D. (1977), *Biochem. Biophys. Res. Commun. 74,* 643-649.
Reynolds, W. F., Peat, I. R., Freedman, M. H., and Lyerla, J. R., Jr. (1973), *J. Am. Chem. Soc., 95,* 328-331.
Robertus, J. D., Kraut, J., Alden, R. A., and Birktoft, J. J. (1972), *Biochemistry 11,* 4293-4303.
Robillard, G., and Shulman, R. G. (1972), *J. Mol. Biol. 71,* 507-511.
Robillard, G., and Shulman, R. G., (1974a), *J. Mol. Biol. 86,* 519-540.
Robillard, G., and Shulman, R. G. (1974b), *J. Mol. Biol. 86,* 541-558.
Rogers, G. A., and Bruice, T. C. (1974), *J. Am. Chem. Soc., 96,* 2473-2481.
Rühlmann, A., Kukla, D., Schwager, P., Bartels, K., and Huber, R. (1973), *J. Mol. Biol. 77,* 417-436.
Scheiner, S., Kleier, D. A., and Lipscomb, W. N. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2606-2610.
Schoellmann, G., and Shaw, E. (1963), *Biochemistry 2,* 252-255.
Shaw, E., Mares-Guia, M., and Cohen, W. (1965), *Biochemistry 4,* 2219-2225.
Shotton, D. M., and Watson, H. C. (1970), *Nature (London) 225,* 811-816.
Shotton, D. M., White, N. J., and Watson, H. C. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 91-105.
Spencer, T., and Sturtevant, J. M. (1959), *J. Am. Chem. Soc., 81,* 1874-1882.
Stroud, R. M., Kay, L. M., and Dickerson, R. E. (1974), *J. Mol. Biol. 83,* 185-208.
Tulinsky, A., Mani, N. V., Morimoto, C. N., and Vandlen, R. L. (1973a), *Acta Crystallogr. Sect. B, 29,* 1309-1322.
Tulinsky, A., Vandlen, R. L., Morimoto, C. N., Mani, N. V., and Wright, L. H. (1973b), *Biochemistry 12,* 4185-4192.
Tung, M. S., and Steiner, R. F. (1974), *Eur. J. Biochem. 44,* 49-58.
Vandlen, R. L., and Tulinsky, A. (1973), *Biochemistry 12,* 4193-4199.
Westler, W. M., and Markley, J. L. (1978), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37,* Abstr. 2882.
Wilcox, P. E. (1970), *Methods Enzymol. 19,* 64-108.
Wright, H. T. (1973a), *J. Mol. Biol. 79,* 1-11.
Wright, H. T. (1973b), *J. Mol. Biol. 79,* 13-23.
Wright, C. S., Alden, R. A., and Kraut, J. (1969), *Nature (London) 221,* 235-242.
Wüthrich, K., Shulman, R. G., and Yamane, T. (1968), *Proc. Natl. Acad. Sci. U.S.A. 61,* 1199-1206.

Active Site in Zymogens. Proton Magnetic Resonance pH Titration Curves of Histidine-57 in Porcine and Bovine Trypsinogens and in Their Complexes with Bovine Pancreatic Trypsin Inhibitor (Kunitz)†

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ABSTRACT: Peaks attributed to the $C^{\epsilon 1}$ protons of the four histidines of porcine trypsinogen have been resolved in 250-MHz ^1H NMR spectra after preexchange of overlapping N-H resonances in ^2H_2O at elevated temperature. One of the histidyl peaks is shifted abnormally far downfield at high pH and is affected by two transitions occurring with pH_{mid} values of 3.2 and 7.7. A similar peak observed in spectra of preexchanged bovine trypsinogen undergoes transitions with pH_{mid} values of 1.7 and 7.7. These peaks have been assigned to the $C^{\epsilon 1}$-H of histidine-57 (chymotrypsinogen numbering system) in the active site. The assignments are based on the perturbation of these peaks in diisopropylphosphoryl derivatives of bovine and porcine trypsinogen. The pK' values of 7.7 are assigned to protonation of histidine-57, and the pK' values of 3.2 and 1.7 are assigned to protonation of aspartate-102 in porcine and bovine trypsinogen, respectively. The titration behavior of histidine-57 in both trypsinogens is modified significantly in complexes with bovine pancreatic trypsin inhibitor (Kunitz). In both complexes, the chemical shift of the peak is similar to that of histidine-57 in spectra of the porcine trypsin-bovine pancreatic trypsin inhibitor complex [Markley, J. L., & Porubcan, M. A. (1976) *J. Mol. Biol. 102,* 487-509]. Calculations based on the environment of histidine-57 in the X-ray crystallographic structure of bovine trypsinogen [Fehlhammer, H., Bode, W., & Huber, R. (1977) *J. Mol. Biol. 111,* 415-438] show that the unusual NMR deshielding of the histidine-57 $C^{\epsilon 1}$ protons in trypsinogen results from magnetic anisotropy about the nearby carbonyl group of serine-214. The ^1H NMR data indicate that the environment and pK' of histidine-57 are different in trypsinogen and trypsin.

Trypsin, an important and extensively studied enzyme, is synthesized and stored by the pancreas in an inactive form known as trypsinogen. This precursor or zymogen is released into the duodenum and is activated by the specific action of another proteinase, enterokinase. Trypsin then acts to catalyze the conversion of additional trypsinogen to trypsin as well as initiating a cascade of other zymogen activations which are necessary for the production and maintenance of proper enzyme levels in the digestive tract.

Trypsinogen and trypsin have been the subject of numerous investigations throughout the years. Early chemical modification studies implicated the involvement of a histidyl and a seryl residue in the catalytic mechanism of trypsin and other

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related serine proteinases. Later these were identified as His^{57} and Ser^{195} in the chymotrypsinogen numbering system.[1] X-ray crystallographic studies of chymotrypsin (Blow et al., 1969), a homologous serine proteinase, implicated a third residue, namely, Asp^{102}, as being essential for catalytic activity. The three-dimensional arrangement of these three residues suggested a hydrogen-bonded structure capable of transferring a developing charge from the serine to the aspartate during enzymatic catalysis. Originally it was believed that formation of this structure or "charge relay" (Blow et al., 1969) was a primary event in the conversion of trypsinogen to trypsin. Subsequent crystallographic studies of chymotrypsinogen (Freer et al., 1970) and trypsinogen (Fehlhammer et al., 1977; Bode et al., 1976; Kossiakoff et al., 1977) revealed that the Asp-His-Ser structure is intact in these zymogens. It is now generally accepted that the large increase in activity upon activation of these zymogens is due to conformational changes in the overall three-dimensional structure of the enzyme and in particular to changes in the region around the specificity pocket of the enzyme. The small differences observed in the relative crystallographic positions of the side chain residues in the region of histidine-57 in trypsinogen and trypsin are not generally believed to be important in the overall activation mechanism.

