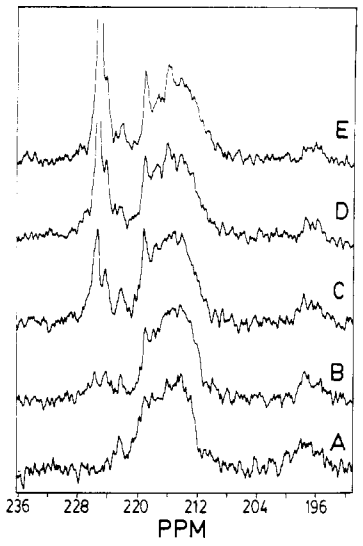

FIGURE 4: Spectra of carboxypeptidase A during titration with 2-benzylsuccinic acid-*1*-^{13}C: (A) no inhibitor; (B) 0.4 equiv of inhibitor; (C) 1.5 equiv of inhibitor; (D) 3 equiv of inhibitor; (E) 7 equiv of inhibitor. Conditions are described in the text.

somewhat higher than these uncorrected values (Glasoe & Long, 1960).[1] In Figure 3, positions of resonances attributed to the protein-bound L and D isomers, indicated by lines B and C, respectively, arre seen to be at an even lower shielding than the resonance of the fully ionized inhibitor in free solution at high pH.

Assignment of Resonances from Bound DL-*2-Benzylsuccinate-1-*^{13}C. Overlapping of resonances rendered assignments of chemical shifts of this inhibitor more difficult than for 2-benzylsuccinate-*4-*^{13}C. Addition of 0.4 equiv of 2-benzylsuccinate-*1-*^{13}C (Figure 4B) gave rise to two new resonances at 224.6 and 226.2 ppm. After addition of 1.5 equiv of inhibitor, the spectrum (Figure 4C) exhibited resonances at 224.6 and 225.6 ppm, with a shoulder that appeared to correspond to the resonance previously observed at 226.2 ppm. The resonance at 225.6 ppm, not present at low inhibitor concentrations, was assigned to unbound benzylsuccinate-*1-*^{13}C. At higher concentrations of inhibitor (Figure 4D, E), resonances at 225.6 and 226.2 ppm could no longer be resolved. However, the resonance at 224.6 ppm was found to be diminished as an excess of inhibitor was added. This resonance was accordingly assigned to the weaker-binding D isomer, and by the process of elimination the resonance at 226.2 ppm was assigned to the L isomer.

pH Effects on Chemical Shifts of 2-Benzylsuccinate-1-^{13}C. The chemical shift of 2-benzylsuccinate-*1-*^{13}C varied from 220 ppm at low pH to 225.5 ppm at high pH (Figure 5).[1] In Figure 5, chemical shifts of the enzyme-bound L and D isomers, indicated by lines B and C, respectively, fall within 1 ppm of the resonance of the fully ionized inhibitor.

Discussion

Resonances of both L and D isomers of benzylsuccinate-*1-*^{13}C, when bound to carboxypeptidase, fall within 1 ppm of the chemical shift of the fully ionized inhibitor in free solution (Figure 5), suggesting that the 1-carboxylic acid groups of these inhibitors are ionized in their enzyme–inhibitor complexes. Positions of these resonances may be slightly influenced by shielding effects from positively charged groups in the neighborhood of the binding site or be deshielding effects exerted by Tyr-248 or the phenyl group of the inhibitor itself.

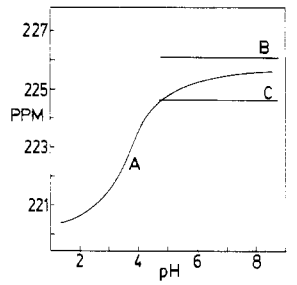

FIGURE 5: ^{13}C chemical shift of 2-benzylsuccinic acid-*1-*^{13}C, plotted as a function of pH: (A) free acid; (B) L isomer bound to the protein; (C) D isomer bound to the protein.

The resonance of the relatively weakly bound D-benzylsuccinate-*4-*^{13}C is somewhat deshielded (1.5 ppm) with respect to the position of the resonance observed for the fully ionized inhibitor (Figure 3C). The strongly bound L isomer is relatively strongly deshielded (3 ppm) as if this inhibitor were "superionized" in its complexes with carboxypeptidase A (Figure 3B). Previous work suggests that minor interactions with the protein, such as hydrogen bonding and ring current effects from nearby aromatic groups, would ordinarily result in perturbations of 1.0 ppm or less. Interactions with the metal atom at the active site would thus appear to provide a more reasonable explanation of the extreme shift observed with L-benzylsuccinate. This possibility is supported by observations involving the carboxyl carbon atoms of ethylenediaminetetraacetic acid. Addition of Zn^{2+} at pH 7.0 results in a downfield shift of the carboxyl resonances of this chelating agent from 218 to 221 ppm; Cd^{2+} produces a comparable shift (A. Palmer, unpublished observations).

