Monoanion Inhibition and ^{35}Cl Nuclear Magnetic Resonance Studies of Renal Dipeptidase†

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ABSTRACT: Kinetic analyses of monoanion inhibition and ^{35}Cl nuclear magnetic resonance at 5.88 MHz were employed to study monoanion interactions with the zinc metalloenzyme, renal dipeptidase. The enzyme-catalyzed hydrolysis of glycyldehydrophenylalanine exhibited competitive inhibition when the reaction rate was determined in the presence of the monovalent anions fluoride, chloride, bromide, iodide, azide, nitrate, or thiocyanate or upon the addition of the divalent anion, sulfate. Competitive inhibition was produced by these anions. One anion was bound per enzyme molecule, and except in the case of fluoride all of the anions appeared to bind at the same site. Cyanide ion produced a much more effective inhibition of renal dipeptidase than the other monoanions, and it was shown that two cyanide ions were bound per enzyme molecule. An investigation of the effect of pH upon monoanion inhibition suggested that the anion inhibitors bind to the group with a pK of approximately 7.8. Complete dissociation of this group (~pH 8.4) eliminates the inhibitory effect of anions. The ^{35}Cl line broadening produced by renal dipeptidase in 0.5 M NaCl solutions was 100 times more effective than that produced by equivalent concentrations of aquozinc(II). The line broadening was dependent upon the concentration of the metalloenzyme and independent of the frequency of the exciting radiation. When zinc ion was removed from the metalloenzyme by dialysis or when chloride was titrated from the metalloenzyme by cyanide, line broadening was decreased. Treatment of renal dipeptidase with saturating concentrations of the competitive inhibitor, guanosine triphosphate, in the presence of 0.5 M NaCl also produced a significant decrease in the ^{35}Cl line width. The ^{35}Cl line broadening produced by renal dipeptidase was shown to decrease with increasing pH through the range pH 5.8–10.8. This line-width variation with pH appeared to result from the titration of a site on the metalloprotein with an approximate pK of 7.4. Temperature studies of ^{35}Cl line broadening by the metalloenzyme in the presence of chloride and cyanide inhibitors suggest that the fast exchange process pertains and that the dominant relaxation mechanism is quadrupolar in nature.

Renal dipeptidase, a membrane-bound enzyme located in porcine kidney cortex, catalyzes the hydrolysis of a variety of dipeptides but does not act upon esters, tripeptides, or proteins (Campbell, 1970). The enzyme has been shown to be a zinc metalloenzyme which contains 2.04 g-atoms of zinc/mol of enzyme of molecular weight 94000 (Armstrong et al., 1974).

Preliminary investigations of the effect of various inhibitors upon the metalloenzyme have indicated that monoanions reduce the rate of enzyme-catalyzed hydrolysis (Harper, 1969) and that phosphate esters such as guanosine triphosphate act as competitive inhibitors (Harper et al., 1971). In the present studies the nature of monovalent anion inhibition of renal dipeptidase was determined by kinetic analyses, and ^{35}Cl nuclear magnetic resonance (NMR) spectroscopy was employed to examine the interaction of chloride ions with the zinc metalloenzyme. The results suggest that monoanions inhibit the enzyme by competing with substrate for a position in the coordination sphere of the enzyme-bound zinc.

Experimental Section

Materials. Renal dipeptidase was purified from porcine kidney cortex according to the method of Armstrong et al. (1974) modified in that the 1-butanol solubilization step was carried out for 48 hr rather than for 2 hr. The purified metalloenzyme was homogeneous as shown by analytical polyacrylamide gel electrophoresis (Ornstein and Davis, 1964) and by high-speed equilibrium centrifugation (Yphantis, 1964). The zinc content of the purified enzyme was 2.0 g-atom of zinc/mol of enzyme of molecular weight 94000. The zinc analysis was obtained by atomic absorption spectroscopy as described by René and Campbell (1969). Glycyldehydrophenylalanine was synthesized by methods previously described (Campbell et al., 1963) and recrystallized from demineralized water. All anions were used in the form of analytical reagent grade sodium salts. Metal-free buffers were prepared by repeated extractions with dithizone (Vallee et al., 1960). Glassware and containers were cleaned and washed as described by Thiers (1957). Dialysis tubing was prepared free of metals as described by Klotz and Hughes (1956).

