15, 660-665.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406-4412.
Wofsy, L., and Singer, S. J. (1963), *Biochemistry 2*, 104-116.
Wold, F. (1967), *Methods Enzymol. 11*, 617-640.
Wood, H. A., and Bozarth, R. F. (1972), *Virology 47*, 604-609.

Postproline Cleaving Enzyme: Identification as Serine Protease Using Active Site Specific Inhibitors†

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ABSTRACT: Postproline cleaving enzyme (PPCE) (EC 3.4.21.-) is an endopeptidase which has a strong specificity for catalyzing the cleavage of the Pro-X peptide bond. The enzyme also exhibits esterase activity. Highly purified PPCE is not inhibited by EDTA or 1,10-phenanthroline and partial inhibition is obtained only with high molar ratios of *p*-hydroxymercuribenzoic acid, sodium tetrathionate, 5,5′-dithiobis(2-nitrobenzoic acid), or 2-iodoacetamide. Both peptidase and esterase activities are lost upon treatment with diisopropyl phosphorofluoridate (iPr_2P-F). Titration with tritiated inhibitor, [^{3}H]iPr_2P-F, reveals that this inhibition is irreversible and occurs on a 1:1 molar basis. PPCE inhibited by treatment with [^{3}H]iPr_2P-F retains the p*I* of 4.8 of unmodified PPCE and has a similar subunit molecular weight (58 000) on sodium dodecyl sulfate electrophoresis as unmodified PPCE, and identical chromatographic properties on the affinity column Z-Pro-D-Ala-poly(Lys)-Sepharose 4B. The pH dependence of incorporation of [^{3}H]iPr_2P-F is similar to that of the enzymatic hydrolysis of the standard substrate Z-Gly-Pro-Leu-Gly. Increasing concentrations of the competitive inhibitor Z-Gly-Pro progressively reduce the amount of [^{3}H]iPr_2P-F incorporated into PPCE and none of the [^{3}H]iPr_2P-F is incorporated when PPCE is preincubated in the presence of 8 M urea or subjected to heat treatment. These results suggest, albeit based on indirect evidence, that a single serine residue plays a vital role in the catalytic process of PPCE. Chloromethyl ketone derivatives of Tos-Pro, Z-Pro, Tos-Gly-Pro, Z-Gly-Pro, and Z-Gly-Gly-Pro inhibit PPCE following a pseudo-first-order rate constant ($k_{obsd}/[I]$ = 0.43, 0.35, 10.3, 55.6, and 109 M^{-1} s^{-1}, respectively), but these inhibitors do not affect trypsin, α-chymotrypsin, elastase, and papain. Pretreatment of PPCE with Z-Gly-ProCH_2Cl prevents the incorporation of [^{3}H]iPr_2P-F into the enzyme. These data are taken as indirect evidence that the chloromethyl ketone inhibitors interact with a critical histidine residue of PPCE. On the basis of the results presented, it is hypothesized that the active site of PPCE has a Asp-His-Ser triad, analogous to other serine proteases.

Postproline cleaving enzyme (PPCE)[1] was first discovered in human uterus, when it was found that preparations of this target organ of oxytocin cleave the prolyl^7-leucyl^8 peptide bond of the nonapeptide hormone (Walter et al., 1971). Subsequent studies seem to suggest that this enzymatic activity is ubiquitous in vertebrates, but is present only at low levels (Walter, 1973). The enzyme has been purified from lamb kidney and characterized as the first known endopeptidase with a high specificity for cleaving the -L-Pro-X-peptide bond except for the -L-Pro-L,D-Pro- bond, which for all practical purposes is not hydrolyzed (Walter, 1976; Koida and Walter, 1976).

In this paper, information on the active site of PPCE is obtained by inhibition and kinetic studies, and the catalytic mechanisms of both the peptidase and esterase activities of the enzyme are investigated. While reagents known to inhibit sulfhydryl proteases (Glazer and Smith, 1971) and metal-activated enzymes (Mildvan, 1970) only reduce the activities of PPCE at exceedingly high molar ratios, the synthetic organophosphorus inhibitor, diisopropyl phosphorofluoridate (Jansen et al., 1949), stoichiometrically reacts with PPCE; this finding may be the first indication that PPCE is a serine protease since this agent is known as a rather specific inhibitor of all serine proteases, such as α-chymotrypsin, trypsin, elastase, and subtilisin BPN′ (Cohen et al., 1967; Hartley, 1960; Walsh and Wilcox, 1970). PPCE was also allowed to react with amino acid and peptide chloromethyl ketones, which have been important in implicating by specific alkylation the functional role of a histidine residue located in the active center of the above serine proteases (Petra et al., 1965; Schoellmann and Shaw, 1963; Thompson and Blout, 1973; Morihara and Oka, 1970). Whereas chloromethyl ketone derivatives of phenylalanine and lysine were ineffective in the case of PPCE, newly synthesized proline-containing chloromethyl ketone derivatives showed a high degree of specificity and only inhibited PPCE irreversibly. The results presented lead to the tentative conclusion that PPCE is a representative member of the well-characterized family of serine proteases.

Materials and Methods

PPCE was purified over 10 000-fold from lamb kidney as described by Koida and Walter (1976). (9-[1-^{14}C]Glycinamide)arginine-vasopressin was from the same batch prepared by Walter and Havran (1971) used previously. α-Chymotrypsin, trypsin, elastase, papain, N^{α}-benzyloxycarbonyl-

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[1] Abbreviations used follow the tentative Rules and Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature for amino acids and peptides ((1972), *J. Biol. Chem. 247*, 977) and for enzyme inhibitors as circulated by the Office of Biochemical Nomenclature on July 17, 1975. All optically active amino acids are of L configuration unless otherwise stated. Additional abbreviations used are: PPCE, postproline cleaving enzyme; Nbs, 5,5′-dithiobis(2-nitrobenzoic acid); iPr_2P-F, diisopropyl phosphorofluoridate.

TABLE I: Analytical and Physicochemical Data of Proline-Containing Chloromethyl Ketone Derivatives.