Matthews et al. (1977) recently have reviewed the known crystal structures of serine proteinases and have suggested that, based on geometric considerations alone, it is unlikely that a significant hydrogen bond can exist between Ser^{195} and His^{57} in most of the serine proteinases that have been studied. Curiously, the hydrogen bond between Ser^{195} and His^{57} is present in chymotrypsinogen (Birktoft et al., 1976). This revised view implies that the presence of the nearby hydrogen-bonded histidine–aspartic acid couple simply serves as an efficient proton acceptor and donor during enzyme catalysis rather than polarizing the hydroxyl in Ser^{195}. The reactivity of Ser^{195} is now ascribed to a favorable orientation of the susceptible peptide bond of bound substrate. The energy of the transition state is lowered further by groups that stabilize the oxyanion (Robertus et al., 1972).

Proton NMR[2] spectroscopy is a useful technique for studying the active site since the $C^{\epsilon 1}$–H resonances of histidyl residues lie in a relatively resonance-free region of the spectrum just downfield from the aromatic envelope (Markley, 1975). In addition, this resonance is sensitive to the protonation state of the histidyl side chain, shifting up to a full ppm during titration. Previous NMR studies of serine proteinases have considered the protonation states of the active site residues in porcine trypsin (Markley & Porubcan, 1976; Markley, 1978), bovine chymotrypsinogen and bovine chymotrypsin (Robillard & Shulman, 1972, 1974a,b; Markley & Ibañez, 1978), and α-lytic proteinase (Hunkapiller et al., 1973; Westler & Markley, 1978). We describe here the ^1H NMR titration behavior of His^{57} in the active site and compare these data to the above studies to gain insight into the nature of the catalytic groups in zymogens and in zymogen–inhibitor complexes.

Experimental Section

Materials. Bovine trypsinogen was purchased from Worthington Biochemical Corp. (TG 35C823). Porcine trypsinogen was isolated from fresh pig pancreas according to the method of Charles et al. (1963). Bovine pancreatic trypsin inhibitor (BPTI[2]) (Trasylol, a registered trademark of Bayer AG) was a generous gift from Bayer, AG. Deuterium oxide, 99.8% isotopically pure, potassium deuteroxide, and Chelex 100 resin were purchased from Bio-Rad Laboratories. Deuterium chloride was from Merck of Canada. *p*-Nitrophenyl *p'*-guanidobenzoate was from Nutritional Biochemical Corp. CM-cellulose was from Whatman. UltraPure potassium chloride and calcium chloride were from Alfa Chemical Co. Sephadex G-25 and PD-10 columns were from Pharmacia. All other chemicals were reagent grade or better.

Assays of Trypsinogen Samples. Trypsinogen samples were assayed by activation to trypsin followed by active site titration using the NPGB method of Chase & Shaw (1967). Activation was carried out at pH 8.0 by addition of 2–5% trypsin at room temperature in 0.1 M Tris buffer containing 0.02 M $CaCl_2$. The value of $E_{280}^{1\%} = 15.0$ for bovine trypsinogen (Walsh, 1970) was used for both bovine and porcine trypsinogen. Potential activities were typically 75–80% using this method.

Preparation of Exchanged Protein Samples. All trypsinogen samples were subjected to exchange in ^2H_2O at pH* 2.8 for 1 h at 10 mg/mL in order to remove slowly exchangeable N–H groups that interfere with resolution of the histidine $C^{\epsilon 1}$–H peaks. Potential activity was reduced to 60–65% by this procedure. Exchanged samples were lyophilized and frozen until use.

Bovine pancreatic trypsin inhibitor was preexchanged at 7 mg/mL in ^2H_2O at 80 °C, pH 3.0, for 75 min. This procedure removes all N–H resonances from the histidyl $C^{\epsilon 1}$–H region of the NMR spectrum (Masson & Wüthrich, 1973; Markley & Porubcan, 1976). BPTI usually was purified and desalted on Sephadex G-25 prior to exchange.

Preparation of Trypsinogen–Pancreatic Trypsin Inhibitor Complexes. Complexes between trypsinogen and BPTI were prepared by addition of an equimolar amount of preexchanged BPTI to preexchanged bovine or porcine trypsinogen at pH* 8.0 in ^2H_2O. The resulting complexes were lyophilized and frozen until use.

Preparation of iPr_2P-Trypsinogens. Diisopropylphosphoryl derivatives of the trypsinogens were prepared by an adaptation of the procedure of Gertler et al. (1974). Bovine or porcine trypsinogen was reacted with a 100–300-fold excess of iPr_2P-F by addition of the appropriate amount of a 1 M solution of iPr_2P-F in isopropyl alcohol to preexchanged trypsinogen at a concentration of 3–5 mg/mL in 0.1 M Tris, 0.02 M $CaCl_2$, pH* 8.0 in ^2H_2O. The pH* was adjusted occasionally back to 8.0 during the course of the 24-h reaction. Reacted samples showed little or no potential activity. The pH was lowered to 3.0 and the small amount of precipitation that formed during the reaction was centrifuged out. The solution was concentrated by ultrafiltration and was desalted on a Sephadex PD-10 column preequilibrated with ^2H_2O. The protein solutions were lyophilized and stored at −20 °C until use.

Solutions Used for NMR Spectroscopy. Typical samples contained 25–30 mg of trypsinogen in 0.5 mL of 0.5 M KCl in ^2H_2O. Insoluble material was removed by centrifugation.

[1] The chymotrypsinogen numbering system is used throughout to specify residues in related serine proteinases.