Kinetic Analyses. Renal dipeptidase was assayed in the presence or absence of inhibitors by means of a spectrophotometric method. The rate of enzyme-catalyzed hydrolysis of the unsaturated dipeptide, glycyldehydrophenylalanine, was measured by observing the fall in absorbance at 275 mμ of a solution of 5.00×10^{-5} M glycyldehydrophenylalanine as reported previously (René and Campbell, 1969). The temperature of the assay was maintained at 37°. Protein concentrations were determined by the method of Lowry et al. (1951). Velocities are reported as micromoles of substrate hydrolyzed per minute per milligram of enzyme.

Nuclear Magnetic Resonance Measurements. The ^{35}Cl nuclear magnetic spectra were measured at 5.88 MHz. The

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Table I: Inhibition of Renal Dipeptidase Activity by Anions.

Inhibitor	K_i (mM)[a]	Inhibitor	K_i (mM)[a]
Sodium fluoride	0.042	Sodium nitrate	0.45
Sodium chloride	0.54	Sodium sulfate	0.12
Sodium bromide	0.48	Sodium thiocyanate	0.17
Sodium iodide	0.59	Sodium cyanide	1.2×10^{-6}
Sodium azide	0.34		

[a] The units of K_i for cyanide are mol/l.

Table II: n Values of Inhibition of Renal Dipeptidases by Various Anions.

Inhibitor	n[a]	Inhibitor	n[a]
Sodium fluoride	1.00	Sodium nitrate	0.95
Sodium chloride	0.99	Sodium sulfate	0.92
Sodium bromide	0.85	Sodium thiocyanate	1.02
Sodium iodide	1.04	Sodium cyanide	2.01
Sodium azide	0.98		

[a] n value as defined in eq 1.

NMR spectrometer was operated in a continuous wave frequency swept mode. The NMR probe contained an "external" proton lock signal which stabilized the magnetic field and allowed time averaging of the NMR signal for appreciable time periods. The radio-frequency field level was low enough so that saturation effects were absent. Routinely the NMR measurements were made on 0.5 M NaCl solutions containing renal dipeptidase. Variable temperature studies were performed by passing cooled N_2 gas over the sample. The temperature was constant to within a few tenths of a degree during the time of the experiment. Ambient temperature studies were carried out at 31.6°. The ^{35}Cl line widths were measured from recorded spectra as the full width at half-maximum amplitude. Values of the transverse relaxation rate, $1/T_{2p}$, were recorded as net line width variations $\Delta\nu_{net}$, where $\Delta\nu_{net} = \Delta\nu_{obsd} - \Delta\nu_{Cl^-}$ and $1/T_{2p} = \pi\Delta\nu_{net}$.

Results

Kinetic Analyses. Dilution experiments demonstrated that renal dipeptidase was inhibited instantaneously and reversibly by the anion inhibitors. Enzyme-catalyzed hydrolysis of 5.00×10^{-5} M glycyldehydrophenylalanine was measured at pH 7.60 in 2 mM Tris-HCl buffer in the presence of 0.10 M fluoride, chloride, bromide, iodide, azide, nitrate, thiocyanate, and sulfate. Total recovery of enzymic activity was obtained when the anion–enzyme solutions were diluted 50-fold. Cyanide was employed at 0.001 M concentration to produce inhibition, and total recovery of enzyme activity was demonstrated following 50-fold dilution of the inhibited enzyme.

The initial velocity of the peptidase-catalyzed hydrolysis of glycyldehydrophenylalanine was measured as a function of anion concentration at various levels of substrate concentration. The data obtained were plotted as the reciprocal of initial velocity vs. the concentration of the inhibiting anion. For all anions except cyanide, straight lines were obtained that intersected in the second quadrant indicating competitive inhibition (Dixon, 1953). The inhibitor dissociation constants, K_i, for the anions were calculated as described by Dixon (1953) and are presented in Table I. When the data for cyanide were plotted as described above curved lines were obtained. However, when the cyanide data were plotted as the reciprocal of initial velocity vs. the concentration of cyanide squared, straight lines were obtained that intersected on the x axis indicating noncompetitive inhibition. The K_i for cyanide, calculated from these data, is given in Table I. These results also suggest that two cyanide ions are bound per active site of renal dipeptidase.

