Chloroketone Hydrolysis by Chymotrypsin and *N*-Methylhistidyl-57-chymotrypsin: Implications for the Mechanism of Chymotrypsin Inactivation by Chloroketones[†,‡]

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ABSTRACT: We have examined the reaction of *N*-(benzyloxycarbonyl)-L-alanyl- L-glycyl-L-phenylalanyl chloromethyl ketone (ZAGFCMK) with chymotrypsin (Cht) and have found that, in addition to irreversible alkylation of the enzyme, some of the corresponding hydroxymethyl ketone is produced. For each molecule of hydroxy ketone formed, 3.6 molecules of chymotrypsin are inactivated. Chloroketone hydrolysis is also observed with chymotrypsin methylated at N-3 of the active site histidine (MeCht). The hydrolysis proceeds slowly (k = 0.14 min^{-1}). Alkylation of the modified enzyme was not observed. An initial burst of free chloride is detected during the MeCht-catalyzed hydrolysis. The magnitude of the chloride burst is proportional to the enzyme concentration in an approximate 1:1 stoichiometry and indicates a relatively rapid chloride-releasing step which gives rise to an intermediate which is more slowly converted to hydroxy ketone. We have also investigated both the solution and MeCht-mediated hydrolysis of the *S* isomer of *N*-acetyl-L-alanyl-L-phenylalanyl chloroethyl ketone (*S*-AcAFCEK). We have concluded that the nonenzymatic hydrolysis proceeds with inversion of configuration at the stereocenter, while the enzymatic process occurs with retention of configuration. The two nucleophilic displacements attending the MeCht-mediated hydrolysis of *S*-AcAFCEK imply the formation of an intermediate, possibly of an epoxy ether, formed by internal displacement of the chloride by the oxyanion of the initially generated enzyme–chloroketone hemiketal adduct.

Chloromethyl ketones (CMKs)[1] are classic affinity labels of serine proteases which inactivate by specifically alkylating at the N-3 position of the active site histidine residue (Powers, 1977). X-ray crystallographic (Poulos et al., 1976; James et al., 1980; Scott et al., 1986) as well as ^{13}C-NMR studies (Scott et al., 1886; Malthouse et al., 1985; Finucane et al., 1989) of several protease–CMK complexes have revealed a second covalent bond between the enzyme active site serine hydroxyl and the inhibitor carbonyl carbon. Yet, while the structure of the enzyme–CMK adduct is well established, the mechanism of inactivation remains to be characterized. Does reaction with the active site serine occur prior to alkylation or after alkylation, and is this reaction an essential component of the inactivation process? Although addition of the serine hydroxyl to the carbonyl group prior to alkylation would greatly decrease the leaving tendency of the chloride of the inactivator, the observation that anhydrochymotrypsin is not alkylated by *N*-tosyl-PheCMK despite its ability to bind substrate analogs with affinities comparable to those of the native enzyme (Weiner et al., 1966) strongly suggests that the participation of the active site serine is integral to the inactivation process. Additional support for this postulate has been provided through kinetic analyses of the inactivation of some serine proteases by chloromethyl ketones, in which the data is suggestive of the formation of an initial covalent, albeit reversible, enzyme–inactivator complex (Powers & Tuhy, 1973; Matthews et al., 1975; McMurray & Dyckes, 1986; Stein & Trainor, 1986).

Figure 1 shows two mechanisms consistent with the above observations: Mechanism A is a one-step displacement of chloride from the initially formed hemiketal intermediate. Mechanism B, initially proposed by Powers (Powers, 1977), is a two-step process, which proceeds via hemiketal formation and subsequent chloride displacement by the hemiketal oxyanion to yield an epoxy ether intermediate. This species then undergoes nucleophilic attack by the active site histidine residue.

Additional support for the intermediacy of the epoxy ether stems from studies in which the pK_a of the active site histidine of chymotrypsin (Cht) is found to increase from 7.2 in the native enzyme to greater than 10.0 in an enzyme–trifluoromethyl ketone tetrahedral complex (Liang & Abeles, 1987). This considerable elevation in the histidine pK_a is partly attributed to the negative charge of the oxyanion of the enzyme–inhibitor hemiketal adduct. This renders mechanism A of Figure 1 suspect, since it does not allow for significant deprotonation of histidine in the neutral pH range in which haloketone-based inactivators are typically studied (Powers, 1977). In the case of mechanism B, the histidine pK_a would be expected to be closer to 7.0 owing to the neutrality of the epoxy ether intermediate. This obviates the histidine pK_a problem.

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[‡] The authors have deposited atomic coordinates for *R*-AcAFCEK with Cambridge Crystallographic Data Centre. The coordinates may be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ U.K.

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[1] Abbreviations: CMK, chloromethyl ketone; CEK, chloroethyl ketone; Cht, chymotrypsin; MeCht, *N*-methylhistidyl-57-chymotrypsin; ZAGFCMK, *N*-(benzyloxycarbonyl)-L-alanyl-L-glycyl-L-phenylalanyl chloromethyl ketone; ZAGF(OH)MK, *N*-(benzyloxycarbonyl)-L-alanyl-L-glycyl-L-phenylalanyl hydroxymethyl ketone; AcAFCEK, *N*-acetyl-L-alanyl-L-phenylalanyl chloroethyl ketone; AcAF(OH)EK, *N*-acetyl-L-alanyl-L-phenylalanyl hydroxyethyl ketone; BTEE, *N*-benzoyltyrosine ethyl ester.

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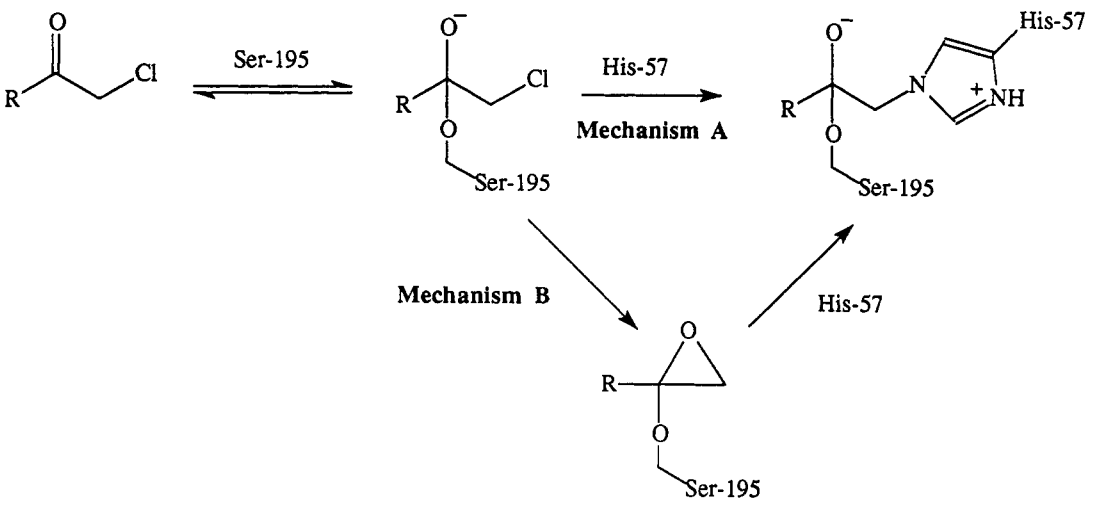

FIGURE 1: Possible mechanisms for the inactivation of a serine protease by a chloroketone.