Chloromethyl ketone	Yield (%)	Mp (°C) (not cor)	R_f[a]	R_f[b,c]	Formula	Mol wt	Calcd (%) C	Calcd (%) H	Calcd (%) N	Found (%)[d] C	Found (%)[d] H	Found (%)[d] N	$[\alpha]_D^{24}$ (c 2, DMF)
Tos-ProCH_2Cl	56	98–100	0.94	0.20[b]	$C_{13}H_{16}ClNO_3S$	301.80	51.7	5.34	4.64	51.7	5.62	4.49	−146
Z-ProCH_2Cl	81	Oil[e]	0.91	0.17[b]	$C_{14}H_{16}ClNO_3$	281.74	59.7	5.72	4.97	60.3	6.10	4.84	−43
Z-Gly-ProCH_2Cl	60	Oil[e]	0.81	0.69[c]	$C_{16}H_{19}ClN_2O_4 \cdot \frac{1}{2}$ ethanol	361.83	56.4	6.12	7.74	56.5	6.18	7.43	−71
Tos-Gly-ProCH_2Cl	24	Oil[e]	0.78	0.67[c]	$C_{15}H_{19}ClN_2O_4S \cdot \frac{1}{3}CHCl_3$	389.64	46.2	4.89	7.03	46.5	5.08	6.90	−55

[a] Solvent composition: 1-butanol–acetic acid–water (4:1:1, v/v/v). [b] Solvent composition: chloroform–benzene (19:1, v/v). [c] Solvent composition: chloroform–methanol (19:1, v/v). [d] Elementary analyses performed by Robertson Lab., Florham Park, N.J. [e] Purified by column chromatography on silica gel.

phenylalanine chloromethyl ketone (Z-PheCH_2Cl), Tos-LysCH_2Cl, *p*-chloromercuribenzoate ($ClHgBzO^-$), phenylmethanesulfonyl fluoride (PheCH_2SO_2F), benzoyltyrosine ethyl ester (Bz-Tyr-OEt), Z-Ala-ONp, 1,10-phenanthroline, 2-iodoactamide, 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs), and ethylenediaminetetraacetic acid were purchased from Sigma Chemical Co. and diisopropyl phosphorofluoridate (iPr_2P-F) was from Aldrich. Sodium tetrathionate was obtained from K & K Labs, Inc. [1,3-^{3}H]iPr_2P-F ([^{3}H]iPr_2P-F) dissolved in propylene glycol (specific activity, 3.9 Ci/mmol) was purchased from Amersham/Searle Corp. Liquid scintillation cocktail was purchased from Research Products International Corp. Z-Gly-Pro-Leu-Gly was bought from the Protein Research Foundation in Osaka, Japan.

Tritiated products were measured with a Beckman liquid scintillation counter LS-335. Esterase activity was determined using a double-beam Beckman spectrophotometer, Model Acta M VI. Isoelectric focusing was performed using the LKB-7900 Uniphor apparatus. Tos-Pro·½benzenate (Pravda and Rudinger, 1955), Z-Gly-Pro (Beecham, 1957), and Z-Gly-Pro-ONp (Goodman and Stueben, 1959) were prepared as described. Tos-ProCH_2Cl, Z-ProCH_2Cl, and Z-Gly-ProCH_2Cl were synthesized as described for Z-PheCH_2Cl (Hayashi et al., 1975). The oily Z-ProCH_2Cl and Z-Gly-ProCH_2Cl were purified on a silica gel column (17 g, 12 × 2 cm) by elution with chloroform; yield and physical constants of these new peptide derivatives are listed in Table I.

Preparation of Tos-Gly-ProCH_2Cl and Z-Gly-Gly-ProCH_2Cl from ProCH_2Cl Hydrochloride. According to Penke et al. (1970) the diazoketone, Boc-ProCHN_2, was prepared from Boc-Pro and diazomethane using a mixed anhydride method. Anhydrous HCl was bubbled through a solution of Boc-ProCHN_2 (7 mmol) in ether for 45 min. The reaction mixture was allowed to stand at 25 °C for 2 h. Isolation of the ProCH_2Cl·HCl involved evaporation to an oil, resuspension of the residual oil in ether (100 mL), and evaporation of the ether. Z-Gly-Gly-ProCH_2Cl was prepared from Z-Gly-Gly (Beecham, 1963), by addition of the oily ProCH_2Cl·HCl using the mixed anhydride method according to Powers and Tuhy (1973). The product was isolated in 21% yield by chromatography on a silica gel column (12 × 2 cm) and eluted with a chloroform–benzene mixture (95:5, v/v).

Since attempts to prepare Tos-Gly-ProCH_2Cl from Tos-Gly-Pro with diazomethane using the mixed anhydride method result in the formation of a diketopiperazine (Gut et al., 1968), the chloromethyl ketone was prepared as follows: to a stirred solution of Tos-Gly (1.83 g, 8 mmol) and 1-hydroxybenzotriazole (2.5 g, 16 mmol) in 6 mL of dimethylformamide and 10 mL of ethyl acetate at 0 °C dicyclohexylcarbodiimide (1.65 g, 8 mmol) was added. After 45 min stirring at 25 °C the precipitated *N,N′*-dicyclohexylurea was removed by filtration and the filtrate added to a suspension of ProCH_2Cl·HCl (7 mmol) in 50 mL of ethyl acetate. The mixture was cooled to −5 °C and the pH adjusted to 8 by the dropwise addition of triethylamine and allowed to stir for an additional 2 h at 25 °C. The organic solution was washed with: 5% aqueous $NaHCO_3$ (60 mL), followed by 1 N HCl (60 mL), and saturated aqueous NaCl (60 mL). The ethyl acetate solution was dried over anhydrous $MgSO_4$. After evaporation the oily Tos-Gly-ProCH_2Cl was isolated from the residue by chromatography on a silica gel column (12 × 2 cm) from which it was eluted with a chloroform–benzene mixture (95:5, v/v) (see Table I for yield, analytical data, and physical constants).