Kinetic data have been employed to differentiate between simple and complex (hyperbolic and parabolic) competitive inhibition (Cleland, 1963; Plowman, 1972). To determine whether the observed competitive inhibition by anions was simple or complex, plots of v_0/v_i vs. [i] were made for each competitive inhibitor at substrate concentrations of 1.05×10^{-5} and 10.5×10^{-5} M. The symbol, v_0, refers to the initial velocity in the absence of inhibitor and v_i refers to the initial velocity in the presence of inhibitor. The concentration of the anion is given by [i]. Linear plots at both substrate concentrations were obtained with fluoride, chloride, bromide, iodide, azide, nitrate, and thiocyanate, and these plots intersected the v_0/v_i axis at values from 1.0 to 1.1. These results indicate simple competitive inhibition (Braun and Schmidt, 1973). The sulfate data exhibited marked curvature at both substrate concentrations indicating complex competitive inhibition.

The number of inhibitor molecules, n, in an enzyme–inhibitor complex:

$$E + nI = EI_n \tag{1}$$

is related to the various kinetic coefficients by the following expression (Ebersole et al., 1944; Bergmann and Segal, 1954):

$$\log\left(\frac{v_0}{v_i} - 1\right) = n \log [i] + \log\left(\frac{K_m^{app}}{K_i\{K_m^{app} + [S_0]\}}\right) \tag{2}$$

where v_0, v_i, [i], and K_i are as previously defined. K_m^{app} refers to the dissociation constant for the enzyme–substrate complex, and $[S_0]$ is the initial substrate concentration. The value of n is obtained from the slope of a plot of log $[(v_0/v_i) - 1]$ against log [i]. Values of n were determined for the anion inhibitors using this graphical technique for renal dipeptidase inhibition at $[S_0] = 1.05 \times 10^{-5}$ M. For each anion inhibitor the slope of the resulting straight line was 1.0 ± 0.1 except for cyanide where the slope was 2.0. These results are given in Table II.

Mixed anion inhibition studies were carried out to determine if the various anions inhibit by combining at an identical site on the enzyme or at independent, noninteracting sites. The equations developed by Braun and Schmidt (1973) were used to calculate the expected velocities in the presence of various mixtures of anions for identical and for independent sites. These calculated values and the experimentally determined reaction rates are presented in Table III.

The effect of pH upon monoanion inhibition of renal dipeptidase was examined by measuring the initial velocity in the presence of 0.2 M chloride or bromide and with no inhibitor present over the pH range 5.00–9.40. The pH of the solutions was buffered using 4 mM tris(hydroxymethyl)-methylaminopropanesulfonic acid and 4 mM piperazine-N,N'-bis(2-ethanesulfonic acid)monosodium monohydrate. The variation of reaction velocity with pH is reported in Figure 1. Plots of v_0/v_i vs. [i] at pH's over the range 5.6–8.0 indicated that both these anions exhibited simple competitive inhibition under these conditions. The intersection of noninhibited and anion-inhibited curves at pH 8.4 shown in

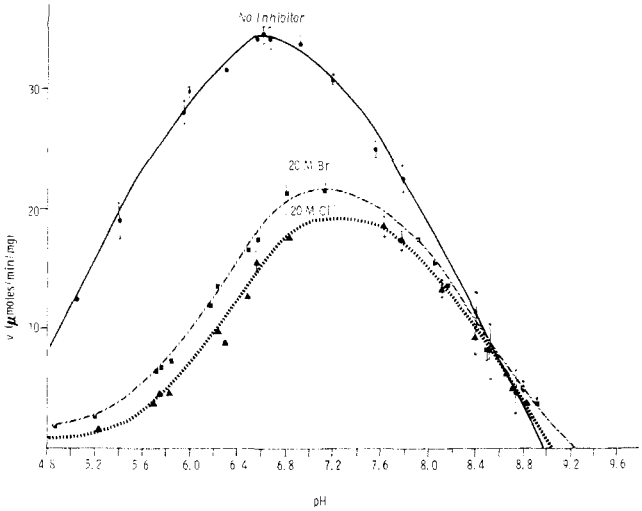

FIGURE 1: Variation of renal dipeptidase catalyzed hydrolysis with pH for inhibited and active systems. Assays were performed at 37° using 10.5×10^{-5} *M* glycyldehydrophenylalanine in a 4 m*M* tris(hydroxymethyl)methylaminopropanesulfonic acid–4 m*M* piperazine-N,N′-bis(2-ethanesulfonic acid) buffer system at the pH's indicated in the presence and absence of anions. Each point represents the average of four determinations of the initial velocity. Error bars show the standard deviation of the mean.