In attempting to discriminate between mechanisms A and B, we elected to carry out studies employing a peptide-based CMK and Cht methylated at the N-3 of histidine 57. This approach has its basis in our finding that, in addition to inactivation, some hydryoxymethyl ketone is formed upon treatment of native Cht with the CMK inactivator. This suggests that some common intermediate may be attacked by either histidine or water. Hence, we entertained the possibility that with the modified enzyme the presence of the methyl group would retard or prevent the alkylation event and enhance the reaction with water. We have found not only that the alkylation of *N*-methyl-57-chymotrypsin (MeCht) by the CMK is completely precluded but that the modified enzyme catalyzes the hydrolytic conversion of the CMK to hydroxymethyl ketone. In addition to examining the reaction of a peptide-based CMK with MeCht, we employed a chloroethyl ketone (CEK) to facilitate investigation of the stereochemical course of the hydrolysis.

EXPERIMENTAL PROCEDURES

α-Cht (type II, 3× crystallized, from bovine pancreas) was purchased from Sigma Chemical Company and used without further purification. The number of active sites per mole of Cht was determined from burst kinetics using *p*-nitrophenyl trimethylacetate (Bender & Marshall, 1968) in 100 mM KP_i, pH 7.8. A value of 0.9 active site per mole of Cht was routinely obtained. MeCht was prepared as previously described (Ryan & Feeney, 1975) and purified on turkey ovomucoid (trypsin inhibitor type II-T from Sigma) coupled to Sepharose 4B (Cuatrecasas & Anfinsen, 1971). *N*-(Benzyloxycarbonyl)-L-alanyl-L-glycine (ZAG), N-acetyl-L-alanine, L-phenylalanine methyl ester hydrochloride, *N*-(benzyloxycarbonyl)-L-phenylalanine, and *N*-benzoyltyrosine ethyl ester (BTEE) were purchased from Sigma Chemical Company. [4-^{3}H]-Phenylalanine (28 Ci/mmol) and [^{3}H]acetic anhydride (500 mCi/mmol) were obtained from Amersham. [1-^{13}C]Phenylalanine (99% enriched) was from MSD Isotopes. All other chemicals were reagent grade and used as received. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian XL-300 spectrophotometer and are reported on the δ scale. Mass spectra were recorded on an HP 5985 GC/MS (chemical ionization) or a Fison's VG-Quattro instrument (fast atom bombardment). TLC was performed on Merck silica gel plates.

N-(Benzyloxycarbonyl)-L-alanyl-L-glycyl-L-phenylalanyl Chloromethyl Ketone (ZAGFCMK). The chloromethyl ketone was prepared from *N*-(benzyloxycarbonyl)-L-alanyl-L-glycyl-L-phenylalanine (ZAGF) according to the method of Shaw (Shaw, 1967). ZAGF was prepared from ZAG and L-phenylalanine methyl ester hydrochloride using the mixed anhydride procedure (Anderson et al., 1976) followed by room temperature saponification and neutralization with HCl. The final product (68% yield) was crystallized from CH_2Cl_2/hexane. TLC: R_f = 0.70 (CH_3CN). ^{1}H-NMR (CD_3OD/acetone-d_6) δ 1.36 (d, 3H, CH_3-Ala), 2.96 (dd, 1H, CH_2-Phe), 3.19 (dd, 1H, CH_2-Phe), 3.75 (d, 1H, CH_2-Gly), 3.90 (d, 1H, CH_2-Gly), 4.11 (q, 1H, CH-Ala), 4.29 (d, 1H, CH_2-Cl), 4.53 (d, J = 16.7, 1H, CH_2Cl), 4.72 (dd, 1H, CH-Phe), 5.04 (d, 1H, CH_2-Z), 5.11 (d, 1H, CH_2-Z), 7.1–7.4 (m, 10H, PH). ^{13}C-NMR (CD_3OD/D_2O) δ 17.66 (CH_3-Ala), 36.43 (CH_2-Phe), (43.92 CH_2-Gly), 48.80 (CH_2Cl), 51.91 (CH-Ala), 59.00 (CH-Phe), 67.37 (CH_2-Z), 127.66, 128.55, 128.90, 129.39, 130.10 (CH,PH), 137.90, 138.50 (C, PH). MS (CI) 488:490 (3:1) M + $C_2H_5^+$; 460:462 (3:1) MH^+.

ZAG[^{3}H]FCK was prepared similarly starting with L-[4-^{3}H]phenylalanine. Conversion to [4-^{3}H]phenylalanine methyl ester was accomplished using 2,2-dimethoxypropane (Rachele, 1963). Final specific activity was 14.77 mCi/mmol. Radiochemical purity (96%) was assessed by quantitating the radioactivity of collected HPLC fractions in a liquid scintillation counter. (The chromatographic protocol used is outlined below under HPLC Analysis of MeCht- and Cht-Mediated Hydrolysis of ZAGFCMK. The retention time of ZAGFCMK under these conditions is 18.8 min.)

N-Acetyl-L-alanyl-L-phenylalanyl Chloroethyl Ketone (AcAFCEK). The synthesis was essentially the same as described for the analogous chloromethyl ketone (Segal et al., 1971) with the following exceptions: (1) 6 equiv of diazoethane prepared from 1-ethyl-3-nitro-*N*-nitrosoguanidine (McKay et al., 1950) was used in place of diazomethane, and (2) deprotection of *Z*-L-phenylalanyl chloroethyl ketone was accomplished with trifluoracetic acid/phenol (Shaw & Ruscica, 1971). The product was obtained practically pure from extraction, as a mixture of isomers at the CHCl center (7:3 ratio) in an overall yield of 23%. TLC: R_f = 0.54 (CH_2Cl_2/MeOH, 9:1). The major isomer crystallized preferentially from ethyl acetate (>95% pure). The minor isomer was purified by HPLC on a semipreparative reverse-phase column (Waters μ-Bondapak C_{18}, 7.8 × 300 mm) using UV detection (250 nm). The following linear gradient of H_2O and CH_3CN was applied (flow rate, 2.2 mL/min): 20% CH_3CN for 20 min, 20 → 30% CH_3CN for 20 min, 30% CH_3CN for 20 min.