[2] Abbreviations used: NMR, nuclear magnetic resonance; DSS, 3-trimethyl-silyl-1-propanesulfonic acid sodium salt (2,2-dimethyl-2-silapentane-5-sulfonate); BPTI, bovine pancreatic trypsin inhibitor (Kunitz); PTg, porcine trypsinogen; BTg, bovine trypsinogen; BCtg, bovine chymotrypsinogen; PTr, porcine trypsin; iPr_2P-F, diisopropyl phosphorofluoridate; iPr_2P (DIP in figures), diisopropylphosphoryl; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate. The symbol pH* is used to indicate the uncorrected pH meter reading of ^2H_2O solutions obtained using a glass electrode standardized with buffers made up in ^1H_2O. Notation used follows the "Recommendations for the Presentation of NMR Data for Publication in Chemical Journals" ((1976) *Pure Appl. Chem. 45,* 219). Because of differences in the numbering of the histidyl ring in the chemical and biochemical literature, the crystallographic convention is utilized here: $C^{\epsilon 1}$ is C(2) in the usual biochemical nomenclature; $N^{\delta 1}$ is N_π, and $N^{\epsilon 2}$ is N_τ.

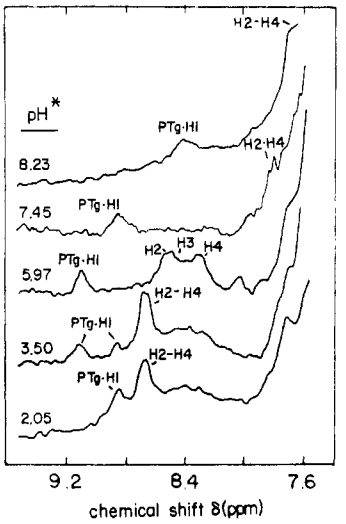

FIGURE 1: The 250-MHz ^1H NMR spectra of the histidyl $C^{\epsilon 1}$-H region of preexchanged porcine trypsinogen (PTg) at various pH* values. Twenty-five milligrams of protein/0.5 mL of 0.5 M KCl in ^2H_2O, 31 °C. Note that at high pH all of the peaks broaden significantly, making distinct resolution of peaks H2-H4 difficult.

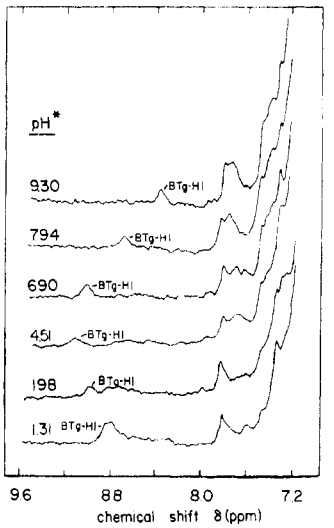

FIGURE 3: The 250-MHz ^1H NMR spectra of preexchanged bovine trypsinogen (BTg) at various pH* values. Twenty-five milligrams of protein/0.5 mL of 0.5 M KCl in ^2H_2O, 31 °C. Peak BTg-H1 is assigned to histidine-57 of the active site. Peaks corresponding to the other two histidines were not resolved.

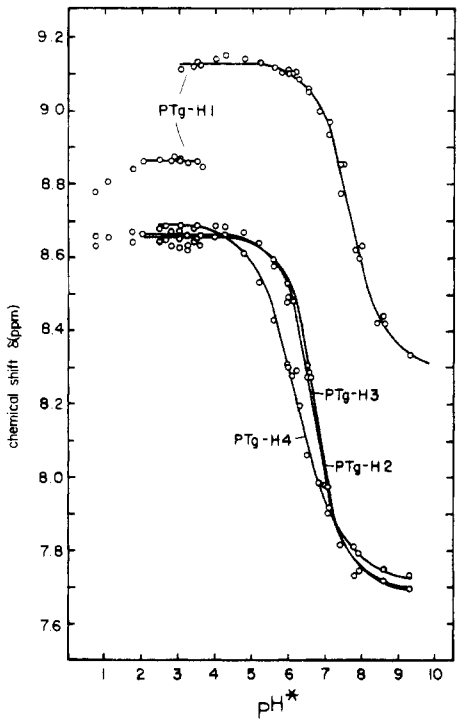

FIGURE 2: ^1H NMR titration curves of the histidyl $C^{\epsilon 1}$-H peaks of porcine trypsinogen (PTg) (Figure 1). Peak PTg-HI is affected by two transitions and is assigned to histidine-57 of the active site. Peaks H2-H4 are not assigned to specific histidines. The pK' values are listed in Table I.

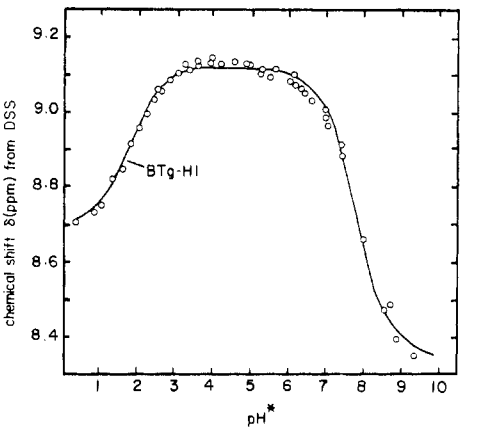

FIGURE 4: ^1H NMR titration curves of the histidyl $C^{\epsilon 1}$-H peak of bovine trypsinogen (BTg) (Figure 3). The peak is affected by two transitions and is assigned to histidine-57 of the active site. The pK' values are listed in Table I.

The pH adjustments were carried out as described previously (Markley & Porubcan, 1976). The notation pH* indicates uncorrected pH meter readings of ^2H_2O solutions made with electrodes standardized using ^1H_2O buffers. The pH* was measured at 25 °C before and after spectra were taken, and spectra were used only if there was agreement within 0.05 pH unit. Trypsinogen is susceptible to autoactivation above pH 6. Activation could be retarded by the addition of 5% preexchanged BPTI to permit acquisition of NMR spectra of the zymogen. The presence of the autoactivation product (trypsin) could be detected by inspecting the ^1H NMR spectra, and samples that had activated were discarded.

NMR Spectroscopy. ^1H NMR spectra were obtained in 15 min at 31 °C using the correlation technique at 250 MHz as described previously (Markley & Porubcan, 1976). A single sample could be used for up to ten spectra depending on the pH range covered. The reversibility of all transitions was checked. Only in cases where there was extensive precipitation at very low pH was there any irreversibility. All chemical shifts are reported in parts per million (ppm) from internal DSS (2,2-dimethyl-2-silapentane-5-sulfonate). These chemical shifts are related to those previously reported referenced to external 5% $(CH_3)_4Si$ by the equation:

$$\delta_{DSS} = \delta_{(CH_3)_4Si} + 0.33$$

Data analysis was as previously described (Markley & Porubcan, 1976).