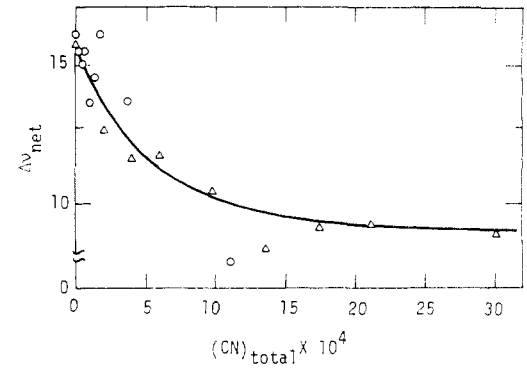

FIGURE 2: Cyanide titration of renal dipeptidase. A solution of 0.5 *M* NaCl containing 6.5 mg/ml of renal dipeptidase at pH 7.6 was titrated with cyanide, and the effect upon the net line broadening was observed. The circles and triangles denote separate titrations of the same stock solution of renal dipeptidase. The solid curve represents a best fit of the data taking into consideration a small pH change during the titration and assuming that chloride and cyanide competed for the zinc. The dissociation constant for the cyanide–enzyme complex giving the best fit was 2×10^{-6}.

Figure 1 indicates that the anions do not inhibit the reaction at pH's above this point. Using the method of Pocker and Stone (1967) the enzyme–inhibitor constant, K_i, was calculated for chloride-inhibited renal dipeptidase as a function of pH. A plot of pK_i vs. pH was then analyzed as described by Dixon (1953). This plot revealed three pK inflection points. Two of the pK's associated with the free enzyme were noted at pH 6.0 and 7.8. A pK associated with the ionization of a group within the enzyme–substrate complex was apparent at pH 6.6.

Nuclear Magnetic Resonance Measurements. The ^{35}Cl nucleus has a spin of $3/2$ and an electric quadrupole moment, Q. The interactions between the electric quadrupole moment and the fluctuating electric field gradients at the nucleus provide the dominant nuclear magnetic relaxation mechanism for ^{35}Cl nuclei in solution. In order to compare the effectiveness of zinc ions in renal dipeptidase with zinc ions in aqueous solution in altering the relaxation rate of chloride nuclei, the quadrupole enhancement parameter, ϵq, was determined (Ward, 1969):

$$\epsilon q = \frac{\Delta\nu^* - \Delta\nu^*_0}{\Delta\nu - \Delta\nu_0} \qquad (3)$$

where $\Delta\nu$ is the observed line width in the presence of zinc ions and $\Delta\nu_0$ is the line width in the absence of zinc ions. The asterisk indicates the presence of protein or chelating agent. It has been reported (Ward and Happe, 1967) that $\Delta\nu - \Delta\nu_0$ for zinc ions in 0.5 *M* NaCl is equal to 2×10^3 Hz mol^{-1} l. The value of $\Delta\nu^* - \Delta\nu^*_0$ for renal dipeptidase at a concentration of 6.5 mg/ml (1.4×10^{-4} *M* Zn) in 0.5 *M* NaCl was 25 Hz giving a quadrupole enhancement parameter, ϵq, equal to approximately 100. Determination of ^{35}Cl line broadening at concentrations of the metalloenzyme equal to 6.5 and 8.2 mg/ml indicated that the magnitude of the line broadening was dependent on the concentration of renal dipeptidase. A zinc-deficient peptidase was prepared by dialysis of 10.35 mg/ml of enzyme against a metal-free 2 m*M* Tris-HCl buffer at pH 7.1. The resulting enzyme contained 40% of the zinc present in the native enzyme, and the net ^{35}Cl line-width broadening, $\Delta\nu_{net}$, was reduced from 28 to 9 Hz. Examination of the ^{35}Cl line width at 3.92 MHz showed no frequency dependence of $\Delta\nu$ within an experimental accuracy of 15%.