The present results suggest that L-benzylsuccinate is bound as a *dianion.* In earlier kinetic experiments, the affinity of this inhibitor for the enzyme was found to become maximal at pH values where this inhibitor bore a *single* negative charge in free solution (Byers & Wolfenden, 1973). These findings can be reconciled with each other by supposing that as the dianionic inhibitor is bound, either a proton is taken up or a hydroxide ion is released by the enzyme. The obligatory connection between these events is noteworthy, suggesting that they occur at positions on the enzyme that are only narrowly separated.

In attempting to decide which of these is the more likely alternative, we may first the possibility consider that a hydroxide ion is released from the active site zinc as the dianion of benzylsuccinate is taken up. The enzyme exhibits an inflection in V_{max} near pH 6 (Auld & Vallee, 1970) that could reflect ionization of a zinc-bound water at the active site (Makinen et al., 1979) whose pK_a is 3–4 units below the value observed for zinc hydrate in free solution. The present findings, along with similar experiments involving the Cd-substituted enzyme, leave little doubt that benzylsuccinate is bonded to the active site zinc in the enzyme–inhibitor complex. Yet binding of benzylsuccinate exhibits no inflection that would indicate ionization of the enzyme in the pH range near 6 (Byers & Wolfenden, 1973). In the absence of such ionization, the possibility that benzylsuccinate displaces a hydroxide ion from the active site zinc appears remote.

[1] Apparent pK_a values of benzylsuccinate, recorded at 22 °C in 30% D_2O containing 3 M NaCl in the present nuclear magnetic resonance experiments, were approximately 3.7 and 4.6. pH values were estimated with a glass electrode calibrated against aqueous buffers and are uncorrected. These apparent pK_a values are substantially lower than potentiometric values recorded earlier at much lower salt concentrations, which were 4.1 and 5.6 (Byers & Wolfenden, 1973). These shifts are expected as a result of the very large increase in ionic strength.

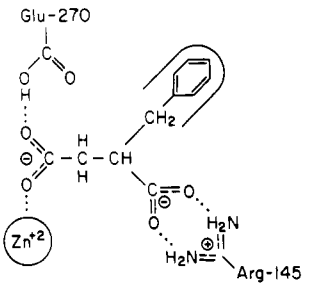

FIGURE 6: Possible mode of binding of the benzylsuccinate dianion by protonated carboxypeptidase A.

An alternative possibility is that as dianionic L-benzylsuccinate is bound by the enzyme, a proton is taken up at a basic site on the enzyme in such a manner as to promote binding of the inhibitor. From studies on the presumably analogous complex that is formed between benzylsuccinate and thermolysin, Bolognesi & Matthews (1979) have inferred a possible binding mode for benzylsuccinate to carboxypeptidase A that would place one of the oxygens of the 4-carboxylate group of the inhibitor in coordination with the active site zinc, the other oxygen projecting directly toward the γ-carboxylate group of Glu-270. Juxtaposition of two carboxylate groups would be expected to create a repulsive electrostatic potential, but this repulsive potential might be eliminated or reversed by addition of a proton to the γ-carboxylate group of Glu-270. The inferred positions of the inhibitor and enzyme would allow formation of a carboxylate–carboxylic acid hydrogen bond as shown in Figure 6. The actual pathway by which this stable complex is formed might involve preliminary protonation of Glu-270, or the proton might "ride in" on the inhibitor. These mechanistic details are irrelevant to the strength of the complex that is ultimately formed. Such a final structure would, however, explain the observed coupling between proton uptake and binding of the dianionic inhibitor. Hydrogen bonds involving a carboxylate and a carboxylic acid group, noteworthy for their apparent stability even in aqueous solution, probably account for the 1000-fold greater acidity of maleic acid than that of fumaric acid (Hunter, 1953) and the 3000-fold difference in acidities between *cis*- and *trans*-caronic acids (Jones & Soper, 1936). There are also indications that such a bond is formed when the trianion of the transition-state analogue 2-phosphoglycollate is bound at the active site of triosephosphate isomerase (Campbell et al., 1978).