Results

Titration Curves of the Histidyl Residues in Porcine Trypsinogen and Bovine Trypsinogen. Preexchange at 80 °C, pH* 2.8, in ^2H_2O permits the resolution of four peaks in the

TABLE I: Transitions Affecting the Histidyl $C^{\epsilon 1}$–H NMR Peaks of Bovine and Porcine Trypsinogens and Their Diisopropylphosphoryl Derivatives.

	histidyl NMR peak	pK' value	Hill coefficient	chemical shift (ppm)[b] $\delta_{low\ pH}$	$\delta_{high\ pH}$
porcine trypsinogen	PTg-H1	3.2 ± 0.1[e]	*c*	8.86 ± 0.05	9.12 ± 0.01
		7.67 ± 0.05	0.90 ± 0.06	9.12 ± 0.01	8.31 ± 0.03
	PTg-H2	6.70 ± 0.02	1.17 ± 0.05	8.66 ± 0.01	7.69 ± 0.01
	PTg-H3	6.70 ± 0.02	1.12 ± 0.06	8.65 ± 0.01	7.69 ± 0.01
	PTg-H4	6.25 ± 0.05	0.69 ± 0.05	8.69 ± 0.02	7.72 ± 0.02
bovine trypsinogen	BTg-H1	1.72 ± 0.06	*a*	8.69 ± 0.01	9.12 ± 0.01
		7.72 ± 0.04	*a*	9.12 ± 0.01	8.36 ± 0.01
porcine iPr_2P-trypsinogen	iPr_2P-PTg-H1	3.4 ± 0.2	*c*	8.83 ± 0.02	8.93 ± 0.02
		4.3 ± 0.2	*d*	8.93 ± 0.02	9.00 ± 0.02
		7.67 ± 0.02	0.97 ± 0.03	9.00 ± 0.02	7.83 ± 0.02
bovine iPr_2P-trypsinogen	iPr_2P-BTg-H1	4.3 ± 0.2	*d*	8.81 ± 0.02	8.91 ± 0.02
		7.99 ± 0.02	0.99 ± 0.03	8.92 ± 0.01	7.99 ± 0.02

[a] Double pK' fit; Hill coefficient was fixed at one. [b] Chemical shifts are referenced to internal DSS. [c] Not applicable. [d] Not calculated. [e] Transition probably cooperative; pH_{mid} rather than pK' value.

histidyl $C^{\epsilon 1}$–H region of 250-MHz ^1H NMR spectra of porcine trypsinogen (Figure 1). Peaks H1, H2, and H3 appear to titrate normally in spite of some broadening at intermediate pHs and yield pK' values between 6.3 and 6.7 (Figure 2). Peak H1 undergoes two transitions. The high pH transition occurring with a pK' of 7.7 appears as a normal histidyl titration curve that has been shifted downfield by 0.6 ppm. The low pH transition (pH_{mid} 3.2) is slow on the NMR time scale, as is shown by the discontinuous curve, and leads to partial normalization of the chemical shift of peak H1.

A similar titrating peak is observed in spectra of preexchanged bovine trypsinogen (Figure 3). The two transitions in bovine trypsinogen occur with pK' values of 1.7 and 7.7 (Figure 4). As in porcine trypsinogen, the high pH transition is characterized by an unusually deshielded chemical shift (~0.6 ppm) indicating an abnormal environment for the histidyl $C^{\epsilon 1}$–H. The low pH transition results in normalization of the chemical shift. In bovine trypsinogen the low pH transition is continuous (fast on the NMR time scale) and occurs with a lower pK' value. Peaks corresponding to the other two histidyl residues of bovine trypsinogen have not been resolved fully. The peaks are not broadened by paramagnetic impurities since exhaustive dialysis against Chelex 100 or addition of EDTA does not lead to appearance of the peaks. Sharpening of histidyl peaks by buffers has been reported for ribonuclease A (Markley, 1975) and carbonic anhydrase (Campbell et al., 1974). Also calcium ion may be expected to stabilize conformational mobility (Fehlhammer et al., 1977). However, the missing peaks do not appear except at low pH in any of the following solvents: 0.5 M acetate; 0.3 M acetate, 0.2 M KCl; 0.5 M KCl, 0.05 M $CaCl_2$; 0.5 M KCl or 0.2 M phosphate. Titration parameters for the observed peaks in bovine trypsinogen are listed in Table I.

Diisopropylphosphoryl Bovine and Porcine Trypsinogen. Spectra of the iPr_2P-F derivatives of both porcine trypsinogen (Figure 5) and bovine trypsinogen (Figure 6) show perturbations in the titration behavior of the abnormally titrating histidyl peak. In iPr_2P-porcine trypsinogen, the high pH transition occurs with a pK' of 7.7 as in the uninhibited zymogen, but the curve is displaced upfield (shielded) by ~0.2 ppm (Figure 7). The low pH transition still results in normalization of the chemical shift of this peak occurring with a pH_{mid} of 3.2. A third unassigned transition occurs with a pK' of 4.3. Resolution of the other three histidines in spectra of iPr_2P-PTg is difficult above pH 6 owing to broadening of the peaks. Consequently,

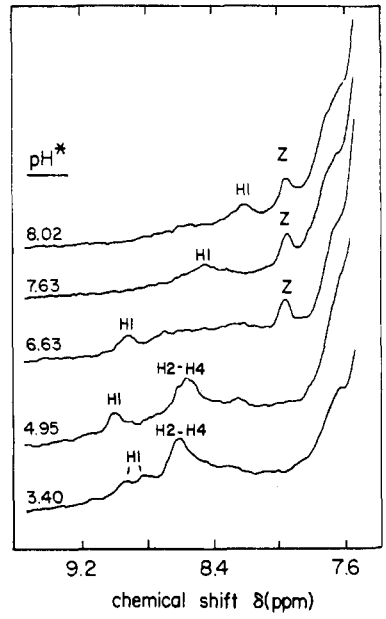

FIGURE 5: The 250-MHz ^1H NMR spectra of the histidyl $C^{\epsilon 1}$–H region of diisopropylphosphoryl porcine trypsinogen. Peak H1 is assigned to histidine-57 and is affected by two transitions as in uninhibited porcine trypsinogen. Peaks H2–H4 are resolvable at low pH only. Peak Z is a nontitrating unassigned peak that appears at high pH.

these are not plotted. The titration behavior of the histidyl peak in iPr_2P-bovine trypsinogen is affected similarly (Figure 8). Again the high pH transition occurs with a pK' of 7.7 as in the free zymogen, but the curve is displaced ~0.2 ppm upfield. The effect on the low pH transition is unclear, since excessive precipitation of the sample below pH 2 prevented resolution of any peaks. A small unassigned transition is observed near pH 4.3 consistent with the behavior of the same peak in iPr_2P-PTg. Peaks corresponding to the other two histidines in iPr_2P-BTg were unresolved as in BTg itself. Further experiments are required to determine why these peaks are absent. Possible reasons include conformational equilibria and sample heterogeneity resulting, for example, from metal ion binding.