Table III: Inhibition of Renal Dipeptidase by Mixtures of Anions.

Inhibitor Mixtures	ν_j (μmol per min per mg) Calcd for Identical Sites	Calcd for Independent Sites	Exptl[a]
NaCl (0.10 *M*) + NaF (0.01 *M*)	13.9	13.5	13.2 ± 0.3 (4)
NaCl (0.10 *M*) + NaBr (0.10 *M*)	13.8	13.4	14.0 ± 0.1 (4)
NaCl (0.10 *M*) + NaI (0.20 *M*)	13.0	12.5	14.3 ± 0.2 (4)
NaCl (0.10 *M*) + NaN_3 (0.10 *M*)	13.4	12.9	14.2 ± 0.3 (4)
NaCl (0.10 *M*) + NaSCN (0.10 *M*)	11.3	10.7	12.4 ± 0.3 (4)
NaCl (0.10 *M*) + $NaNO_3$ (0.10 *M*)	14.0	13.6	14.2 ± 0.3 (4)
NaCl (0.10 *M*) + Na_2SO_4 (0.05 *M*)	12.4	11.9	13.8 ± 0.6 (4)

[a] Reported is the average ± standard deviation (number of determinations).

The effect of anion inhibitors upon ^{35}Cl line broadening was observed in solutions containing renal dipeptidase. In Figure 2 is presented the results of a cyanide titration of a 0.5 *M* NaCl solution containing 6.5 mg/ml of renal dipeptidase at pH 7.6. The data demonstrate a decrease in ^{35}Cl line broadening as cyanide was added. The solid curve represents a best fit line calculated for a competitive reaction of chloride and cyanide ions for the zinc site assuming a cyanide–enzyme dissociation constant of 2×10^{-6} *M*. The curve has been corrected for a small pH change which occurred during the titration. The assumed value of the dissociation constant agrees quite well with the value of 1.2×10^{-6} *M* determined by kinetic analyses of peptidase inhibition (Table I). It has been previously established that guanosine triphosphate is an effective competitive inhibitor of renal dipeptidase (Harper et al., 1971). When saturating concentrations of guanosine triphosphate were added to a

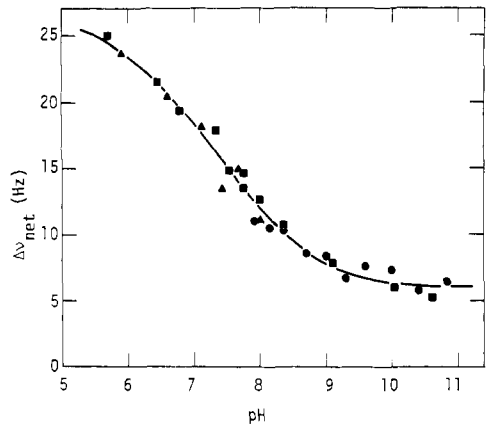

FIGURE 3: pH dependence of the normalized ^{35}Cl line width for renal dipeptidase. The ^{35}Cl net line broadening of a 0.5 *M* NaCl solution containing either 6.5 or 8.2 mg/ml of renal dipeptidase was determined as a function of pH. The symbols refer to different enzyme preparations and to different pH titrations of the same preparation.

0.5 *M* NaCl solution containing 6.5 mg/ml of renal dipeptidase, the net ^{35}Cl line-broadening effect was reduced by 67%.

The normalized ^{35}Cl line-width variation with pH for a 0.5 *M* NaCl solution containing either 6.5 or 8.2 mg/ml of renal dipeptidase is shown in Figure 3. A marked dependence of line broadening upon pH was observed, and the decrease in line broadening with increasing pH occurred with an apparent p*K* of 7.4. The effect of temperature upon the ^{35}Cl line width was determined for chloride and cyanide-inhibited enzyme. The quantity $\pi\Delta\nu_{net}/f$ was measured as a function of the absolute temperature, and the data are presented in Figure 4.