Enzyme Assays. The peptidase activity of PPCE was measured either by release of radioactively labeled Arg-Gly-NH_2 from (9-[1-^{14}C]glycinamide)arginine-vasopressin or by ninhydrin determination of Leu-Gly released from Z-Gly-Pro-Leu-Gly at 37 °C by a procedure described earlier (Walter, 1976), except that 50 mM phosphate buffer, pH 7.0, was used. Esterase activity of PPCE was assayed using Z-Gly-Pro-ONp as substrate by the general method of Martin et al. (1959), slightly modified from the adopted procedure of D. V. Saňti (personal communicaton): enzyme solution (10 μL) was added to 1 mL of 10 mM phosphate buffer, pH 7.0, containing 1 mM DTT and EDTA. Z-Gly-Pro-ONp (2.1 mM) dissolved in 50 μL of 1,4-dioxane was added and incubated for various periods of time at 25 °C. The initial velocity was assayed using a double-beam spectrophotometer. The reference contained 10 μL of phosphate buffer in place of enzyme solution. The esterase activity of elastase was determined using the same experimental conditions except that Z-Ala-ONp served as substitute and that the buffer used was Tris, pH 7.0. Esterase activities of α-chymotrypsin and trypsin were determined spectrophotometrically according to Hummel (1959) using the substrate Bz-Tyr-OEt, and Schwert and Takenaka (1955) using Bz-Arg-OEt, respectively. The peptidase activity of papain was assayed by the casein-Cl_3CCOOH method (Arnon and Shapira, 1967).

Definition of Enzyme Concentration and Activities. Enzyme concentration was calculated on the basis of a specific peptidase activity of 45 units/mg for purified PPCE (Koida and Walter, 1976). One unit of peptidase or esterase activity is defined as the cleavage of 1 μmol of the respective substrate/min by the particular enzyme. Peptidase reactions were carried out at 37 °C and esterase reactions at 25 °C.

Effect of Chemical Inhibitors. Enzyme solution (5 μL, 0.06

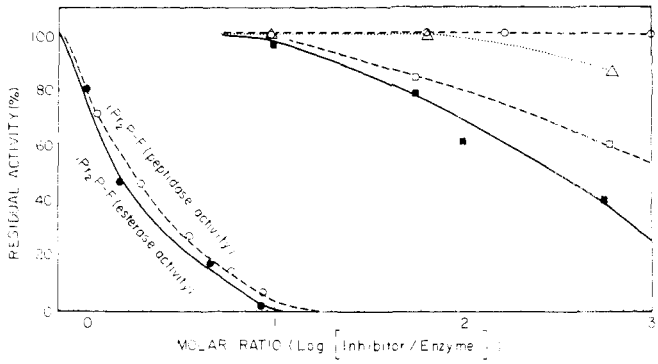

FIGURE 1: Effect of chemical reagents on the peptidase and esterase activities of PPCE. The enzyme (0.06 μM) was preincubated in phosphate buffer, pH 7.0, at 25 °C for 30 min in the absence or presence of increasing concentrations of reagents. Residual peptidase activity was determined using Z-Gly-Pro-Leu-Gly as substrate; residual esterase activity (only in case of iPr_2P-F) was measured using Z-Gly-Pro-ONp. The chemical reagents were 2-iodoacetamide, EDTA, and 1,10-phenanthroline (○ - - ○); 5,5′-dithiobis(2-nitrobenzoic acid) (△ . . . △); sodium tetrathionate (□ - - □); *p*-chloromercuribenzoic acid (■ — ■); diisopropyl phosphorofluoridate (●—● for esterase activity and ○ - - ○ for peptidase activity).

TABLE II: Rate of Inhibition of Postproline Cleaving Enzyme, α-Chymotrypsin, and Trypsin by Active-Site-Directed Reagents.[a]

	$k_{obsd}/[I]$ (M^{-1} s^{-1}) at 25 °C, pH 7.0		
	Postproline cleaving enzyme	α-Chymotrypsin	Trypsin
iPr_2P-F	64.6	40.6	2.5
$PheCH_2SO_2F$	0.07	703	2.4

[a] Enzyme concentration used: PPCE, [E] = 1.0×10^{-5} M; α-chymotrypsin, [E] = 1.6×10^{-5} M; trypsin, [E] = 2.1×10^{-5} M.

μM) was mixed with 5 μL of phosphate buffer, pH 7.0, in the absence or presence of increasing concentrations of the respective chemical inhibitor (iPr_2P-F, $ClHgBzO^-$, Nbs, sodium tetrathionate, 2-iodoacetamide, EDTA, or 1,10-phenanthroline) and preincubated at 25 °C for 30 min. Residual peptidase activity was then determined after incubation with 1 mM Z-Gly-Pro-Leu-Gly dissolved in 50 μL at 37 °C for 5 min. Residual esterase activity was measured after incubation at a final concentration of 0.1 mM Z-Gly-Pro-ONp (1.06 mL) at 25 °C for not more than 5 min.

Effect of Active Site-Directed Inhibitors on the Peptidase and Esterase Activities of PPCE, α-Chymotrypsin, Trypsin, and Papain. Inhibition experiments using chloromethyl ketone derivatives, iPr_2P-F and $PheCH_2SO_2F$, were carried out under conditions identical with those used with the chemical inhibitors, except that in the case of the active-site directed inhibitors the time courses of residual peptidase and esterase activities were followed for as long as 24 h. Note that the starting concentration of the various enzymes was different. If higher concentrations of chloromethyl ketones were required ($2.5-5 \times 10^{-4}$ M), the inhibitor was initially dissolved in 1,4-dioxane and then diluted with buffer to a final concentration of 5% 1,4-dioxane. Control experiments were carried out under identical conditions in the absence of chloromethyl ketone. Pseudo-first-order rates for enzyme inactivation (k_{obsd}) were calculated from the initial rates of inhibition according to the equation

