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Kinetic Analysis by Stopped-Flow Radiationless Energy Transfer Studies: Effect of Anions on the Activity of Carboxypeptidase A†

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ABSTRACT: We have utilized a highly sensitive radiationless energy transfer (RET) assay to investigate the effect of anions on the activity of carboxypeptidase A (CPD-A). The RET kinetic method visualizes the ES complex directly and thus enables both the mode of action of anions and the quantitation of their effect to be determined at a single substrate concentration. In marked contrast to the activating effect of anions on the closely related metalloprotease, angiotensin converting enzyme, Cl^-, and other anions inhibit CPD-A catalysis. NaCl inhibits the hydrolysis of Dns-Ala-Ala-Phe throughout the pH range 6-10. Other di- and tripeptides are similarly inhibited while their ester analogues are affected only slightly. Changes in the type of cation [e.g., Na^+, Li^+, K^+, Ca^{2+}, and $(CH_3)_4N^+$] at a constant [Cl^-] of 0.1 M showed no difference in the extent of inhibition, whereas with anion substitution the differences were marked. In all cases, the inhibition was partially competitive. At pH 5.9, the K_i values for the free enzyme are 51 (Cl^-), 17 (N_3^-), 2.1 (SO_4^{2-}), and 0.21 mM ($H_2PO_4^-$), and for the ES complex, the K_I' values are 1000, 720, 42, and 13 mM, respectively. The other anions were shown to act at the chloride site. The results indicate that investigations of anion inhibition in 1 M NaCl, a typical assay condition, may be greatly hindered by the presence of Cl^-. Thus, the competitive binding mode of phenylacetate toward peptide hydrolysis is greatly decreased by the presence of 1 M Cl^- ion while its noncompetitive component is unaffected. The site of anion interaction is not likely the metal atom since spectroscopic studies have indicated that binding to this site is much weaker than is observed here. Since Cl^- and PO_4^{2-} can decrease the rate of inactivation of the enzyme by butanedione, an arginine residue is a likely anion binding site. The results of the study are discussed in terms of the effect of salt on the solubility of the enzyme and the reduced catalytic activity of the crystals.

The active site of carboxypeptidase A contains several cationic groups (Arg-145, -127, -124, and -71 and Zn^{2+}). Its substrates, which are carboxylates, and some of its most potent inhibitors, such as β-phenylpropionate (Kaufman & Neurath, 1949) and benzyl succinate (Palmer et al., 1982), are known to be negatively charged. Until now, it has been impossible to evaluate the effect of anions on the enzyme's activity because it is generally assayed in 0.5-1 M NaCl due to its limited solubility in the absence of NaCl. We have now used a highly sensitive radiationless energy transfer (RET)[1] assay to overcome the problem. The RET assay was used in this study because it provides a means of observing submicromolar concentrations of enzyme-substrate complexes and greatly simplifies analysis of modifier effects (Auld et al., 1972, 1977).

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[1] Abbreviations: CPD-A, carboxypeptidase A; ACE, angiotensin converting enzyme; RET, radiationless energy transfer; ammediol, 2-amino-2-methyl-1,3-propanediol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Dns, dansyl [5-(dimethylamino)naphthalene-1-sulfonyl]; Tris, tris(hydroxymethyl)aminomethane; OPhe, L-β-phenyllactate.

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The transfer of energy between the dansyl substrate and the enzyme tryptophans only occurs when the substrate is bound to the enzyme so the formation and breakdown of the ES complex can be directly monitored. Thus, the effect of inhibitors on binding and catalysis can be observed directly (Auld et al., 1972). A preliminary account of this work has been reported (Williams & Auld, 1982).

Materials and Methods

Bovine carboxypeptidase A (Cox) was obtained from Sigma Chemical Co. and further purified by affinity chromatography (Peterson et al., 1976). Protein concentration was determined by using a molar absorptivity of 6.42×10^4 M^{-1} cm^{-1} at 278 nm (Simpson et al., 1963). The substrates used were dansylated oligopeptides and depsipeptides synthesized and characterized as described (Auld & Holmquist, 1974; Galdes et al., 1983). Furoylacryloyl (FA) substrates were gifts from B. Holmquist. Buffers were made up as required from stock solutions of Mes, Hepes, Tris, and ammediol. All stock solutions were extracted with 0.001% dithizone in carbon tetrachloride in order to remove extraneous metals. As a further precaution against metal contamination, all glassware was washed with 30% nitric acid and deionized, distilled water.