The major and minor isomers eluted at 43.6 and 45.8 min, respectively. The fractions corresponding to each of these isomers were pooled, and the CH_3CN was removed on a rotary evaporator. The resulting aqueous solutions were lyophilized to dryness. Purity of the individual diastereomers was confirmed using an analytical C_{18} HPLC column as delineated under HPLC Analysis of MeCht-Mediated Hydrolysis of *S*-AcAFCEK. Under these conditions the major and minor isomers eluted at 25.6 and 26.5 min, respectively. The absolute configuration of the major diastereomer was determined by X-ray crystallography to be the *R* configuration at the CHCl center (*vide infra*). Therefore, the major and minor isomers will be referred to as the *R* and *S* isomers, respectively. *R* isomer: ^{1}H-NMR ($CDCl_3$) δ 1.32 (d, 3H, CH_3-Ala), 1.54 (d, 3H, CH_3-CHCl), 1.96 (s, 3H, CH_3-Ac), 3.01 (dd, 1H, CH_2-Phe), 3.20 (dd, 1H, CH_2-Phe), 4.31 (q, 1H, CH-Cl), 4.45 (quintet, 1H, CH-Ala), 5.24(m, 1H, CH-Phe), 5.91(d, 1H, NH-Ala), 6.73 (d, 1H, NH-Phe), 7.15–7.35 (m, 5H, PH). ^{13}C-NMR ($CDCl_3$) δ 18.07 (CH_3-Ala), 19.17 (CH_3-Ac), 23.02 (CH_3-CHCl), 37.94 (CH_2-Phe) 48.64 (CHCl), 55.38 (CHN), 56.45 (CHN), 127.17, 128.68, 129.03 (CH,PH), 135.69 (C,PH), 170.05, 172.14 (CON), 202.75 (C=O). *S* isomer: ^{1}H-NMR ($CDCl_3$) δ 1.27 (d, 3H, CH_3-Ala), 1.59 (d, 3H, CH_3-CHCl), 1.91 (s, 3H, CH_3-Ac), 2.92 (dd, 1H, CH_2-Phe), 3.26 (dd, 1H, CH_2-Phe), 4.43 (quintet, 1H, CH-Ala), 4.61 (q, 1H, CH-Cl), 4.94 (m, 1H, CH-Phe), 6.06 (d, 1H, NH-Ala), 7.00(d, 1H, NH-Phe), 7.15–7.35 (m, 5H, PH). ^{13}C-NMR ($CDCl_3$) δ 17.71 (CH_3-Ala), 19.61 (CH_3-Ac), 22.98 (CH_3CCl), 36.84 (CH_2-Phe), 48.37 (CHCl), 56.51 (CHN), 56.82 (CHN), 127.12, 128.702, 129.17, (CH,PH), 136.07 (C,PH), 170.13, 172.38(CON), 202.68 (C=O). MS of 7:3 *R*:*S* mixture (CI) 353:355 (3:1) M+$C_2H_5^+$; 325:327 (3:1) MH^+; 289.

S-[^{3}H]AcAFCEK was prepared similarly using [^{3}H]acetyl-L-alanine. The latter was prepared by acetylation of L-alanine with 1.1 equiv of [^{3}H]acetic anhydride in hot glacial acetic acid. Final specific activity was 208 μCi/mmol. The compound was determined to be 98% radiochemically pure by HPLC. *S*-AcA[3-^{13}C]FCEK was prepared as described above from *Z*-L-[1-^{13}C]phenylalanine.

N-(Benzyloxycarbonyl)-L-alanyl-L-glycyl-L-phenylalanyl Hydroxymethyl Ketone (ZAG(OH)MK). ZAGFCMK (14.1 mg, 0.31 mmol) was dissolved in 3 mL of 75% DMF. One equivalent of NaOH was added, and the reaction mixture was stirred at room temperature for 1.5 h. Dilute HCl was added until a pH of 2–3 was obtained. The solution was evaporated to dryness, and the hydroxyketone product was purified on a silica column using CH_3CN as eluent; 5.6 mg of ZAG(OH)MK was obtained (41% yield). TLC: R_f = 0.40 (CH_3CN). ^{1}H-NMR (CD_3OD/acetone-d_6) δ 1.35 (d, 3H, CH_3-Ala), 2.94 (dd, 1H, CH_2-Phe), 3.18 (dd, 1H, CH_2-Phe), 3.82 (m, 2H, CH_2-Gly), 3.90 (d, 1H, CH_2-Gly), 4.07 (q, 1H, CH-Ala), 4.13 (d, 1H, CH_2Cl), 4.34 (d, J = 16.7, 1H, CH_2Cl), 4.72 (dd, 1H, CH-Phe), 5.03 (d, 1H, CH_2-Z), 5.09 (d, 1H, CH_2-Z), 7.1–7.4 (m, 10H, PH). MS (FAB) 442 MH^+.

N-Acetyl-L-alanyl-L-phenylalanyl Hydroxyethyl Ketone (AcAF(OH)EK). AcAFCEK (345 mg, 1.1 mmol) (*R*:*S* = 7:3) was dissolved in 60 mL of 75% THF/H_2O and cooled to 0 °C. NaOH, 0.9 equiv, was added. After 15 min, the solution was acidified to pH 2–3 with dilute HCl and the THF was removed in vacuo. The remaining aqueous suspension was lyophilized to dryness. Purification was effected on a silica column (CH_2Cl_2/MeOH, 9:1; R_f = 0.4) to yield 58 mg of a 1:1 diastereomeric mixture of hydroxyethyl ketones (17% yield). Separation of the diastereomers was carried out on a Waters μBondapak C_{18} preparative column (19 × 300 mm). A 70-min linear gradient from 10% CH_3CN/90% H_2O to 50% CH_3CN/50% H_2O (flow rate, 6.0 mL/min) was implemented immediately following injection. The diastereomers eluted at 39.5 and 41.2 min and are designated respectively as the *S* and *R* isomers on the basis of the nucleophilic displacement studies described in Results. *S* isomer: ^{1}H-NMR ($CDCl_3$) δ 1.32 (d, 3H, CH_3-Ala), 1.30 (d, 3H, CH_3-CH(OH)), 1.97 (s, 3H, CH_3-Ac), 2.98 (dd, 1H, CH_2-Phe), 3.11 (dd, 1H, CH_2-Phe), 4.13 (q, 1H, CH-OH), 4.24 (br s, 1H, OH), 4.48 (m, 1H, CH-Ala), 5.09 (m, 1H, CH-Phe), 5.99(d, 1H, NH-Ala), 6.87 (d, 1H, NH-Phe), 7.10–7.35 (m, 5H, PH). *R* isomer: ^{1}H-NMR ($CDCl_3$) δ 1.26 (d, 3H, CH_3-Ala), 1.29 (d, 3H, CH_3-CH(OH)), 1.94 (s, 3H, CH_3-Ac), 2.93 (dd, 1H, CH_2-Phe), 3.19 (dd, 1H, CH_2-Phe), 4.12 (q, 1H, CH-OH), 4.35 (br s, 1H, OH), 4.43 (m, 1H, CH-Ala), 5.08 (m, 1H, CH-Phe), 5.96 (d, 1H, NH-Ala), 6.93 (d, 1H, NH-Phe), 7.10–7.35 (m, 5H, PH). MS of 1:1 diastereomeric mixture (CI) 335 M + $C_2H_5^+$; 307 MH^+.