$$k_{obsd} = \frac{\ln(E_1/E_2)}{T_2 - T_1}$$

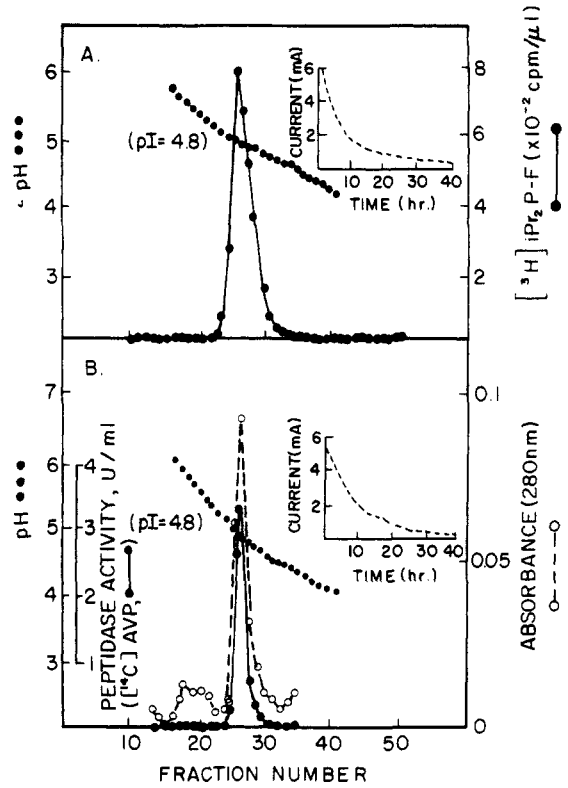

FIGURE 2: Isoelectric focusing of unmodified PPCE and enzyme inhibited by [^{3}H]iPr_2P-F. The samples (480 μg of native PPCE and 174 μg of [^{3}H]iPr_2P-F-inhibited PPCE) were applied onto a electrophoretic column (250 mL) using ampholite (pH 3.5–6) and a sucrose density gradient (0–50%). Electrophoresis was performed at 4 °C for 40 h at 400 V. Currents as a function of time are given in inserts. Panel A: [^{3}H]iPr_2PF-inhibited PPCE (●—●, tritium activity). Panel B: native PPCE (●—●, enzyme activity by using (9-[1-^{14}C]glycinamide)arginine-vasopressin ([^{14}C]AVP); (○ - - - - ○) protein absorbance at 280 nm); pH gradient (• • •).

where E_1 and E_2 are the observed activities at time T_1 and T_2.

Preparation of [^{3}H]iPr_2P-F-Inhibited PPCE. The enzyme was inhibited with tenfold molar excess of [^{3}H]iPr_2P-F in phosphate buffer, pH 7.0, containing 1 mM EDTA and DTT for 1 h at 25 °C and for an additional 20 h at 4 °C. The irreversibly labeled enzyme was separated from excess unbound [^{3}H]iPr_2P-F by gel filtration on a Sephadex G-25 column (0.7 × 20 cm) equilibrated with 50 mM phosphate buffer, pH 6.8, containing 1 mM EDTA and DTT.

Titration of PPCE with [^{3}H]iPr_2P-F. PPCE (20 pmol in 50 mM phosphate buffer, pH 6.8) in 24 μL was incubated with 5, 20, 40, 80, 100, 250, and 320 pmol of [^{3}H]iPr_2P-F (in 24 μL) at 25 °C for 15 min. After the incubation 4-μL aliquots were assayed for residual peptidase activity. Free [^{3}H]iPr_2P-F was immediately removed from a 40-μL aliquot of incubation mixture as described above using a 0.7 × 20 cm Sephadex G-25 column. The amount of [^{3}H]iPr_2P-F that reacted with the enzyme was calculated from the first radioactive peak eluted.

Determination of the Molecular Weight of Unmodified PPCE and [^{3}H]iPr_2P-F-Incubated PPCE. The molecular weight was estimated by sodium dodecyl sulfate gel electrophoresis as described by Weber and Osborn (1969). A total volume of 75 μL containing 20 μg of unmodified or labeled enzyme was applied. A current of 2.5 mA per gel (5.5 × 0.5 mm) was used. Bovine serum albumin (mol wt 63 000), oval-

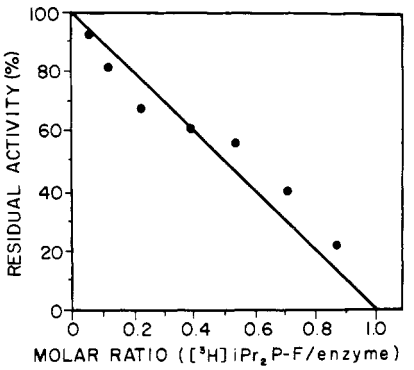

FIGURE 3: Quantitative relationship between incorporation of [^{3}H]-iPr_2P-F and residual peptidase activity of PPCE. The enzyme (20 pmol in 24 μL) was incubated with increasing concentrations of [^{3}H]iPr_2P-F (pmol in 24 μL) at 25 °C for 15 min. Aliquots (4 μL) were used to determine the residual peptidase activity. A 40-μL aliquot of the same sample was immediately passed through a 0.7 × 20 cm column of Sephadex G-25 using 50 mM phosphate buffer, pH 6.8, containing 1 mM EDTA and DTT. The amount of [^{3}H]iPr_2P-F-inhibited enzyme was determined by measuring the radioactivity in the first peak emerging from the column.

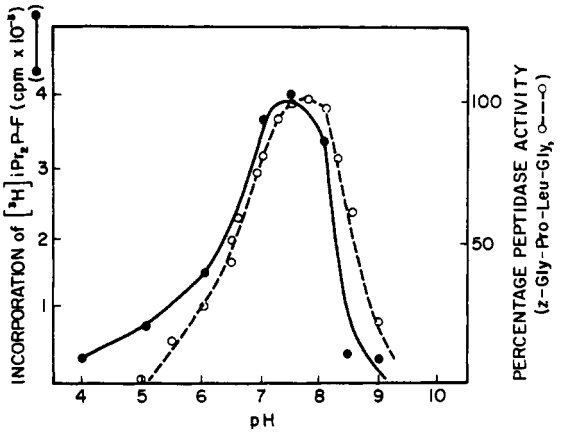

FIGURE 4: The pH dependence of the peptidase activity of PPCE. The enzyme (10 pmol in 5 μL) was mixed with [^{3}H]iPr_2P-F (100 pmol in 15 μL) dissolved in 20 mM buffer of different pHs containing 1 mM EDTA and DTT (acetate buffer, pH 4-6; phosphate buffer, pH 6-7; Tris buffer, pH 7-8; borate buffer, pH 8-9). After an incubation period for 30 min at 25 °C, the incorporation of [^{3}H]iPr_2P-F was determined following the separation of free and enzyme-bound label by gel filtration as described. Peptidase activity of PPCE as a function of pH using the standard substrate Z-Gly-Pro-Leu-Gly (O - - - - O). Incorporation of labeled [^{3}H]iPr_2P-F into PPCE as a function of pH (●—●).

bumin (43 000), rabbit muscle aldolase (40 000), chymotrypsinogen (25 700), and ribonuclease (13 700) were used as standards.