Complexes of Bovine Pancreatic Trypsin Inhibitor (Kunitz) with Bovine and Porcine Trypsinogens. Proton NMR spectra of a 1:1 mixture of bovine pancreatic trypsin inhibitor (BPTI) with bovine trypsinogen (Figure 9) show large changes in the

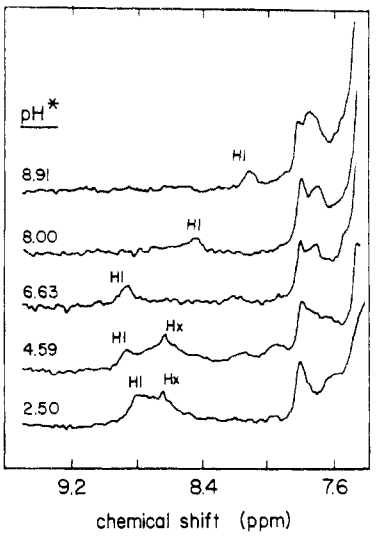

FIGURE 6: The 250-MHz ^{1}H NMR spectra of the histidyl C$^{\epsilon 1}$-H region of diisopropylphosphoryl bovine trypsinogen. Peak H1 is affected by two transitions and is assigned to histidine-57. Peak Hx may represent the sharpening of poorly resolved histidyl C$^{\epsilon 1}$-H peaks at low pH.

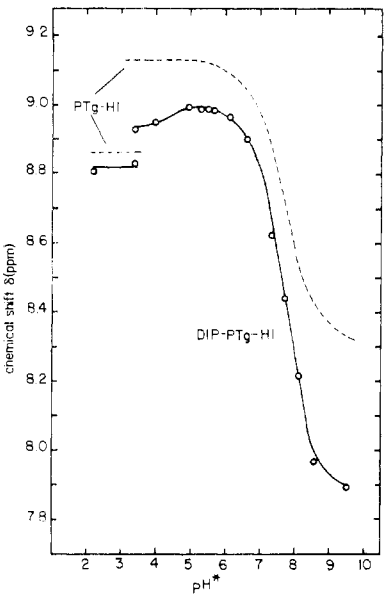

FIGURE 7: ^{1}H NMR titration curve of peak H1 of diisopropylphosphoryl porcine trypsinogen (solid line). The titration curve of peak H1 of porcine trypsinogen is included for comparison (dashed line). The pK' values are listed in Table I. Sample spectra are shown in Figure 5.

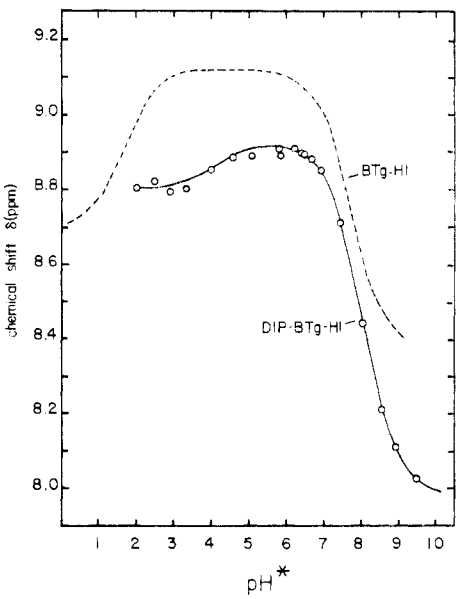

FIGURE 8: ^{1}H NMR titration curve of peak H1 in diisopropylphosphoryl bovine trypsinogen (solid line). The titration curve of peak H1 of bovine trypsinogen is included for comparison (dashed line). The pK' values are listed in Table I. Sample spectra are shown in Figure 6.

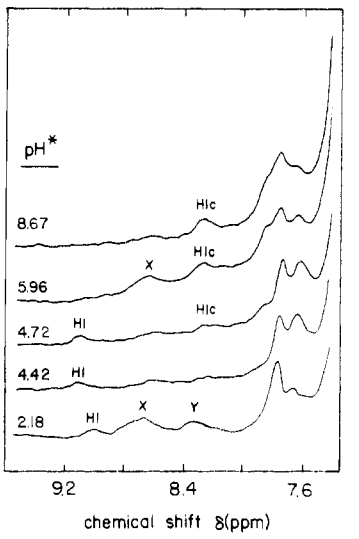

FIGURE 9: The 250-MHz ^{1}H NMR spectra of the histidyl C$^{\epsilon 1}$-H region of the 1:1 complex between bovine trypsinogen and bovine pancreatic trypsin inhibitor. Peak H1c is assigned to histidine-57 in the complex. Peak H1 corresponds to histidine-57 at low pH where the complex dissociates. Peak Y is an unassigned N-H peak that sharpens up at low pH. Peak X may correspond to resonances from the two unresolved histidyl peaks.

chemical shift of the observed histidyl peak compared with that of free bovine trypsinogen. This occurs over the pH range where a complex between the two species is known to occur (Vincent & Lazdunski, 1976; Bode et al., 1978). In the complex, the histidyl peak is shifted upfield to a position similar to that of His^{57} in the BPTI complex with porcine trypsin (Markley and Porubcan, 1976). Below pH 5.3 the titration behavior of the peak is the same as in the free zymogen (Figure 10). Similar results are found for peak H1 in spectra of the BPTI complex with porcine trypsinogen (Figure 11). The chemical shift of the peak is independent of pH above pH 6.1. Below this pH, peak H1 in the complex titrates identically with H1 in the uninhibited zymogen (Figure 12). The titration behavior of the other three histidines of porcine trypsinogen does not seem to be affected by complex formation.