The stopped-flow studies were carred out by using a Durrum-Gibson instrument equipped with Durrum fluorescence accessory 16400, a 75-W xenon–mercury lamp [Model X75-2089 (Illumination Industries) or Model HX75-7415 (Durrum)], and an EMI 9526B photomultiplier. A low-noise amplifier with reference voltage offset faciliated the observation of small changes in light level above a large background (Hanahan & Auld, 1980). The signal to root mean square noise ratio for the system was typically in the range of 2000–15 000. Fluorescence intensities were digitized and stored on floppy disks (BSAF) by using a PDP 11/34 computer (DEC) equipped with an AR11 A/D converter and a VT-55 Decscope.

Pertinent parameters were calculated from the 1000 time points stored per experiment. The fluorescent intensity at any given time point t (F_t) was determined with a resolution of 1 part per 1000. Areas were calculated as previously described (Lobb & Auld, 1980, 1984). Turnover numbers (k) were computed from the measured values of maximum fluorescence (F_{max}) and the area under the curve (A_0) or from the known values of initial substrate concentration ($[S_0]$) and total enzyme concentration ($[E_T]$). The values of k_{cat} and K_m were determined by using a least-squares analysis of double-reciprocal plots of either $1/k$ vs. $1/[S_0]$ or $1/k_t$ vs. $1/[S_t]$. The values of k_t were determined by taking a series of intensities F_t and associated areas A_t from a single substrate concentration using every tenth time point from the reaction curve. F_t and A_t values were used to generate a series of rate constants k_t and substrate concentrations $[S_t]$. About 100–500 data points were used to generate 10–50 k_t and $[S_t]$ values. All values of k_{cat} and $K_{m_{app}}$ shown in the figures were an average of three or four determinations. Analyses and their associated errors were recorded and stored on floppy disks.

The theoretical plots[2] of $K_{m_{app}}$ and $k_{cat}/K_{m_{app}}$ vs. anion concentration could be fitted to eq 1 (see Results), permitting the parameters to be obtained by successive approximation as described previously (Auld & Vallee, 1970).

Arginine modification was performed according to the procedures developed by Riordan (1973). Butanedione (10%) was made up fresh and added to an enzyme solution (1 μM) maintained at pH. 7.8 with a 50 mM borate and 25 mM Hepes buffer to give a reaction concentration of 0.5 mM butanedione. Aliquots of the reaction mixture were removed at assigned times and diluted 20-fold into an assay containing the substrate FA-Gly-Phe (5×10^{-5} M) in 1 M NaCl and 5 mM Hepes, pH 7.5 at 25 °C.

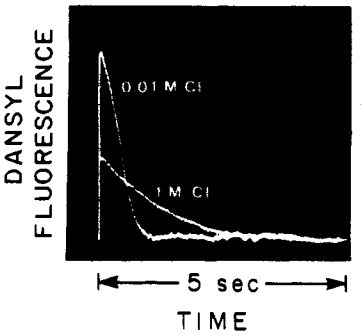

FIGURE 1: Stopped-flow fluorescence observation of the formation and breakdown of α-CPD-A (0.6 μM) and Dns-Ala-Ala-Phe (10 μM) under steady-state conditions. Excitation was at 285 nm, and the dansyl emission >420 nm was observed. Assay conditions: 1 or 0.01 M NaCl and 5 mM Mes, pH 5.9, 25 °C.

Results

Previous RET studies have shown that the mode of action of an inhibitor on the ES complex can be determined by the effect it has on the height and area of the stopped-flow trace (Auld et al., 1972). For example, a competitive inhibitor reduces the initial height of the signal and does not affect the area. In contrast, a noncompetitive inhibitor leaves the height of the signal unchanged, but the area under the curve is increased. Figure 1 compares the effect of 1 M NaCl with that of 0.01 M NaCl on the formation and breakdown of the ES complex formed between 10 μM Dns-$(Ala)_2$-Phe and 0.5 μM carboxypeptidase A. The initial height of the signal is decreased in 1 M salt, but the area under the curve is unchanged which is indicative of competitive inhibition (Auld et al., 1972).

RET kinetic analysis may also be used to quantitate the inhibition. Lineweaver–Burk plots can be generated either from multiple initial substrate concentrations or from a single initial substrate concentration by analysis of the time-dependent changes in fluorescent intensities and areas under the curve (Lobb & Auld, 1980, 1984). In both cases, a series of Lineweaver–Burk plots can be obtained by varying the inhibitor concentration (Figure 2A,B). Both plots indicate that k_{cat} is unchanged while K_m depends markedly on sodium chloride concentration, a characteristic of competitive inhibition.