Affinity Chromatography of Unmodified and [^{3}H]iPr_2-P-F-Inhibited PPCE. The experimental conditions were the same as described by Koida and Walter (1976) using Z-Pro-D-Ala-poly(Lys)-Sepharose 4B as affinity column (0.9 × 7 cm). The unmodified (1.2 nmol) or [^{3}H]iPr_2P-F-inhibited enzyme (70 pmol) was dissolved and applied in 1 mL each of 50 mM phosphate buffer, pH 6.8, containing 1 mM DTT and 1 mM EDTA. After washing with 25 mL of buffer, the column was eluted with 25 mL of buffer containing Z-Pro-Phe (4 mg/mL). The flow rate was 25 mL/h. The eluted, unmodified enzyme was detected by its peptidase activity using the standard substrate. Inhibited PPCE was detected on the basis of the ^{3}H label.

Isoelectric Focusing of Native PPCE and Enzyme Irreversibly Inhibited with iPr_2P-F. Native (4 mL, 480 μg) and inhibited enzyme (4 mL, 174 μg) samples were applied onto an electrophoretic column (250 mL in volume) according to the method of Vesterberg and Svensson (1966) using carrier ampholites containing 1 mM EDTA and DTT, pH range 3.5 to 6 (prepared by mixing equal volumes of pH 3.5 to 5 and pH 4 to 6 carrier ampholites), and sucrose to form the density gradient (from 0 to 50%). The column was then subjected to isoelectric focusing by applying 400 V for 40 h at 4 °C.

Results

Esterase Activity of PPCE. Using the substrate Z-Gly-Pro-ONp, a K_m of 0.08 mM and k_{cat} of 20.8 s^{-1} were determined.

Effect of Chemical Inhibitors on the Peptidase and Esterase Activities of PPCE. Figure 1 shows that 2-iodoacetamide, EDTA, and 1,10-phenanthroline failed to inhibit PPCE even when applied in a 1000-fold molar excess. Using these ratios Nbs inhibited PPCE by only 20%, sodium tetrathionate by about 40%, and $ClHgBzO^-$ by about 75%. However, the peptidase and esterase activities of PPCE were very sensitive to iPr_2P-F, and 100% inhibition was already achieved with a 10-fold molar excess of inhibitor.

Rate of Inhibition of PPCE, α-Chymotrypsin, and Trypsin. PPCE is inhibited faster by iPr_2P-F than α-chymotrypsin or trypsin, as revealed by the high value for the pseudo-first-order rate constant (Table II). $PheCH_2SO_2F$ inhibited α-chymotrypsin rapidly, but PPCE not to any significant degree.

Molecular Weight Estimates and Isoelectric Points of Unmodified and [^{3}H]iPr_2P-F-Inhibited PPCE. Gel electrophoresis in the presence of sodium dodecyl sulfate indicated an average mol wt of 58 000 for monomers of both unmodified PPCE and enzyme inhibited by [^{3}H]iPr_2P-F. The isoelectric points for both unmodified and inhibited PPCE were 4.8 (Figure 2).

Behavior by Affinity Chromatography of Unmodified and [^{3}H]iPr_2P-F-Inhibited PPCE. Affinity chromatography using the method described by Koida and Walter (1976) with a smaller affinity column revealed that unmodified and [^{3}H]-iPr_2P-F-inhibited PPCE showed identical elution profiles. Fractions 15-19 contained the enzyme preparations with highest concentration in fraction 17.

Titration of PPCE with [^{3}H]iPr_2P-F. PPCE was titrated with [^{3}H]iPr_2P-F in order to determine the molar concentration of nucleophilic centers per mole of enzyme capable of reacting. By extrapolation it was found that the incorporation of 1 mol of [^{3}H]iPr_2P-F/mol of PPCE (mol wt 58 000) completely inhibited the enzyme (Figure 3). The reaction of [^{3}H]iPr_2P-F was pH dependent and optimum incorporation occurred at pH 7.5. The pH profile of the [^{3}H]iPr_2P-F incorporation into PPCE was similar, although shifted slightly to lower pH values as compared with that obtained for the enzymatic hydrolysis of the standard substrate Z-Gly-Pro-Leu-Gly by unmodified PPCE (Figure 4).

Effect of Chloromethyl Ketone Derivatives. In Table III the pseudo-first-order rate constants of the inhibition of PPCE, α-chymotrypsin, trypsin, elastase, and papain by a series of chloromethyl ketone derivatives of Z-Phe, Tos-Lys, Z-Pro, Tos-Pro, Z-Gly-Pro, Tos-Gly-Pro, and Z-Gly-Gly-Pro are compared. PPCE was not inhibited by $Z\text{-}PheCH_2Cl$ and $Tos\text{-}LysCH_2Cl$, but was inhibited by chloromethyl ketone derivatives of Tos-Pro, Z-Pro, Tos-Gly-Pro, Z-Gly-Pro, and Z-Gly-Gly-Pro ($k_{obsd}/[I]$ = 0.43, 0.35, 10.3, 55.6, and 109 M^{-1} s^{-1}, respectively). The above chloromethyl ketone de-

TABLE III: Rate of Inhibition of Postproline Cleaving Enzyme (PPCE), α-Chymotrypsin, Trypsin, Elastase, and Papain by Chloromethyl Ketone Derivatives.[a]

Inhibitor	PPCE [I] × 10^4 (M)	PPCE $k_{obsd}/[I]$ (M^{-1} s^{-1})	α-Chymotrypsin [I] × 10^4 (M)	α-Chymotrypsin $k_{obsd}/[I]$ (M^{-1} s^{-1})	Trypsin [I] × 10^4 (M)	Trypsin $k_{obsd}/[I]$ (M^{-1} s^{-1})	Elastase [I] × 10^4 (M)	Elastase $k_{obsd}/[I]$ (M^{-1} s^{-1})	Papain [I] × 10^4 (M)	Papain $k_{obsd}/[I]$ (M^{-1} s^{-1})
Z-PheCH$_2$Cl	5	0.00[b]	0.01–0.05	73.0	5	0.00	5	0.05	0.01–0.1	50.1
Tos-LysCH$_2$Cl	5	0.00[b]	5	0.00	0.1–0.5	7.70	5	0.00	0.01–0.1	46.0
Tos-ProCH$_2$Cl	5	0.43[b]	5	0.00	5	0.00	5	0.00	5	0.00
Z-ProCH$_2$Cl	2.5–5	0.35[b] 0.56[c]	5	0.00	5	0.00	5	0.01	5	0.00
Z-Gly-ProCH$_2$Cl	0.01–0.1	55.6[b] 53.0[c]	5	0.00	5	0.03	5	0.00	5	0.00
Tos-Gly-Pro-CH$_2$Cl	0.06–1	10.3[b] 15.0[c]	5	0.00	5	000	5	0.00	5	0.00
Z-Gly-Gly-Pro-CH$_2$Cl	0.01–0.1	109[b] 144[c]	5	0.09	5	0.00	5	0.03	5	0.00