Discussion

Assignment of the Unusually Titrating ^{1}H NMR Peaks of Bovine and Porcine Trypsinogen. We assign the peak designated H1 in spectra of bovine and porcine trypsinogen (Figures 1–4) to the C$^{\epsilon 1}$-H of His^{57} in the active site. The major evidence for this assignment comes from the titration curves of the iPr$_2$P derivatives of these zymogens (Figures 7 and 8). The bulky diisopropylphosphoryl group on Ser^{195} is expected to have a greater effect on the adjacent His^{57} than on the other histidines in the molecule. It is possible that an isopropyl chain could contact His^{40} (the next nearest histidine); however, the spectra of porcine iPr$_2$P-trypsinogen and bovine iPr$_2$P-chymotrypsinogen (Markley & Ibañez, 1978) indicate that only one histidyl C$^{\epsilon 1}$-H peak is affected in each zymogen. Fur-

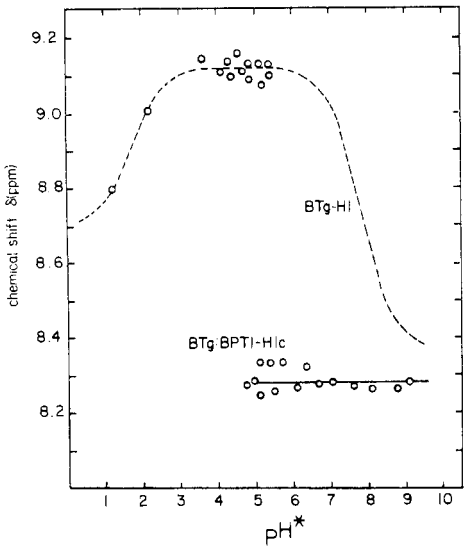

FIGURE 10: Titration behavior of peak H1 in the bovine trypsinogen-bovine pancreatic trypsin inhibitor complex (Figure 9). The chemical shift of histidine-57 is identical with that of histidine-57 of trypsin in the complex with bovine pancreatic trypsin inhibitor (see text). Below pH 5.3 where the complex dissociates, peak H1 titrates identically with corresponding peak in uninhibited bovine trypsinogen (dashed line).

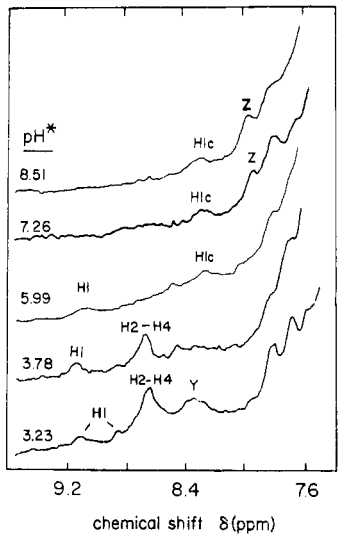

FIGURE 11: The 250-MHz ^{1}H NMR spectra of the histidyl C$^{\epsilon 1}$-H region of the complex between porcine trypsinogen and bovine pancreatic trypsin inhibitor. Peak H1c is assigned to histidine-57 in the complex. Peak H1 corresponds to histidine-57 at low pH where the complex dissociates. Peak Y is an unassigned N-H peak that sharpens up at low pH. Peak Z is a nontitrating unassigned peak that shows up at high pH.

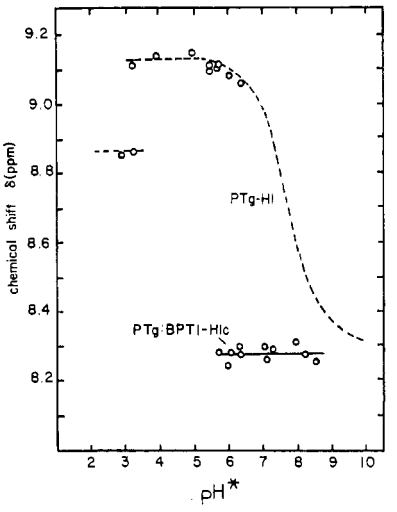

FIGURE 12: ^{1}H NMR titration behavior of peak H1 of the porcine trypsinogen-bovine pancreatic trypsin inhibitor complex (Figure 11). In the complex peak (H1c) has a chemical shift identical with that of histidine-57 in the complex with porcine trypsin (see text). When the complex breaks up below pH 6.1, peak H1 titrates normally as in uninhibited porcine trypsinogen (dashed line).

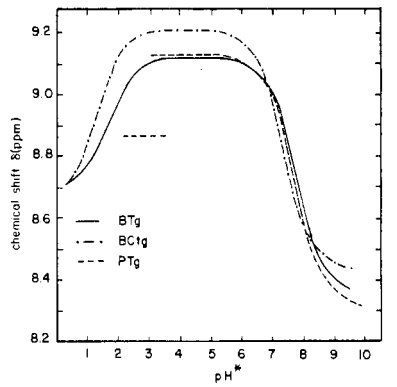

FIGURE 13: Comparison of the titration behavior of histidine-57 in bovine trypsinogen (—), porcine trypsinogen (- - -), and bovine chymotrypsinogen (• — •) (Markley & Ibañez, 1978).

thermore, ^{31}P NMR studies of these iPr_2P derivatives (Porubcan, Ibañez, & Markley, to be published) show that the ^{31}P resonance is affected by transitions with pK' values equivalent to those of the ^{1}H NMR peaks assigned to His^{57}.

Additional lines of evidence are consistent with these assignments: (i) There is a strong similarity in the titration behavior of peak H1 of both porcine and bovine trypsinogen to the peak assigned to His^{57} in the homologous serine proteinase bovine chymotrypsinogen (Markley & Ibañez, 1978). The chemical shift of this peak in all three zymogens suggests an unusual deshielded environment for the C$^{\epsilon 1}$-H of His^{57} (Figure 13). (ii) A distinctive feature of curve PTg-H1 is the discontinuity in the second protonation step. It is reasonable to correlate this peak with peak PTr-H4 of porcine trypsin (Markley & Porubcan, 1976) which also shows two inflections, the first continuous and the second discontinuous (Figure 14). The other 3 histidines of porcine trypsinogen and porcine trypsin yield normal, continuous, single-step titration curves. Peak PTr-H4 of porcine trypsin has been assigned to His^{57} (Markley & Porubcan, 1976). (iii) All the peaks assigned to His^{57} in the zymogens exhibit two inflections (Figure 13). This is expected of His^{57} because of its proximity to Asp^{102}; however, His^{40} also should show two inflections if it interacts with Asp^{194}. The X-ray structure of bovine chymotrypsinogen indicates a hydrogen bond between the His^{40} imidazole and Asp^{194} carboxylate (Freer et al., 1970). The existence of this hydrogen bond in trypsinogen is uncertain because it has been reported to be present in one X-ray structure (Fehlhammer et al., 1977) but not in another (Kossiakoff et al., 1977). The ^{1}H NMR peak assigned to the C$^{\epsilon 1}$-H of His^{40} of bovine chymotrypsinogen is abnormally shielded and exhibits two inflections (Markley & Ibañez, 1978) as expected from the X-ray structure (Freer et al., 1970). On the other hand, although all four His C$^{\epsilon 1}$-H peaks of porcine trypsinogen are resolved (Figures 1 and 2), none of them is shielded, and only H1 (assigned to His^{57}) shows two inflections. The results agree with the X-ray structure of trypsinogen that shows no interaction between His^{40} and Asp^{194} (Kossiakoff et al., 1977).