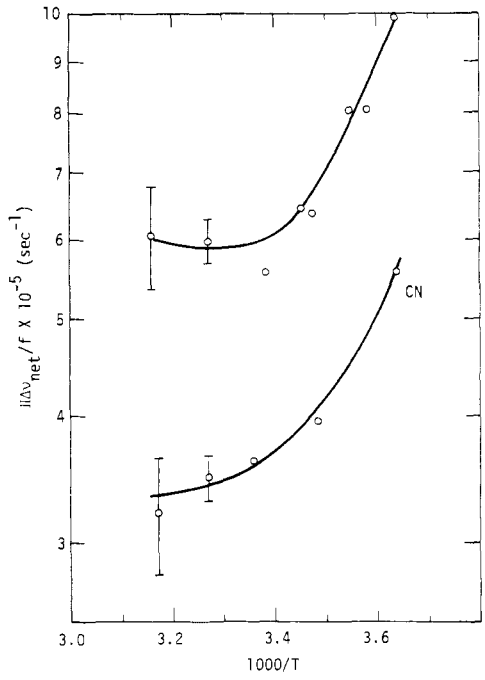

FIGURE 4: Temperature dependence of ^{35}Cl line broadening for chloride and cyanide-inhibited enzyme. The net line broadening of the ^{35}Cl line at half-maximum was determined as a function of temperature for a 0.5 *M* NaCl solution containing 6.5 mg/ml of renal dipeptidase either in the presence or absence of saturating concentrations of cyanide.

Discussion

The data demonstrating that fluoride, chloride, bromide, iodide, azide, nitrate, and thiocyanate exhibit simple competitive inhibition with renal dipeptidase indicate that these monoanions compete with substrate for a group within the active site of the enzyme. The *n* values presented in Table II show that these peptidase–anion combinations occur when 1 mol of anion binds per active site. However, evidence presented in Table II indicates that two cyanide ions react per active site to produce a much more effective inhibition than the other anions.

The K_i values reported for renal dipeptidase in Table I are, in general, an order of magnitude greater than the K_i values obtained for protein–anion interactions with firefly luciferase (Denburg and McElroy, 1970), carbonic anhydrase (Pocker and Stone, 1967), fumarylacetoacetate fumarylhydrolase (Braun and Schmidt, 1973), hemerythrin (Garbett et al., 1971), and acetoacetate decarboxylase (Fridovich, 1963). The halide ions chloride, bromide, and iodide produce approximately the same level of inhibition, but fluoride is a much stronger inhibitor than the other halides. Similar results have been obtained by Braun and Schmidt (1973) for fumarylacetoacetate fumarylhydrolase. This difference in fluoride inhibition was also observed in the mixed anion experiments reported in Table III. These results suggest that chloride, bromide, iodide, azide, thiocyanate, nitrate, and sulfate all bind at the same site on the enzyme. Although the data presented in Table III appear to indicate that fluoride acts at a site different from that which binds the other anions, the evidence is not conclusive. Future binding experiments should provide direct evidence to support the kinetic data presented in Table III.

The variation of anion inhibition with pH indicated that an enzymic site with a p*K* of approximately 7.8 was implicated in the inhibitor–enzyme interaction. When this group was completely dissociated at pH 8.4, inhibition by chloride or bromide ions was no longer detected (Figure 1). This effect could be the result of the dissociation of a zinc-coordinated water molecule to produce a hydroxyl group which would not exchange with the anion inhibitor, or it could be produced by the ionization of a histidine residue which could then coordinate with the group in the active site normally available for binding substrate or anion inhibitors.

The longitudinal and transverse relaxation rates of quadrupolar nuclei such as ^{35}Cl are increased by coordination to metal ions such as zinc which distort the symmetrical distribution of electrons about the chloride ions and thereby increase the electric field gradient at the chloride nucleus. When the zinc ion is bound within the conformation of a protein the effect on the relaxation rate of chloride is further enhanced due to an increase in the correlation time, τ_c, the time constant of that process which modulates the zinc–chloride interaction. The correlation time, τ_c, is related to the transverse relaxation rate, $1/T_{2p}$, and hence to the line width of the nuclear magnetic resonance absorption, $\Delta\nu$. The line width for a ^{35}Cl nucleus which can exist in two environments, i.e., in the coordination sphere of a zinc ion and in the bulk solvent, in the absence of a chemical shift between the environments, is given by (Swift and Connick, 1962)

$$\frac{1}{T_{2p}} = \frac{1}{T_2} - \frac{1}{T_{2(0)}} = \frac{f}{T_{2M} + \tau_M} \qquad (4)$$

where $1/T_{2p}$ is as previously defined, $1/T_2$ is the observed relaxation in the presence of bound chloride, $1/T_{2(0)}$ is the observed relaxation rate in the absence of bound chloride, f

is the fraction of the total chloride bound, T_{2M} is the transverse relaxation time of bound chloride, and τ_M is the exchange lifetime of bound chloride.