[a] $K_{obsd}/[I]$ was determined at pH 7 and at 25 °C. Enzyme concentrations used are as follows. For determination of peptidase activity: PPCE, [E] = 2.5 × 10^{-6} M, and papain, [E] = 5 × 10^{-6} M. For esterase activity PPCE: [E] = 1.2 × 10^{-6} M; α-chymotrypsin, [E] = 1.8 × 10^{-6} M; trypsin, [E] = 2.1 × 10^{-5} M; elastase, [E] = 8.0 × 10^{-6} M. [b] Peptidase activity. [c] Esterase activity.

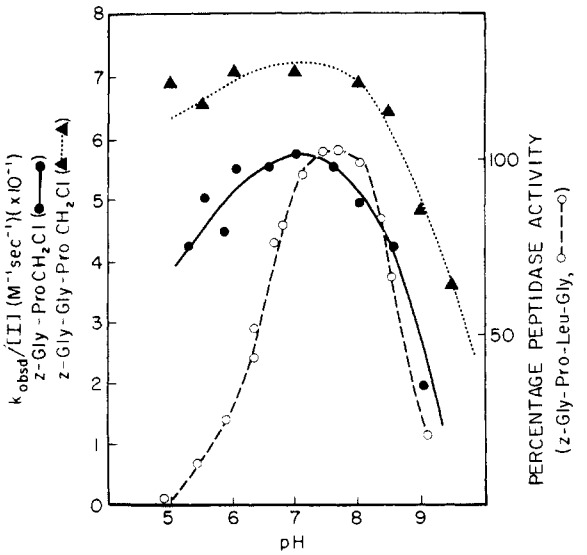

FIGURE 5: Rate of inactivation of PPCE by the chloromethyl ketones Z-Gly-ProCH$_2$Cl and Z-Gly-Gly-ProCH$_2$Cl as a function of pH. PPCE (1 × 10^{-6} M, 20 μL) was mixed with chloromethyl ketone (5 × 10^{-6} M, 20 μL) dissolved in 200 mM buffers of different pH values (acetate buffer, pH 5.5–6; phosphate buffer, pH 6–7; Tris pH 7.5–8; borate buffer, pH 8–9). Aliquots (5 μL) of the mixture were assayed for residual peptidase activity after 0, 20, 40, and 60 min incubation time. PPCE incubated under identical experimental conditions but in the absence of chloromethyl ketone served as control. Rate of inactivation of PPCE by Z-Gly-ProCH$_2$Cl (●—●) and by Z-Gly-Gly-ProCH$_2$Cl (▲ . . . ▲). Peptidase activity of PPCE from Figure 5 as a function of pH has been retraced.

rivatives containing proline did not inhibit α-chymotrypsin, trypsin, elastase, or papain. PPCE inactivated by Z-Gly-ProCH$_2$Cl was not reactivated by gel filtration on Sephadex G-25 or by dialysis. The pH dependences of the inactivation of PPCE by given concentrations of Z-Gly-ProCH$_2$Cl and Z-Gly-Gly-ProCH$_2$Cl are shown in Figure 5. The maximal rate of inhibition was seen between pH 6.5 and 7.5. While the pH dependence of inactivation in the region higher than pH 7 was similar to the pH dependence of the peptidase activity using Z-Gly-Pro-Leu-Gly as substrate (Figure 5), the pH dependence of the rate of inactivation below pH 7 differed and remained considerably higher. At pH 5 the enzyme was still capable of reacting with Z-Gly-ProCH$_2$Cl but exhibited no peptidase activity.

TABLE IV: Effect of Various Pretreatments on Postproline Cleaving Enzyme on the Incorporation of [^{3}H]iPr$_2$P-F.

	Pretreatment of PPCE	Incorp of [^{3}H]iPr$_2$P-F mol of ([^{3}H]iPr$_2$P-F) / mol of PPCE	Rel value
(1)	Control[a]	0.77	1.00
(2)	8 M urea[b]	0.01	0.01
(3)	Heat[c]	0.00	0.00
(4)	10 μM Z-Gly-ProCH$_2$Cl[b]	0.02	0.02
(5)	100 μM Z-Gly-ProCH$_2$Cl[b]	0.00	0.00
(6)	10 μM Z-Gly-Pro[b]	0.80	1.04
(7)	100 μM Z-Gly-Pro[b]	0.62	0.81
(8)	1 mM Z-Gly-Pro[b]	0.33	0.42

[a] Native or pretreated PPCE (8.4 μM) incubated at 25 °C for 30 min with 64 μM [^{3}H]iPr$_2$P-F and subjected to gel filtration on a Sephadex G-25 column (0.7 × 20 cm). [b] PPCE is kept for 1 h in the presence of the reagent at 25 °C and then exposed to [^{3}H]iPr$_2$P-F for analysis as in *a*. [c] Storage in a boiling water bath for 10 min and analysis; see *a* and *b*.

Effect of Various Pretreatments of PPCE on the Incorporation of Labeled Diisopropyl Phosphorofluoridate. When PPCE was treated under standard conditions at 25 °C for 15 min with [^{3}H]iPr$_2$P-F, an incorporation of 0.77 mol of inhibitor per mol of PPCE was found; this degree of incorporation was assigned a relative value of 1.0. When the enzyme was first exposed at 25 °C for 30 min to 8 M urea or was denatured in a boiling water bath for 10 min and then allowed to react with [^{3}H]iPr$_2$P-F, in both instances no label was incorporated. While pretreatment with 10 or 100 μM Z-Gly-Pro-CH$_2$Cl likewise resulted in no incorporation of label, there was considerable incorporation of [^{3}H]iPr$_2$P-F after pretreatment with Z-Gly-Pro, which decreased as the dipeptide concentration was raised from 10 to 100 μM and finally to 1 mM (Table IV).