Calculations of the Chemical Shift of the C$^{\epsilon 1}$-H of Histi-

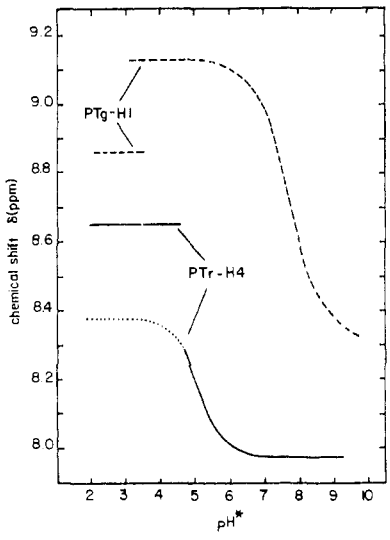

FIGURE 14: Comparison of the titration behavior of histidine-57 in porcine trypsin (PTr-H4, solid line) and porcine trypsinogen (PTg-H1, dashed line) showing the presence of discontinuous curves at low pH and the differences in pK' values in the zymogen and enzyme.

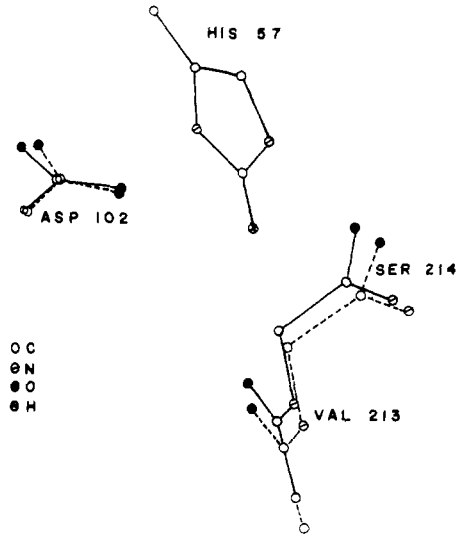

FIGURE 15: Comparison of the three-dimensional structure around histidine-57 in trypsinogen (—) and trypsin (- - -). The coordinates were rotated to superimpose the histidyl rings. Differences in the orientation of the serine-214 carbonyl provide a mechanism for the chemical shift differences of the histidyl $C^{\epsilon 1}$–H in trypsinogen compared with trypsin.

dine-57. The availability of highly refined atomic coordinates from X-ray crystallographic studies of bovine trypsinogen (Fehlhammer et al., 1977; Kossiakoff et al., 1977) and bovine chymotrypsinogen (Freer et al., 1970; Matthews et al., 1977) permits an investigation into possible mechanisms for the unusual ^{1}H NMR deshielding of the His^{57} $C^{\epsilon 1}$–H in zymogens (Figure 13). Upon examination of the bovine trypsinogen structure (Fehlhammer et al., 1977), it appears that the only group close enough to the $C^{\epsilon 1}$–H of His^{57} to cause its deshielding is the carbonyl of Ser^{214} (see Figure 15). Calculations based on the known anisotropy about a carbonyl double bond (Mital & Gupta, 1970) predict a downfield shift of 0.7 ppm in bovine trypsinogen (see Table II). No other carbonyl or aromatic ring is close enough to contribute either a shielding or deshielding interaction. The predicted shielding of 0.7 ppm is in excellent agreement with the experimental value of 0.6 ppm.

TABLE II: Chemical Shift Anisotropy Calculations.

	θ (deg)[a]	r (Å)[b]	σ_d[c]	$\Delta\delta$[d]
trypsin	69.0	2.88	-1.42×10^{-6}	
trypsinogen	60.6	2.78	-7.15×10^{-7}	0.7
trypsin-BPTI	71.2	2.85	-1.65×10^{-6}	−0.3

[a] Angle between the serine-214 carbonyl and a line drawn from the histidine $C^{\epsilon 1}$ proton to a point midway between the carbon and oxygen of the serine-214 carbonyl. [b] Distance from the histidine-57 $C^{\epsilon 1}$ proton to a point midway between the carbon and oxygen of the carbonyl bond. [c] Shielding factor (Homer & Callaghan, 1968) calculated from the equation: $\sigma_d = \Delta\chi^2(1 - 3\cos^2\theta)/3r^3$. [d] Predicted chemical shift perturbation relative to trypsin.

Similar calculations were carried out using the X-ray coordinates for bovine trypsin (Bode & Schwager, 1975) and for the bovine trypsin–BPTI complex (Huber et al., 1974). The structure of the region around His^{57} $C^{\epsilon 1}$ of bovine trypsin is shown in Figure 15. Results of the calculations are given in Table II. The conclusion is that in free trypsin the relationship between Ser^{214} carbonyl and His^{57} $C^{\epsilon 1}$–H is different from that in trypsinogen so that the predicted perturbation of the $C^{\epsilon 1}$–H chemical shift is negligible. Calculations based on the published coordinates of bovine chymotrypsinogen and chymotrypsin yielded less satisfactory agreement with the NMR results, probably because of larger errors in the atomic coordinates for these crystallographic structures. Qualitatively it is clear that an interaction between the Ser^{214} carbonyl and the $C^{\epsilon 1}$ region of His^{57} is present in the zymogen (Freer et al., 1970) but not in the enzyme (Tulinsky et al., 1973).