Mesyl Derivative of N-Acetyl-L-alanyl-L-phenylalanyl Hydroxyethyl Ketone. A diastereomeric mixture (11 mg, 36 μmol; 73:27%, *S*:*R*) of the hydroxyethyl ketones was mesylated according to a previously described procedure (Crossland & Servis, 1970); 6.5 mg of the mesylated compound was obtained (47% yield). TLC: R_f = 0.5 (CH_2Cl_2/MeOH, 9:1). *S* isomer: ^{1}H-NMR ($CDCl_3$) δ 1.30 (d, 3H, CH_3-Ala), 1.58 (d, 3H, CH_3-CH(OMs)), 1.92 (s, 3H, CH_3-Ac), 2.87 (dd, 1H, CH_2-Phe), 3.11 (s, 3H, OMs), 3.22 (dd, 1H, CH_2-Phe), 4.45 (q, 1H, CH-Ms), 4.90 (m, 1H, CH-Ala), 5.29 (m, 1H, CH-Phe), 5.92(d, 1H, NH-Ala), 6.89 (d, 1H, NH-Phe), 7.10–7.40 (m, 5H, PH).

Configuration of R-AcAFCEK by X-ray Crystallography. Single crystals were grown by slow cooling of a saturated ethyl acetate solution. One crystal was transferred to a Supper No. 455 goniometer and optically centered on a Syntex $P2_1$ diffractometer. Routine operations were performed as described previously (Foxman, 1978; Foxman & Mazurek, 1979). The analytical scattering factors of Cromer & Waber were used; real and imaginary components of anomalous scattering were included in the calculations (Cromer & Waber, 1974; Cromer & Ibers, 1974). The structure was solved using the structure-solution program SHELXS-86 (Institut für Anorgische Chemie der Universitat, Gottingen, Germany); all other computational work was carried out on a VAX 8650 computer using the Enraf-Nonius SDP software package. At the conclusion of least-squares refinement of positional and anisotropic thermal parameters for all nonhydrogen atoms (H atoms included as fixed contributions to F_c), R = 0.062 and R_w = 0.049. The absolute configuration was established by reference to the *S*-phenylalanine residue as well as by a Hamilton *R*-factor test 2.

Inhibition of Cht with ZAGFCMK and R- and S-AcAFCEK. Cht was incubated at 25 °C in 50 mM KP_i, pH 7.0, with either ZAGFCMK (2–12 μM), *S*-AcAFCEK (5–100 μM), or *R*-AcAFCEK (200–1000 μM) added from a concentrated CH_3CN stock solution. (The total volume of CH_3CN did not exceed 4% of the buffer volume.) Periodically, aliquots were removed and diluted into assay solution containing BTEE, and the remaining activity was measured by monitoring the increase in absorbance at 256 nm (Hummel, 1959). Values of k_{obs} were calculated from a least-squares fit of semilogarithmic plots of percent residual enzyme activity *versus* time.

HPLC Analysis of MeCht- and Cht-Mediated Hydrolysis of ZAGFCMK. ZAGFCMK was incubated with either MeCht or Cht in 50 mM KP_i, pH 7.0, at 25 °C. At selected intervals a 100-μL aliquot was removed and added to 800 μL of ice-cold 5:3 acetone/ether. After at least 10 min on ice, the resulting suspension was centrifuged and the supernatant liquid was removed. The volatiles were evaporated under a stream of nitrogen. One hundred microliters of 2:3 CH_3CN/H_2O was added to the remaining residue, and the resulting solution was injected onto an analytical reverse-phase HPLC column (Waters μBondapak C_{18}, 3.9 × 300 mM) equilibrated in 30% CH_3CN/70% H_2O. Flow rate was maintained at 1.0 mL/min. Immediately following injection, a 30-min linear gradient from 30% to 80% CH_3CN was implemented. Under these conditions, the chloromethyl and hydroxymethyl ketones were found to elute at retention times of 18.8 and 12.1 min, respectively. Reaction progress was calculated on the basis of peak height at a detection wavelength of 220 nm.

HPLC Analysis of MeCht-Mediated Hydrolysis of S-AcAFCEK. Incubation, quench, and workup of *S*-AcAFCEK incubated with MeCht were as described above for ZAGFCMK. The samples were applied to the above HPLC column equilibrated in 10% CH_3CN/90% H_2O (flow rate, 1.0 mL/min; UV detection at 220 nm). A 30-min linear gradient from 10% to 50% CH_3CN was immediately applied. The *S*-CEK diastereomer elutes at 26.5 min under these conditions. The retention times and stereochemistry of the hydroxyethyl ketone isomers are discussed in the Results and Discussion section. As before, reaction progress was calculated from peak heights.

Proflavin Displacement Measurements. Determination of the K_d of proflavin for Cht and MeCht was carried out spectrophotometrically as previously described (Brady & Abeles, 1990) in 50 mM KP_i, pH 7.0, at 25 °C. Proflavin concentration was varied from 10 to 50 μM. Enzyme concentration ranged from 4.8 to 20.8 μM.

Potentiometric Measurement of Chloride Release. Chloride release was measured on an Orion 701A pH/mV meter with a chloride-specific microelectrode (Ag/AgCl) made from Ag wire soaked in dilute HOCl. The reference electrode was constructed from a Pasteur pipette filled with 100 mM KP_i, 100 mM KCl, 0.5 mM EDTA, and 1.5% agar. Electrode operation was checked immediately before use with chloride concentrations ranging from 10 μM to 6.2 mM in a total volume of 0.5 mL (100 mM KP_i buffer, pH 7.0). Solutions were thermostated in a 25 °C water bath in order to minimize electrode drift due to temperature fluctuation. A slope of −54 mV/decade was routinely obtained. Chloride-release experiments were conducted at pH 7.0 in 100 mM KP_i buffer containing 300 μM KCl and MeCht in a total volume of 0.5 mL. Upon attainment of a stable baseline, 10 μL of a concentrated stock solution of ZAGFCMK in CH_3CN was added. A parallel experiment lacking MeCht was also performed in order to quantitate the amount of chloride present in the ZAGFCMK stock solution. Prior to these experiments, chloride was removed from our MeCht preparations using a Penefsky column (Penefsky, 1979) equilibrated in 100 mM KP_i, pH 7.0.