The transverse relaxation time of a quadrupolar ion is given by the expression (Bull et al., 1973):

$$\frac{1}{T_{2M}} = \frac{2\pi}{5}(e^2qQ)^2 g(\tau_c) \quad (5)$$

where e is the electronic charge, q is the electric field gradient at the bound chloride site, Q is the electric quadrupole moment, and $g(\tau_c)$ is the correlation function which is of the form

$$g(\tau_c) = 0.6\tau_c + \frac{\tau_c}{1 + \omega_I^2\tau_{c2}} + \frac{0.4\tau_c}{1 + 4\omega_I^2\tau_c} \quad (6)$$

where τ_c is the correlation time and ω_I is the nuclear resonance frequency of the exciting radiation.

The correlation time, τ_c, is related to the rotational lifetime of the protein, τ_r, and to the exchange lifetime of the zinc-chloride complex, τ_M:

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_M} \quad (7)$$

When the chloride exchange process is slow compared to the relaxation rate of the chloride bound in the metalloenzyme, $\tau_M \gg T_{2M}$, then $1/T_{2p} \simeq f/\tau_M$ and the correlation time, τ_c depends on the rate of chloride exchange. When the chloride exchange process is fast compared to the relaxation rate of the bound chloride, $T_{2M} \gg \tau_M$, then $1/T_{2p} \simeq 1/T_{2M}$ and the correlation time, τ_c, depends on the rotational lifetime of the chloride-metalloprotein complex.

The measured quadrupole enhancement parameter, ϵq, indicates that renal dipeptidase is approximately 100-fold more effective than aquozinc(II) ions in broadening the ^{35}Cl line. A reasonable choice of the lifetime of chloride bound to aqueous zinc ion is 10^{-6}–10^{-8} sec (Ward and Cull, 1972). To produce an enhancement of 100, the lifetime of chloride bound to zinc in renal dipeptidase would have to be 10^{-4}–10^{-6} sec if the rate of chloride exchange modulated the correlation time. This would be entirely too long for the fast exchange region, and, in fact, exceeds the values obtained for carbonic anhydrase where the slow exchange region pertains (Ward and Cull, 1972). The effective correlation time, τ_c, must, therefore, be rotational in nature for renal dipeptidase. Since the rotational lifetime of the aqueous zinc-chloride complex is of the order of 10^{-11} sec (Mildvan and Cohn, 1970), the rotational lifetime of the chloride-metalloprotein complex must be of the order of 10^{-9} sec. Since this is rather short for a protein of molecular weight of 94000, it may be that the effective correlation time is related to motion of the component subunits that comprise the quaternary structure of the macromolecule (Ferren et al., 1975).

The fact that the observed relaxation is independent of the frequency within an experimental error of 15% reduces eq 6 to $g(\tau_c) = 2\tau_c$. A study of the temperature effect on $1/fT_{2p}$ or $\pi\Delta\nu_{net}/f$ can then be employed to indicate the dominant component of the effective correlation time, τ_c. If chloride exchange is slow and $1/fT_p \simeq 1/\tau_M$, $\pi\Delta\nu_{net}/f$ should vary directly with temperature. If chloride exchange is fast, $1/fT_p \simeq 1/T_{2M}$, $\pi\Delta\nu_{net}/f$ should vary inversely with temperature. In Figure 4 the ^{35}Cl line width is shown to vary inversely with temperature indicating a rapid exchange of chloride into the zinc peptidase binding sites, and again indicating that the dominant relaxation mechanism is rotational in nature. These results differ from those obtained for carbonic anhydrase, where the slow exchange region pertains, but are similar to the results obtained for DNA polymerase where fast exchange was observed (Springgate et al., 1973). In this discussion we have neglected differences in the electric field gradient experienced by the chloride bound to the zinc ion of renal dipeptidase as compared with aqueous zinc ion. Studies on small zinc chelates indicate that these differences are probably not large (Happe, 1973).