The present results indicate that the binding affinity between protonated carboxypeptidase A (a rare species at pH 7) and benzylsuccinate dianion is substantially greater than the apparent K_i value observed for the monoanion in our earlier study, 6.4×10^{-9} M, would suggest (Byers & Wolfenden, 1973). The higher of the two pK_a values of benzylsuccinate is 5.6.[1] If the pK_a values of the enzyme carboxylic acid group inferred to be involved in binding were 4.0, for example, then the true dissociation constant of the protonated enzyme from the dianionic inhibitor would be approximately 10^{-10} M. The states of ionization of two new inhibitors of carboxypeptidase A, containing sulfhydryl and phosphonamide groups (Ondetti et al., 1979; Jacobsen & Bartlett, 1981), remain to be determined.

The fact that benzylsuccinate is bound in its most highly charged state by carboxypeptidase A may be of general interest for inhibitor design. NMR studies indicate that triosephosphate isomerase binds 2-phosphoglycollate in its rare trianionic form, with simultaneous uptake of a proton by the enzyme in a process that mimics general base catalysis (Campbell et al., 1978; Wolfenden, 1980). Active sites of these enzymes demonstrate a remarkable capacity to bind small, highly charged, reaction intermediates and their analogues very tightly in dilute aqueous solution, despite vigorous competition by solvent water.

References

Auld, D. S., & Vallee, B. L. (1970) *Biochemistry 9*, 4352–4359.

Bolognesi, M. C., & Matthews, B. W. (1979) *J. Biol. Chem. 254*, 634–639.

Byers, L. D., & Wolfenden, R. (1972) *J. Biol. Chem. 247*, 606–608.

Byers, L. D. & Wolfenden, R. (1973) *Biochemistry 12*, 2070–2078.

Campbell, I. D., Jones, R. B., Klener, P. A., Richards, E., Waley, S. G., & Wolfenden, R. (1978) *Biochem. Biophys. Res. Commun. 83*, 347–352.

Cohen, S., & Milovanovič, A. (1968) *J. Am. Chem. Soc. 90*, 3495–3502.

Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1977) *Biochemistry 16*, 5484–5491.

Eichelberger, W. C., & La Mer, V. K. (1933) *J. Am. Chem. Soc. 55*, 3633–3634.

Elkins-Kaufman, E., & Neurath, H. (1949) *J. Biol. Chem. 178*, 645–655.

Glasoe, P. K., & Long, F. A. (1960) *J. Phys. Chem. 64*, 188–200.

Hunter, L. (1953) *Chem. Ind. (London)* 155–162.

Jacobsen, N. E., & Bartlett, P. A. (1981) *J. Am. Chem. Soc. 103*, 654–657.

Jones, I., & Soper, F. G. (1936) *J. Chem. Soc. 1936*, 133–142.

Makinen, M. W., Kuo, L. C., Dymowski, J. J., & Jaffer, S. (1979) *J. Biol. Chem. 254*, 356–366.

Natelson, S., & Gottfried, S. (1955) *Organic Syntheses*, Collect. Vol. III, pp 381–384, Wiley, New York.

Ondetti, M. A., Condon, M. E., Reid, J., Sabo, E. F., Cheung, H. S., & Cushman, D. W. (1979) *Biochemistry 18*, 1427–1430.

Patchett, A. A., Harris, E., Tristram, E. W., Wyvratt, M. J., Wu, M. T., Taub, D., Peterson, E. R., Ikeler, T. J., ten Broeke, J., Payne, L. G., Ondeyka, D. L., Thorsett, E. D., Greenlee, W. J., Lohr, N. S., Hoffsommer, R. D., Joshua, H., Ruyle, W. V., Rothrock, J. W., Aster, S. D., Maycock, A. L., Robinson, F. M., Hirschmann, R., Sweet, C. S., Ulm, E. H., Gross, D. M., Vassil, T. C., & Stone, C. A. (1980) *Nature (London) 288*, 280–283.

Peterson, L. M., Sokolovsky, M., & Vallee, B. L. (1976) *Biochemistry 15*, 2501–2511.

Simpson, R. T., Riordan, J. F., & Vallee, B. L. (1963) *Biochemistry 2*, 616.

Ward, C. F. (1922) *J. Chem. Soc. 121*, 1161–1165.

Weizman, A. (1943) *J. Org. Chem. 8*, 285–293.

Wolfenden, R. (1972) *Acc. Chem. Res. 5*, 10–18.

Wolfenden, R. (1980) in *Chemical Recognition in Biology* (Chapeville, F., & Haenni, A.-L., Eds.) pp 43–61, Springer, Berlin.