Plots of $K_{m_{app}}$ vs. chloride concentration at pH values of 5.9 and 7.5 show that initially $K_{m_{app}}$ increases proportionally with increasing sodium chloride concentration but reaches a maximum at high concentrations (Figure 3A,B). Since k_{cat} is constant, the decrease in $k_{cat}/K_{m_{app}}$ with increasing sodium chloride concentration reflects the change in $K_{m_{app}}$. This phenomenon has been called partially competitive inhibiton (Segel, 1975).

Scheme I of Figure 4 shows that this type of inhibition differs in mechanism from pure competitive inhibition because an ESI complex is formed which breakds down to give products at the same rate as the ES complex.

The value of $K_{m_{app}}$ varies according to the equation:[2]

$$K_{m_{app}} = K_m \frac{1 + [I]/K_I}{1 + [I]/K_I'} \quad (1)$$

[2] Since k_{cat} is constant as a function of anion concentration, the values of $k_{cat}/K_{m_{app}}$ vary according to the reciprocal of eq 1 multiplied by k_{cat}.

Table I: Kinetic Constants for Chloride and Other Anion Inhibitors of the α-CPD-A-Catalyzed Hydrolysis of Dns-Ala-Ala-Phe[a]

anion	pH	K_I (mM)	K_m (μM)	K_m' (μM)	K_I' (mM)	D (N)[c]
Cl^-	10.0	120	25.0	88	420	3.7 (8)
Cl^-	8.7	100	9.5	35	370	3.5 (9)
Cl^-	7.5	45	3.8	25	300	4.1 (9)
Cl^-	6.9	45	3.8	27	320	3.7 (11)
Cl^-	5.9	51	4.3	84	1000	3.4 (10)
N_3^-	5.9	17.0	4.1	170	720	5.5 (6)
SO_4^{2-}	5.9	2.1	4.0	80	42	4.2 (9)
$H_2PO_4^-$[b]	5.9	0.21	4.0	250	13	7.5 (7)
HPO_4^{2-}[b]	7.5	0.40	5.2	130	10	5.3 (6)

[a] Assay conditions: α-CPD-A (1 μM), Dns-Ala-Ala-Phe (100 μM), and buffers of 5 mM Mes, pH 5.9 and 6.9, 5 mM Hepes, pH 7.5, 5 mM Tris, pH 8.7, and 5 mM ammediol, pH 10.0, all at 25 °C. [b] Predominate phosphate species present at this pH. [c] The average percent deviation (D) between calculated values (C_i) and observed values (O_i) was made a minimum where N is the number of data points (Auld & Vallee, 1970): $D = (100/N)\sum_{i=1}^{N}(|C_i - O_i|)/O_i$.

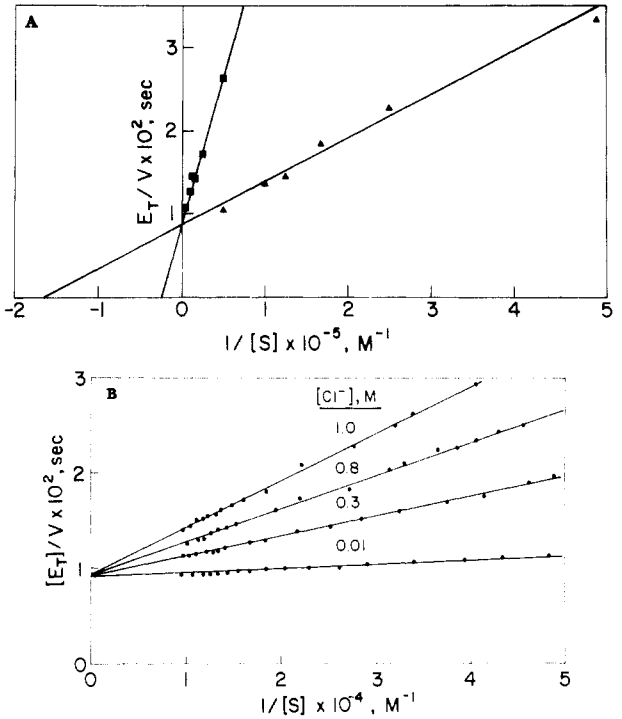

FIGURE 2: Lineweaver–Burk plots for carboxypeptidase-catalyzed hydrolysis of the tripeptide Dns-Ala-Ala-Phe. (A) The plots are determined from multiple initial substrate concentrations at 1 (■) and 10 mM NaCl (▲). (B) The plots are determined from a single initial substrate concentration (100 μM) at the indicated NaCl concentrations. Conditions of the assays were 5 mM Mes, pH 5.9, 25 °C.