RESULTS AND DISCUSSION

Cht-Mediated Hydrolysis of ZAGFCMK. ZAGFCMK (500 μM) was added to Cht (150 μM) at 25 °C in a total volume of 100 μL of 50 mM KP_i, pH 7.0. The reaction was quenched and analyzed by reverse-phase HPLC as described in Experimental Procedures. A peak corresponding to unreacted ZAGFCMK was noted at 18.8 min in addition to a smaller peak with a retention time of 12.1 min. The peak possessing the lower retention time coeluted with an authentic sample of ZAGF(OH)MK. When the experiment was repeated at a Cht concentration of 300 μM, the peak corresponding to ZAGF(OH)MK was doubled in height. Cht was found to be completely inactivated after 20 min in both experiments. Control samples containing only inactivator or enzyme were subjected to the same protocol and did not present any peaks in the 12-min range of the chromatogram. On the basis of peak height it was calculated that the ratio of hydroxy ketone to alkylated enzyme was 1:3.6. These results are consistent with the formation of enzyme-bound intermediates which can either react with water to form a hydroxy ketone or alkylate the active site histidine. For each molecule of hydroxy ketone formed, 3.6 Cht molecules are inactivated.

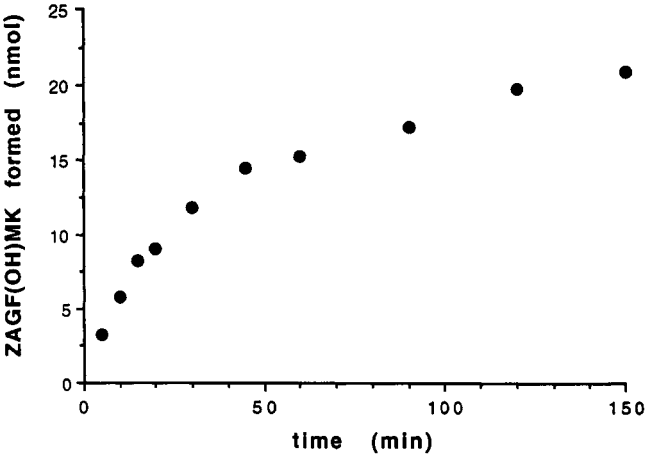

FIGURE 2: Progress curve for the MeCht-catalyzed conversion of ZAGFCMK to ZAGF(OH)MK. MeCht (50 μM) was incubated with 800 μM ZAGFCMK at 25 °C in 50 mM KP_i, pH 7.0. One hundred microliter aliquots were withdrawn, quenched, and assayed by HPLC as described in Experimental Procedures.

MeCht-Mediated Hydrolysis of ZAGFCMK. The observation that Cht catalyzes the conversion of ZAGFCMK to its hydroxy ketone counterpart prompted us to look for conditions more favorable to hydroxy ketone formation. We examined the action of MeCht on ZAGFCMK with the possibility in mind that alkylation of the active site histidine might be less favorable than with the native enzyme, and a corresponding increase of the hydrolysis reaction might be observed. MeCht (50 μM) was incubated with 800 μM ZAGFCMK at 25 °C in 50 mM KP_i, pH 7.0. At various time points, up to 150 min, aliquots were assayed by reverse-phase HPLC as described in Experimental Procedures. Time-dependent disappearance of the CMK substrate (R_f, 18.8 min) was attended by the appearance of a peak eluting at 12.1 min. This peak coeluted with authentic ZAGF(OH)MK. No decomposition of substrate in the absence of enzyme was detected over the assay period. The progress curve for the MeCht-catalyzed hydrolysis of the chloroketone is shown in Figure 2. The reaction appears to be biphasic and proceeds with an initial rate constant ($k_{obs1(HPLC)}$) of 0.14 min^{-1}. The slower phase corresponds to a rate constant ($k_{obs2(HPLC)}$) of 0.014 min^{-1}. The progress curve at a ZAGFCMK concentration of 500 μM is essentially identical to the one depicted in Figure 2, indicating saturating concentrations of the CMK. Experiments were carried out to determine whether ZAGFCMK covalently modifies MeCht. MeCht (70 μM) was incubated with 200 μM ZAG[4-^{3}H]-FCMK (1400 μCi/mmol) for 3.5 h in a total volume of 1 mL (50 mM KP_i, pH 7.0). (During this incubation period, a control lacking MeCht was followed by reverse-phase HPLC.

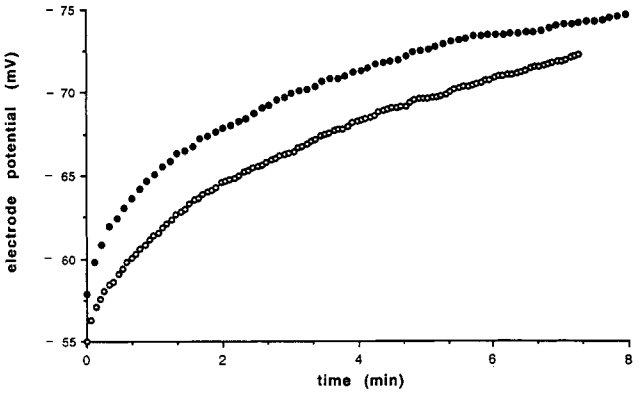

FIGURE 3: Time course of chloride release during hydrolysis of ZAGFCMK (final concentration, 410 μM) by MeCht at enzyme concentrations of 100 (○) and 150 μM (●). The reaction was monitored potentiometrically as described in Experimental Procedures.

No detectable decomposition of inactivator occurred.) The solution was then chromatographed on Sephadex G-25. The radioactivity associated with protein was 25% of that obtained when Cht was used in place of MeCht. Fractions containing both protein and radioactivity were combined and dialyzed at 4 °C for 42 h against 3 × 1 L of H_2O. Only 10% of the original protein-bound counts was recovered. As a control, Cht (70 μM) was incubated at 25 °C with 200 μM radiolabeled ZAGFCMK in 1 mL of the above buffer. After 20 min, the enzyme was assayed and found to be 100% inactivated. After 45 min the solution was applied to a 1.5 × 28 cm bed of Sephadex G-25 equilibrated in 50 mM KP_i, pH 7.0, to separate free and bound radiolabeled material. As expected, the stoichiometry of radiolabeled material migrating with protein was found to be 1:1. Fractions corresponding to radiolabeled protein were combined and subjected to dialysis. Ninety-three percent of the radioactivity was retained in the dialysis bag. Irreversible labeling of MeCht with ZAGFCMK, if it occurs at all, occurs at a low rate compared to labeling of Cht.