Discussion

Similar to other serine proteases (Walsh and Wilcox, 1970), PPCE, in addition to its peptidase activity, was also found to exhibit esterase activity.

One approach to identifying the active center of an enzyme is by the use of chemically reactive reagents which cause an irreversible inhibition of the enzyme by combining with a

single, specific amino acid residue of the protein (Shaw, 1970). When PPCE was treated with $ClHgBzO^-$, sodium tetrathionate, Nbs and 2-iodoacetamide, all of which are reagents known to inhibit sulfhydryl enzymes (Glazer and Smith, 1971), inhibition was only observed at high inhibitor–enzyme ratios or not at all (Figure 1). On the basis of these data, it is unlikely that PPCE is a sulfhydryl enzyme. Since the chelating agents EDTA and 1,10-phenanthroline fail to reduce the enzyme activity, it may also be justified to assume that PPCE is not a metal-activated enzyme (Mildvan, 1970).

However, iPr_2P-F is a powerful irreversible inhibitor of PPCE. This inhibition is common to serine proteases comprising a large group of enzymes which is distinguished by the reactivity of a single serine residue located in the active site (Jansen et al., 1949; Hartley, 1960; Walsh and Wilcox, 1970). In fact, titration of PPCE with [^{3}H]iPr_2P-F reveals that the enzyme is inhibited on a 1:1 molar ratio by iPr_2P-F (Figure 3), suggesting that PPCE possesses such a serine residue critical to the expression of both the peptidase and esterase activities of PPCE (Figure 1).

The next series of experiments was performed in order to substantiate the fact that [^{3}H]iPr_2P-F functions as an active-site-directed inhibitor and to rule out the possibility that the loss of enzymatic acitivity is due to disruption of the interaction of groups comprising the active center caused by a change of the secondary or tertiary enzyme structure evoked by the reaction of [^{3}H]iPr_2P-F with functional groups distant from the active center. First, the similarity of the pH dependence of the peptidase activity of PPCE in cleaving the standard substrate Z-Gly-Pro-Leu-Gly and the incorporation of [^{3}H]iPr_2P-F suggest that the inhibitor reacts with a functional group located in the active site. Furthermore, the isoelectric points of 4.8 of active or [^{3}H]iPr_2P-F-inhibited PPCE are identical (Figure 3). The irreversibly inhibited enzyme still exhibits an identical binding pattern to the same affinity column, Z-Pro-D-Ala-poly(Lys)-Sepharose 4B, as the active enzyme (Koida and Walter, 1976); this finding seems to indicate that the disruption of the active center of PPCE by treatment with the irreversible inhibitor does not perturb the binding site(s) of the enzyme for the affinity column. Moreover, PPCE inhibited by [^{3}H]iPr_2P-F was shown by sodium dodecyl sulfate electrophoresis to possess a molecular weight of 58 000, which is in close agreement with the molecular weight found for the subunits from noninhibited PPCE determined under the same experimental conditions; it was reported earlier that PPCE has a molecular weight of 115 000 and is comprised of two subunits, each with a molecular weight of 57 000 (Koida and Walter, 1976).

Nevertheless, iPr_2P-F is not a specific inhibitor for PPCE, although the rate constant of inhibition was found to be greater for PPCE than for α-chymotrypsin and particularly trypsin (Table II). Another irreversible inhibitor of the serine residue situated in the active site of serine proteases, $PheCH_2SO_2F$, rapidly inactivated α-chymotrypsin (Shaw, 1970), but for all practical purposes failed to reduce the peptidase activity of PPCE (Table II).

Since there exists strong evidence that, in addition to a serine residue, the active site of serine proteases contains also a histidine group (Koshland, 1959), the approach by Schoellman and Shaw (1962, 1963) to use chloromethyl ketone derivatives as specific alkylating reagents was adopted. In addition to those chloromethyl ketone derivatives, Z-$PheCH_2Cl$ and Tos-$LysCH_2Cl$, with high specificity for α-chymotrypsin and trypsin, several new ones were specifically prepared for this study (Table I) in order to evaluate the specificity of PPCE, α-chymotrypsin, trypsin, and elastase for proline-containing chloromethyl ketone derivatives. One of the chloromethyl ketone derivatives, Z-Gly-$ProCH_2Cl$, was actually obtained from the competitive inhibitor Z-Gly-Pro ($K_i = 0.55 \times 10^{-3}$ M, unpublished). The group on the N^{α} terminus of the proline residue of the chloromethyl ketone derivatives was also varied in order to obtain preliminary information on additional structural requirements for initial substrate recognition. Papain was included because it has been reported to be inhibited by Z-$PheCH_2Cl$ and Tos-$LysCH_2Cl$ (for review, see Glazer and Smith, 1971). The data in Table III clearly reveal the high specificity of these proline-containing chloromethyl ketones for inhibiting the peptidase and esterase activities of PPCE. In fact, the specificity of these derivatives was so great that they virtually failed to affect any of the other proteases tested. The observation that proline-containing chloromethyl ketones with increased chain length exhibit greater inhibition may be a reflection of the endopeptidase character of PPCE. These results can be seen as an extension and independent confirmation of earlier substrate specificity studies with PPCE (Shlank and Walter, 1972; Walter, 1976; Koida and Walter, 1976).

The capability of the chloromethyl ketones, Z-Gly-$ProCH_2Cl$ and Z-Gly-Gly-Pro-CH_2Cl, to inhibit the peptidase activity of PPCE as a function of pH is identical at the pH range above 7 with the rate of hydrolysis of the standard substrate Z-Gly-Pro-Leu-Gly by PPCE; however, this parallel behavior is not observed below pH 7 (Figure 5). This finding is dissimilar to results obtained with α-chymotrypsin (Schoellman and Shaw, 1963) and subtilisin BPN′ (Morihara and Oka, 1970), where the pH dependence of the inactivation of the enzymes and the rates of hydrolysis of the substrates exhibit a parallel pattern, but the data are analogous to those obtained with the serine protease carboxypeptidase Y (Hayashi et al., 1975).