The agreement of the theoretical curve with the experimental data presented in the cyanide titration curve (Figure 2) indicate that cyanide ions compete with chloride ions for the anion-binding site. The effect of the competitive inhibitor, guanosine triphosphate, in reducing ^{35}Cl line broadening also suggests that chloride inhibits by competing with substrate at the active site of the enzyme. Furthermore, the dependence of ^{35}Cl line broadening upon the zinc content of the metalloenzyme suggests that chloride inhibits renal dipeptidase by competing with the substrate for a position in the coordination sphere of the zinc at the active site. The ^{35}Cl line-width variation with pH shown in Figure 3 is quite similar to that which was reported for ^{35}Cl NMR studies with bovine carbonic anhydrase (Ward, 1969). The pK of 7.4 determined for this curve is in the range of pK = 7.8 observed in the kinetic analysis of chloride inhibition (Figure 1). It is possible that dissociation of the same group modulates both ^{35}Cl line broadening and chloride inhibition of renal dipeptidase. It is not possible with the available data to distinguish the identity of the dissociating group between a water molecule coordinated to the zinc atom and an imidiazole group in the region of the active site.

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Gas Chromatography–Mass Spectrometry for Probing the Structure and Mechanism of Action of Enzyme Active Sites. The Role of Glu-270 in Carboxypeptidase A†

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ABSTRACT: A new technique for the study of the mechanism of enzymes has been developed. An enzyme, modified by an active-site directed reagent, is digested by one or more proteases. The resulting mixture of oligopeptides is then analyzed directly by gas chromatography–mass spectrometry *without* the use of separation or isolation procedures. A comparison with unmodified enzyme identifies the modified residue as well as quantifies the reaction. This approach has been applied to the identification of Glu-270 in the active site of carboxypeptidase A using a carbodiimide as modification reagent. Studies on the possible incorporation of ^{18}O (from ^{18}O-enriched water) into Glu-270 or other acidic residues near the active site of carboxypeptidase A show that the oxygens of the carboxyl groups of these residues are not exchangeable.

Many methods have been developed for determining which amino acid residues in enzymes are involved in the catalytic process—serving either as substrate binding sites or in the bond-making and -breaking steps (Vallee and Riordan, 1969). One chemical approach involves the selective labeling of functional residues in an enzyme by active-site directed reagents. Subsequent digestion of the modified enzyme by proteases, isolation of the labeled peptide, and identification of the particular residue modified is a prerequisite to an eventual mechanistic interpretation. Such procedures, if possible, are often tedious, time consuming, and best suited to easily detectable labels, e.g., radioisotopes or chromophores.

We have developed a technique by which the digest of the labeled enzyme is transformed into a corresponding mixture of volatile peptide derivatives that can be analyzed directly by a gas chromatography–mass spectrometry (GC–MS)–computer system *without* further purification or isolation steps. Derivatization procedures are used which retain the label or transform it quantitatively and predictably. The resulting mixture of derivatives is amenable to gas chromatographic separation and the structure of the labeled oligopeptide is subsequently determined by mass spectrometry. Knowledge of the amino acid sequence of the enzyme permits the positioning of the modified residue.

This approach has been applied to the identification of Glu-270 in the active site of carboxypeptidase A using a water-soluble carbodiimide as the active-site directed reagent. Furthermore, it has been shown that because of the specificity of mass spectrometry, conditions can be used for labeling enzymes which are identical with those in biological systems, e.g., by the use of stable isotopes. We have applied this technique to the study of the possible incorporation of ^{18}O into side chain carboxyl groups by incubation in ^{18}O-enriched water. The results bear on the mechanism of action of carboxypeptidase A.[1]

Methods and Materials

Pyridine, dimethoxyethane (both "distilled in glass", Burdick and Jackson Lab.), and acetic anhydride ("Baker Analyzed" Reagent) were all redistilled before use. Lithium aluminum deuteride (Alpha-Ventron), methanol, chloro-

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[1] A preliminary account of portions of this work has been presented (Nau and Riordan, 1974).