Kinetics of Chloride Release during MeCht-Mediated Hydrolysis of ZAGFCMK. Upon addition of ZAGFCMK (final concentration, 410 μM) to a solution of MeCht (final concentration, 100 μM), a rapid initial release of chloride ($k_{obs1(Cl)}$ = 1.92 min^{-1}) was observed followed by a slow linear phase corresponding to a rate constant ($k_{obs2(Cl)}$) of 0.17 min^{-1} (Figure 3). Extrapolation of the linear portion of the curve to zero time, followed by subtraction of the free chloride contribution of the ZAGFCMK stock solution (99 μM), gave an intercept corresponding to 84 μM chloride. When the experiment was repeated at an enzyme concentration of 150 μM (Figure 3), a $k_{obs1(Cl)}$ = 1.76 min^{-1}, a $k_{obs2(Cl)}$ = 0.14 min^{-1}, and an intercept corresponding to 138 μM chloride were noted. Stoichiometries of 0.84 and 0.92 mol of chloride released per mole of enzyme were obtained from the first and second experiments, respectively. These observations are consistent with a minimal mechanism in which the enzyme rapidly reacts with the CMK in a chloride-releasing step to give an enzyme-bound intermediate, possibly the epoxy ether (mechanism B, Figure 1). This intermediate is slowly converted to product. The rate constant associated with this slow step ($k_{obs2(Cl)}$) as determined potentiometrically is in good agreement with the faster of the two rate constants for hydroxy ketone formation ($k_{obs1(HPLC)}$) obtained from the HPLC assay. (Appreciable electrode drift was noted for analysis times greater than 10–15 min. Hence, detection of the slower phase in the HPLC assay, which is detectable at approximately 30 min (Figure 2), was not amenable to the potentiometric method.) The reaction proceeding with $k_{obs2(HPLC)}$ = 0.014 min^{-1} was not identified.

Stereochemistry of MeCht-Mediated Hydrolysis of AcAFCEK. The stereochemical outcome of the hydrolysis of a chloroketone will contribute to an understanding of the reaction mechanism. A determination of the stereochemical course of the MeCht-catalyzed hydrolysis of a chloroketone requires assignment of the absolute configuration of the hydroxyketone. We observed that in 100 mM KP_i, pH 7.0 at 25 °C, *S*-AcAFCEK slowly hydrolyzes to the hydroxyethyl ketone isomer having an HPLC retention time of 14.3 min, while the *R* isomer (whose absolute configuration was established by X-ray crystallography) hydrolyzes to the 13.5-min species (k_{obs} for the solution hydrolysis at pH 7.0 for the *R* and *S* isomers = 2.7 × 10^{-4} min^{-1}). In the assignment of an absolute configuration to these hydroxy ketone isomers, it is tempting to assume that a simple S_N2 reaction is occurring with inversion of configuration at the stereocenter. However, in light of the long-known observation that α-haloketones display markedly enhanced reactivities toward nucleophiles compared to their alkyl counterparts (Conant et al., 1925), the possibility of alternative mechanisms cannot be excluded. To address the stereochemical outcome of nucleophilic substitution at C-2 of the CEK, our initial efforts were centered on obtaining crystals of a pure isomer of the hydroxyethyl ketone for X-ray crystallographic analysis. Because attempts to grow crystals of adequate quality were unsuccessful, we decided to approach the question through an examination of the stereochemistry of nucleophilic substitution at the stereocenter of interest in the hydroxyethyl ketone. From a mixture of 73% "earlier" eluting and 27% "later" eluting isomers of AcAF(OH)EK (Figure 4a), the hydroxyl groups were mesylated in order to provide a facile leaving group at C-2. The HPLC chromatogram of the mixture of mesyl derivatives (retention times = 22.4 and 22.8 min) is shown in Figure 4b. Also present is some unreacted hydroxyethyl ketone representing 13% of the mixture. A 60% THF/40% H_2O solution of the mesylated mixture was cooled to 0 °C, and 1 equiv of NaOH was added. After 10 min, an aliquot was injected into the HPLC column and presented the chromatogram shown in Figure 4c. The mesylated species have completely reacted to reform the hydroxyethyl ketones. These are present as 38% of the earlier eluting isomer and 62% of the later eluting isomer. After subtraction of the contribution of the unreacted hydroxyethyl ketones shown in Figure 4b, the percentages of earlier and later eluting isomers resulting from the nucleophilic displacement of the mesyl group by hydroxide are 34% and 66%, respectively. This corresponds to 90% inversion and shows that displacement reactions at C-2 of an α-chloroethyl ketone occur with inversion of configuration. Given the observation that *R*-AcAFCEK spontaneously hydrolyzes to the earlier eluting hydroxy ketone and the *S* isomer yields the later eluting species, the above results allow us to designate the stereochemistry of the earlier and later eluting hydroxyethyl ketones as *S* and *R*, respectively.

A solution comprising 730 μM *S*-AcAFCEK and 300 μM MeCht was incubated at 25 °C in 50 mM KP_i buffer, pH 7.0. Aliquots were removed for HPLC analysis over a 4-h time period. During this incubation period, a decrease in the height of the *S*-AcAFCEK peak (retention time, 26.5 min) was noted, while two peaks appeared at 13.5 and 14.3 min. A control lacking enzyme yielded only the species eluting at 14.3 min. Therefore, the 13.5-min peak results from the action of MeCht on *S*-AcAFCEK. The ratio of the peaks eluting at 13.5 and 14.3 min is 9:1. The MeCht-catalyzed hydrolysis