Protonation State of the Asp-His Couple in Trypsinogen. The chemical shift of the peak assigned to the $C^{\epsilon 1}$–H of His^{57} in both bovine and porcine trypsinogen is affected by two transitions. In each case, the high pH transition is assigned to protonation of the histidine on the basis of the direction and magnitude of the chemical shift change, which is similar to that observed for histidyl residues in a variety of proteins (Markley, 1975). The low pH transition occurring in both zymogens results in normalization of the chemical shift for the $C^{\epsilon 1}$–H of His^{57}. A change in the structure about the active site occurs at low pH which cancels the deshielding effect of the Ser^{214} carbonyl. It is probable that the low pH transition results from the protonation of Asp^{102} which might be linked to the movement of Ser^{214}. The crystallographic structure of bovine trypsinogen (Fehlhammer et al., 1977) shows a hydrogen bond between the hydroxyl of Ser^{214} and a side chain O^{δ} of Asp^{102}. Protonation of Asp^{102} should weaken or destroy this hydrogen bond and could lead to movement of the backbone chain disrupting the deshielding interaction between the Ser^{214} carbonyl and the His^{57} $C^{\epsilon 1}$–H.

The X-ray structure of bovine chymotrypsinogen (Freer et al., 1970) is not consistent with a Ser^{214} O^{γ} to Asp^{102} O^{δ} hydrogen bond. This difference in the X-ray structures of trypsinogen and chymotrypsinogen is not reflected in the ^{1}H NMR titration curves which show similar low pH inflections (Figure 13).

State of the Catalytic Triad in Trypsinogen:Bovine Pancreatic Trypsin Inhibitor Complexes. Complexation of BPTI with either of the trypsinogens leads to a change in the environment around His^{57}. The chemical shift of the histidine-57 $C^{\epsilon 1}$–H is nearly identical in complexes between BPTI and porcine trypsin (Markley & Porubcan, 1976), porcine trypsinogen, or bovine trypsinogen. This is in agreement with X-ray results which indicate that binding of BPTI to trypsinogen induces a conformational change leading to an active site structure which resembles that of trypsin (Bode et al., 1976,

1978). The similarity in structures of trypsin:BPTI and trypsinogen:BPTI is also supported by a ^1H NMR study of hydrogen-bonded N-H complexes (Markley, 1978). Calculations of the anisotropic contributions to the chemical shift of the $C^{\epsilon 1}$-H of His^{57} (Table II) are consistent with at least a partial positive charge on His^{57} of trypsin in the complex with BPTI (Markley & Porubcan, 1976). If the calculated anisotropic contribution of −0.3 ppm is added to the titration shift of 0.9 ppm expected for protonation of the $N^{\epsilon 2}$, the resulting value is 0.6 ppm. The experimental shift on complex formation at neutral pH is 0.4 ppm (Markley & Porubcan, 1976). The remaining 0.2 ppm shielding may be the result of additional environmental effects including partial transfer of the positive charge to Asp^{102}. If it is assumed that the similarity of the trypsin:BPTI and trypsinogen:BPTI complexes (Bode et al., 1976, 1978) includes all environmental contributions to the His $C^{\epsilon 1}$-H chemical shift, the present NMR results suggest that His^{57} is positively charged in the trypsinogen:BPTI complexes.

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References

Birktoft, J. J., Kraut, J., & Freer, S. T. (1976) *Biochemistry 15,* 4481-4485.

Blow, D. M., Birktoft, J. J., & Hartley, B. S. (1969) *Nature (London) 221,* 337-340.

Bode, W., & Schwager, P. (1975) *J. Mol. Biol. 98,* 693-717.

Bode, W., Schwager, P., & Huber, R. (1976) *Miami Winter Symp. 11,* 43-76.

Bode, W., Schwager, P., & Huber, R. (1978) *J. Mol. Biol. 118,* 99-112.

Campbell, I. D., Lindskog, S., & White, A. I. (1974) *J. Mol. Biol. 90,* 469-489.

Charles, M., Rovery, M., Guidoni, A., & Desnuelle, P. (1963) *Biochim. Biophys. Acta 69,* 115-129.

Chase, T., Jr., & Shaw, E. (1967) *Biochem. Biophys. Res. Commun. 29,* 508-514.

Fehlhammer, H., Bode, W., & Huber, R. (1977) *J. Mol. Biol. 111,* 415-438.

Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T., & Xuong, Ng. H. (1970) *Biochemistry 9,* 1997-2009.

Gertler, A., Walsh, K. A., & Neurath, H. (1974) *Biochemistry 13,* 1302-1310.

Homer, J., & Callaghan, D. (1968) *J. Chem. Soc. A,* 439-444.

Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., & Steigemann, W. (1974) *J. Mol. Biol. 89,* 73-101.

Hunkapiller, M. W., Smallcombe, S. H., Whitaker, D. R., & Richards, J. H. (1973) *Biochemistry 12,* 4732-4743.

Kossiakoff, A. A., Chambers, J. L., Kay, L. M., & Stroud, R. M. (1977) *Biochemistry 16,* 654-664.

Markley, J. L. (1975) *Acc. Chem. Res. 8,* 70-80.

Markley, J. L. (1978) *Biochemistry* 17 (following paper in this issue).

Markley, J. L., & Ibañez, I. B. (1978) *Biochemistry 17* (preceding paper in this issue).

Markley, J. L., & Porubcan, M. A. (1976) *J. Mol. Biol. 102,* 487-509.

Masson, A., & Wüthrich, K. (1973) *FEBS Lett. 31,* 114-118.

Matthews, D. A., Alden, R. A., Birktoft, J. J., Freer, S. T., & Kraut, J. (1977) *J. Biol. Chem. 252,* 8875-8883.

Mital, R. L., & Gupta, R. R. (1970) *Z. Phys. Chem. 243,* 121-126.

Porubcan, M. A., Ibañez, I. B., & Markley, J. L. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* Abstr. 2586.

Robertus, J. D., Kraut, J., Alden, R. A., & Birktoft, J. J. (1972) *Biochemistry 11,* 4293-4303.

Robillard, G., & Shulman, R. G. (1972) *J. Mol. Biol. 71,* 507-511.

Robillard, G., & Shulman, R. G. (1974a) *J. Mol. Biol. 86,* 519-540.

Robillard, G., & Shulman, R. G. (1974b) *J. Mol. Biol. 86,* 541-558.

Tulinsky, A., Vandlen, R. L., Morimoto, C. N., Mani, N. V., & Wright, L. H. (1973) *Biochemistry 12,* 4185-4192.

Vincent, J.-P., & Lazdunski, M. (1976) *FEBS Lett. 63,* 240-244.

Walsh, K. A. (1970) *Methods Enzymol. 19,* 41.

Westler, W. M., & Markley, J. L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37,* Abstr. 2882.