The lack of incorporation of [^{3}H]iPr_2P-F into PPCE denatured by either urea or heat treatment shows that the intact secondary and tertiary structures of the enzyme are required for maintenance of the strongly nucleophilic moiety in the active site of the enzyme. Pretreatment of PPCE with the irreversible alkylating agent, Z-Gly-$ProCH_2Cl$, completely prevents the subsequent incorporation of [^{3}H]iPr_2P-F. Assuming that the chloromethyl ketone alkylated a histidine residue in the active center of PPCE, this would have brought about a disruption of the active site with a concomitant reduction of the nucleophilicity of the hydroxyl group of serine in the active site, which may explain the lack of incorporation of [^{3}H]iPr_2P-F. Pretreatment with increasing concentrations of the competitive inhibitor Z-Gly-Pro progressively reduces the incorporation of label.

Based on the close similarity of the catalytic properties as peptidase and esterase as well as similarity in reactivity toward various irreversible inhibitors, it is hypothesized that PPCE possesses a Asp-His-Ser catalytic triad analogous to that established by x-ray crystallography for α-chymotrypsin (Matthews et al., 1967; Sigler et al., 1968), trypsin (Bode and Schwager, 1975), elastase (Shotton and Watson, 1970), subtilisin BPN′ (Wright et al., 1969), for chloromethyl ketone-inhibited α-chymotrypsin (Segal et al., 1971), and subtilisin BPN′ (Robertus et al., 1972) as well as kinetic studies of these enzymes (Schoellman and Shaw, 1963; Shaw et al., 1965; Kurachi et al., 1973; Thompson and Blout, 1973; Powers and Tuhy, 1973) and carbon-13 nuclear magnetic resonance investigation of α-lytic protease (Hunkapiller et al., 1973). Hence, PPCE appears to belong to the family of serine proteases and could be classified as EC 3.4.21.-.

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References

Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1964), *J. Am. Chem. Soc. 86,* 1839.
Arnon, R., and Shapira, E. (1967), *Biochemistry 6,* 3942.
Beecham, A. F. (1957), *J. Am. Chem. Soc. 79,* 3257.
Beecham, A. F. (1963), *Aust. J. Chem. 16,* 160.
Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1964), *Nature (London) 221,* 337.
Bode, W., and Schwager, P. (1975), *J. Mol. Biol. 98,* 693.
Cohen, J. A., Oosterbaan, R. A., and Berends, F. (1967), *Methods Enzymol. 11,* 686.
Glazer, A. N., and Smith, E. L. (1971), *Enzymes, 3rd Ed. 3,* 501.
Goodman, M., and Stueben, K. C. (1959), *J. Am. Chem. Soc. 81,* 3980.
Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, Vol. 2, New York, N.Y., Wiley, p 891.
Hartley, B. S. (1960), *Annu. Rev. Biochem. 29,* 45.
Hayashi, R., Bai, Y., and Hata, T. (1975), *J. Biol. Chem. 250,* 5221.
Hummel, B. C. (1959), *Can. J. Biochem. Physiol. 37,* 1393.
Hunkapiller, M. W., Smallcombe, S. H., Whitaker, D. R., and Richards, J. H. (1973), *Biochemistry 12,* 4732.
Jansen, E. F., Nutting, M. D. F., Jang, R., and Balls, A. D. (1949), *J. Biol. Chem. 179,* 189.
Koida, M., and Walter, R. (1976), *J. Biol. Chem. 251,* 7593.
Koshland, D. E., Jr. (1959), *Enzymes, 2nd Ed. 1,* 317.
Kurachi, K., Powers, J. C., and Wilcox, P. E. (1973), *Biochemistry 12,* 771.
Martin, C. J., Golubow, J., and Axelrod, A. E. (1959), *J. Biol. Chem. 243,* 294.
Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature (London) 214,* 652.
Mildvan, A. S. (1970), *Enzymes, 3rd Ed. 2,* 445.
Morihara, K., and Oka, T. (1970), *Arch. Biochem. Biophys. 138,* 526.
Penke, B., Czombos, J., Balaspiri, L., Peters, J., and Kavacs, K. (1970), *Helv. Chim. Acta 53,* 1057.
Petra, P. H., Cohen, W., and Shaw, E. N. (1965), *Biochem. Biophys. Res. Commun. 21,* 612.
Powers, J. C., and Tuhy, P. M. (1973), *Biochemistry 12,* 4767.
Pravda, Z., and Rudinger, J. (1955), *Collect. Czech. Chem. Commun. 20,* 1.
Robertus, J. D., Alden, R. A., Birktoft, J. J., Kraut, J., Powers, J. C., and Wilcox, P. E. (1972), *Biochemistry 11,* 2439.
Schoellmann, G., and Shaw, E. N. (1962), *Biochem. Biophys. Res. Commun., 7,* 36.
Schoellman, G., and Shaw, E. N. (1963), *Biochemistry 2,* 252.
Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16,* 570.
Segal, D. M., Powers, J. C., Cohen, G. H., Davies, D. R., and Wilcox, P. E. (1971), *Biochemistry 10,* 3728.
Shaw, E. (1970), *Enzymes, 3rd Ed. 1,* 91.
Shaw, E., Mares-Guia, M., and Cohen, W. (1965), *Biochemistry 4,* 2219.
Shlank, H., and Walter, R. (1972), *Proc. Soc. Exp. Biol. Med. 141,* 452.
Shotton, D. M., and Watson, H. C. (1970), *Philos. Trans. R. Soc. London, Ser. B 257,* 111.
Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R. (1968), *J. Mol. Biol. 35,* 143.
Thompson, R. C., and Blout, E. R. (1973), *Biochemistry 12,* 44.
Vesterberg, O., and Svensson, H. (1966), *Acta Chem. Scand. 20,* 820.
Walsh, K. A., and Wilcox, P. E. (1970), *Methods Enzymol. 19,* 31.
Walter, R. (1973), *Proc. Eur. Pept. Symp., 12th 1972,* 363.
Walter, R. (1976), *Biochim. Biophys. Acta 422,* 138.
Walter, R., and Havran, R. T. (1971), *Experientia 27,* 645.
Walter, R., Shlank, H., Glass, J. D., Schwartz, I. L., and Kerenyi, T. D. (1971), *Science 173,* 827.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Wright, C. S., Alden, R. A., and Kraut, J. (1969), *Nature (London) 221,* 235.