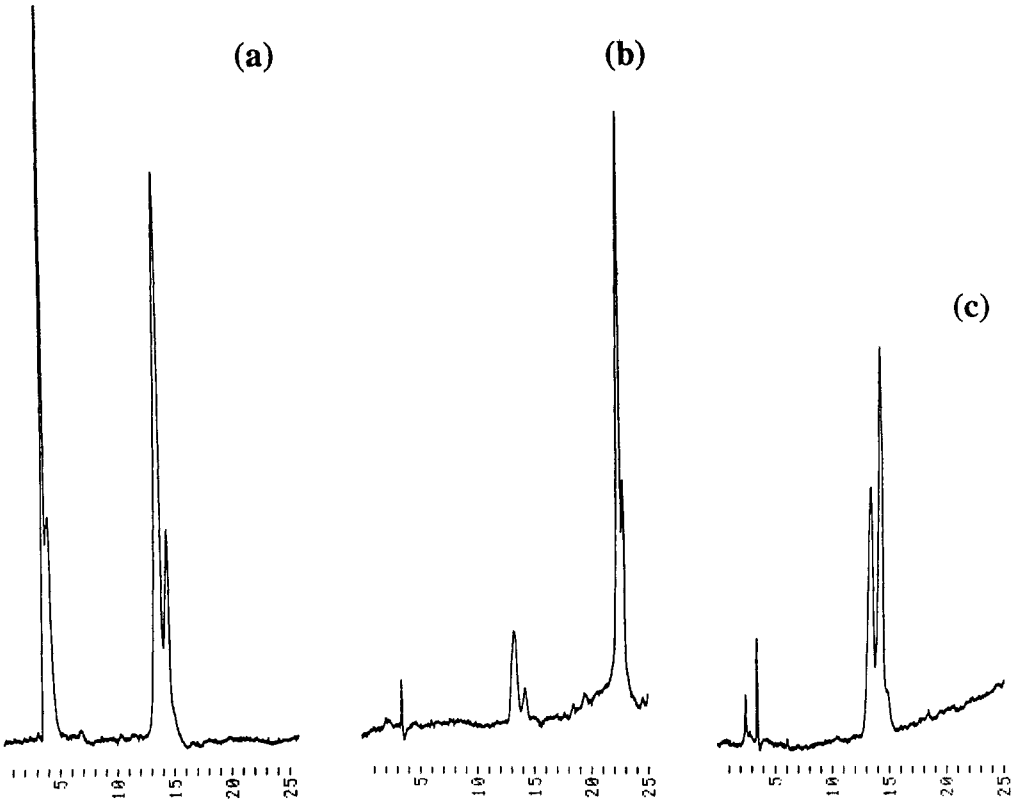

FIGURE 4: HPLC chromatograms of AcAF(OH)EK diasteromers: chromatograms of (a) a 73%/23% mixture of AcAF(OH)EK diastereomers and of the same mixture (b) following mesylation and (c) after treatment of the mesylates with 1 equiv of NaOH. The chromatographic analyses were performed using a Waters μBondapak C_{18} reverse-phase column (3.9 × 300 mm) equilibrated in 10% CH_3CN/90% H_2O at a flow rate of 1.0 mL/min. Following injection, a 30 min-gradient to 50% CH_3CN/50% H_2O was applied. Approximately 100 nmol of sample (dissolved in 2:3 CH_3CN/H_2O) was injected per chromatographic experiment. Peaks eluting before 5 min are ascribable to void volume and traces of solvent present in the samples.

of CEK is 8.1-fold faster than the spontaneous hydrolysis of CEK. Therefore the 14.3-min peak is a nonenzymatic event. The 13.5-min peak coeluted with authentic *S*-AcAF(OH)-EK, while the 14.3-min peak was found to coelute with the *R* diastereomer. Thus, the hydrolysis of *S*-AcAFCEK catalyzed by MeCht proceeds with retention of configuration. The stereochemical course of the reaction suggests a two-step process and is therefore consistent with mechanism B (Figure 1).

The progress curve for the MeCht-mediated conversion of *S*-AcAFCEK to *S*-AcAF(OH)EK is linear over the time course of the experiment and proceeds with a k_{obs} of 2.2×10^{-3} min^{-1}. The hydrolysis as mediated by MeCht is accelerated 8.1-fold over the spontaneous hydrolysis at pH 7.0. When the above experiments were repeated with *R*-AcAFCEK, no evidence was obtained for the MeCht-mediated hydrolysis of this diastereomer.

Inactivation of Cht with a Chloroethyl Ketone. The results reported above establish that MeCht catalyzes the hydrolysis of ZAGFCMK and *S*-AcAFCEK. For comparison, the action of ZAGFCMK and the two CEK diastereomers on Cht at pH 7.0 was investigated. All compounds inactivate Cht through a pseudo-first-order process. For the R-CEK isomer, a replot of $1/k_{obs}$ versus $1/[I]$ yields a straight line from which the kinetic parameters K_I = 5.2 mM, $k_2 = 2.3 \times 10^{-3}$ s^{-1}, and $k_2/K_I = 0.43$ M^{-1} s^{-1} are obtained. Saturation of the enzyme with S isomer and ZAGFCMK was not attainable due to their high rates of reaction. Over the accessible concentration range the inactivation with *S*-AcAFCEK proceeds with a second-order rate constant of 28.2 M^{-1} s^{-1}. The *S* isomer is approximately 65 times more potent an inactivator than the *R* isomer. A $k_{obs}/[I]$ value of 270 M^{-1} s^{-1} was obtained for the inactivation of Cht with ZAGFCMK.

Histidine Content, Chloride Release, and Stoichiometry of Inactivation of Cht Treated with S-AcAFCEK. A determination of histidine content of native Cht and Cht inactivated with *S*-AcAFCEK was carried out according to the method of Pauly (Stewart & Young, 1984). An average value of 1.1 mol of histidine per mole of inactivated Cht was obtained after assigning native Cht a histidine content of 2.0 mol per mole of enzyme. Similarly, amino acid analysis of the *o*-phthalaldehyde-derivatized (Jones et al., 1981) total acid hydrolysate of inactivated enzyme indicated the presence of only 1.0 mol of histidine per mole of enzyme. Accompanying inactivation is the release of 1.0 mol of chloride (determined colorimetrically (Gelb & Abeles, 1984)). Lastly, using radiolabeled inactivator (*S*-[^{3}H]AcAFCEK), a stoichiometry of 1.2 mol of bound radiolabel per mole of inactivated enzyme was established. From these data it is apparent that one histidine, most certainly histidine 57, is alkylated by the chloroethyl ketone via a mechanism that involves displacement of chloride. These data are consistent with the labeling properties of virtually all other substrate-based halomethyl ketones (Powers, 1977). From these results it can be concluded that the presence of the "extra" methyl group of the CEK inactivator does not impart any gross alterations to its active site binding and inactivating conformation relative to its CMK counterpart.

K_d Determinations of Proflavin for Cht and MeCht. To assess the preservation of the active site topography of MeCht, the dissociation constants of proflavin for both native and modified enzyme were determined. A K_d of 35 μM was obtained with Cht, while MeCht yielded a value of 47.5 μM. These constants are in qualitative agreement with those

obtained by Schultz and associates (Schultz et al., 1977) who reported proflavin K_d values of 27 and 25.7 μM for Cht and MeCht, respectively, at pH 7.8 and 25 °C.

CONCLUSION

Two mechanisms were considered for the alkylation of histidine 57 of Cht by chloroketones. One mechanism involves a direct displacement of halogen by histidine 57 (mechanism A, Figure 1). The alternate mechanism (mechanism B, Figure 1) involves a two-step process in which an epoxy ether is first formed, which then alkylates histidine. The reaction of Cht with CMK leads to hydrolysis of CMK as well as alkylation of the active site histidine. This suggests the occurrence of an intermediate which can react either with histidine to inactivate the enzyme or with solvent to produce the hydroxy ketone. MeCht catalyzes only the hydrolysis of CMK. Apparently the methyl group precludes alkylation. Chloride release kinetics show an initial burst followed by a slower reaction. This indicates the rapid formation of an intermediate concomitant with Cl^- release and its subsequent slow hydrolysis, phenomena which are consistent with mechanism B. The steric course of the hydrolysis of CEK by MeCht proceeds with retention of configuration as would be expected if mechanism B is operative. The data we have presented support a two-step mechanism which involves an intermediate. It is likely that this intermediate is the epoxy ether, although no direct evidence for its involvement has been submitted.

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SUPPLEMENTARY MATERIAL AVAILABLE

Tables SI through SVI, listing experimental details, atomic coordinates, bond lengths and angles, and anisotropic thermal parameters for nonhydrogen atoms, and an ORTEP structural diagram for *R*-AcAFCEK (9 pages); calculated structure factors (3 pages). Ordering information is given on any current masthead page.

REFERENCES

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1967) *J. Am. Chem. Soc. 89*, 5012.
Bender, M. L., & Marshall, T. H. (1968) *J. Am. Chem. Soc. 90*, 201.
Brady, K., & Abeles, R. H. (1990) *Biochemistry 29*, 7608.
Brayer, G. D., Delbaera, M. N. G., James, C. A., & Thompson, R. C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 76*, 96.
Chem, R., Gorenstein, D. G., Kennedy, W. P., Lowe, G., Nurse, D., & Schults, R. M. (1979) *Biochemistry 18*, 921.
Conant, J. B., Kirner, W. R., & Hussey, R. E. (1925) *J. Am. Chem. Soc. 47*, 488.
Cromer, D. T., & Waber, J. T. (1974) in *International Tables for X-ray Crystallography* (Ibers, J. A., & Hamilton, W. C., Eds.) Vol. IV, pp 99–101, Kynoch Press, Birmingham, England.
Cromer, D. T., & Ibers, J. A. (1974) in *International Tables for X-ray Crystallography* (Ibers, J. A., & Hamilton, W. C., Eds.) Vol. IV, pp 148–150, Kynoch Press, Birmingham, England.
Crossland, R. K., & Servis, K. L (1970) *J. Org. Chem. 35*, 3195.
Cuatrecasas, P., & Anfinsen, C. B. (1971) *Methods Enzymol. 22*, 345.
Finucane, M. D., Hudson, E. A., & Malthouse, J. P. G. (1989) *Biochem. J. 258*, 853.
Foxman, B. M. (1978) *Inorg. Chem. 17*, 1932.
Foxman, B. M., & Mazurek, H. (1979) *Inorg. Chem. 18*, 113.
Gelb, M. H., & Abeles, R. H. (1984) *Biochemistry 23*, 6596.
Hummel, B. C. W. (1959) *Can. J. Biochem. Physiol. 37*, 1393.
Imperiali, B., & Abeles, R. H. (1986) *Biochemistry 25*, 3760.
James, M. N. G., Brayer, G. D., Delbaere, L. T. J., Sielcki, A. R., & Gertler, A. (1980) *J. Mol. Biol. 139*, 423.
Jones, B. N., Paabo, S., & Stein, S. (1981) *J. Liq. Chromatogr. 4*, 565.
Liang, T.-C., & Abeles, R. H. (1987) *Biochemistry 26*, 7603.
Lowe, G., & Nurse, D. (1977) *J. Chem. Soc., Chem. Commun.*, 815.
Malthouse, J. P. G., Primrose, W. U., Mackenzie, N. E., & Scott, I. A. (1985) *Biochemistry 24*, 3478.
Matthews, D. A., Adler, R. A., Birktoft, J. J., Freer, S. T., & Kraut, J. (1975) *J. Biol. Chem. 250*, 7120.
McKay, A. F., Orr, W. L., Taylor, G. W., Buchanan, M. N., & Crooker, J. F. (1950) *Can. J. Res. 28B*, 683.
McMurray, J. S., & Dyckes, D. F. (1986) *Biochemistry 25*, 2298.
Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527.
Poulos, T. L., Alden, R. A., Freer, S. Y., Birktoft, J. J., & Kraut, J. (1976) *J. Biol. Chem. 251*, 1097.
Powers, J. C. (1977) *Chem. Biochem. Amino Acids, Pept., Proteins 4*, 65.
Powers, J. C., & Tuhy, P. M. (1973) *Biochemistry 12*, 4767.
Rachele, J. R. (1963) *J. Org. Chem. 28*, 3898.
Ryan, D. S., & Feeney, R. F. (1975) *J. Biol. Chem. 250*, 843.
Schultz, R. M., Konevessi-Panayotatos, A., & Peters, J. R. (1977) *Biochemistry 16*, 2194.
Scott, A. I., Mackenzie, N. E., Malthouse, J. P. G., Primrose, W. V., Fagerness, P. E., Brisson, A., Qi, L. Z., Bode, W., Carter, C., & Jang, Y. J. (1986) *Tetrahedron 42*, 3269.
Segal, D. M., Powers, J. C., Cohen, G. H., Davies, D. R., & Wilcox, P. E. (1971) *Biochemistry 10*, 3728.
Shah, D. O., Lai, K., & Gorenstein, D. G. (1984) *J. Am. Chem. Soc. 106*, 4272.
Shaw, E. (1967) *Methods Enzymol. 11*, 684.
Shaw, E., & Ruscica, J. (1971) *Arch. Biochem. Biophys. 145*, 484.
Shaw, E., & Glober, G. (1990) *Arch. Biochem. Biophys. 139*, 298.
Stein, R. L., & Trainor, D. A. (1986) *Biochemistry 25*, 5414.
Stewart, J. M., & Young, J. D. (1984) *Solid Phase Peptide Synthesis*, 2nd ed., p 114, Pierce Chemical Co., Rockford, IL.
Weiner, H., White, W. N., Hoare, D. G., & Koshland, D. E., Jr. (1966) *J. Am. Chem. Soc. 88*, 3851.
Wyeth, P., Sharmon, R. P., & Akhtar, M. (1980) *Eur. J. Biochem. 105*, 581.