At low concentrations of inhibitor (i.e., $[I]/K_I' << 1$), eq 1 reduces to the equation for simple competitive inhibition. Under these conditions, approximate values for K_m and K_I are obtained from the y and $-x$ intercepts (Figure 4). At very high concentrations of inhibitor, the value of $K_{m_{app}}$ approaches that of K_m' (Figure 4), again yielding a good trial value for K_m'. The trial values of K_m, K_I, and K_m' were varied over specified ranges, and the best-fit technique used (see Materials and Methods) yielded the values given in Table I for chloride inhibition at several pH values. All the data (8–11 points for each value of pH) fit to within 3–8% of the theoretical curve generated with eq 1 and the parameters in Table I.

The inhibition by sodium chloride was shown to be due to the anion and not the cation by varying the type of cation at constant chloride concentrations. Table II shows that the kinetic parameters for Dns-Ala-Ala-Phe hydrolysis, when measured in the presence of different cations, do not vary for cations of varying size and charge. When the anion is varied, however, the apparent K_m changes as a function of anion

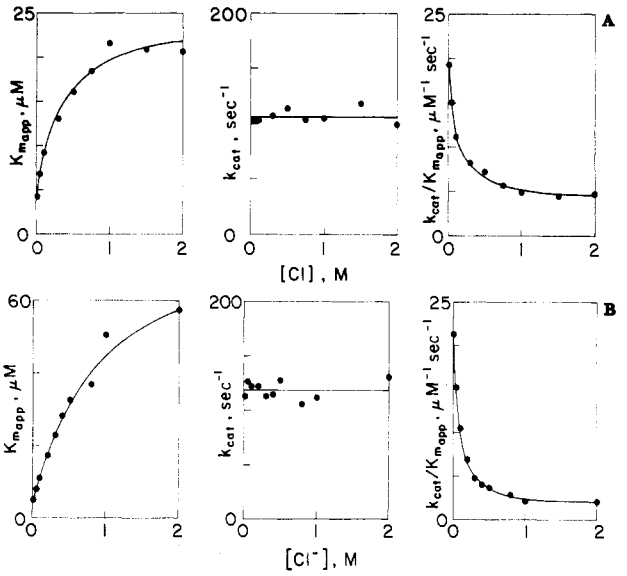

FIGURE 3: Dependence of $K_{m_{app}}$, k_{cat}, and $k_{cat}/K_{m_{app}}$ on chloride concentration for the CPD-A-catalyzed hydrolysis of Dns-Ala-Ala-Phe. Assay conditions are for 5 mM Mes, pH 5.9, 25 °C (A), and for 5 mM Hepes, pH 7.5, 25 °C (B). The lines are generated from eq 1, and the values for K_m, K_m', and K_I' are given in Table I.

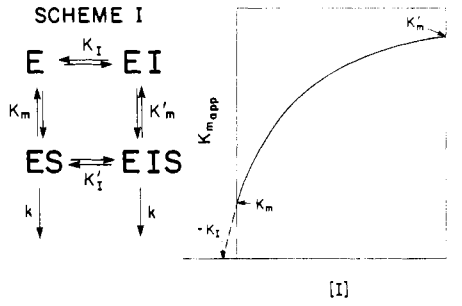

FIGURE 4: Schematic description of the mechanism for partial competitive inhibition.

Table II: Kinetic Parameters for CPD-A-Catalyzed Hydrolysis of Dns-Ala-Ala-Phe for Various Cations[a]

salt	$K_{m_{app}}$ (μM)	k_{cat} (s^{-1})	$k_{cat}/K_{m_{app}}$ (μM^{-1} s^{-1})
LiCl	11.4	96	8.4
NaCl	12.1	87	7.2
KCl	12.0	91	7.6
$N(CH_3)_4Cl$	12.7	91	7.2
$CaCl_2$[b]	12.8	83	6.5

[a] Assay conditions: CPD-A (1 μM), Dns-Ala-Ala-Phe (100 μM), 5 mM Mes, pH 5.9, 0.1 M salt, 25 °C. [b] The concentrations of the calcium and chloride ions were 0.05 and 0.1 M, respectively.

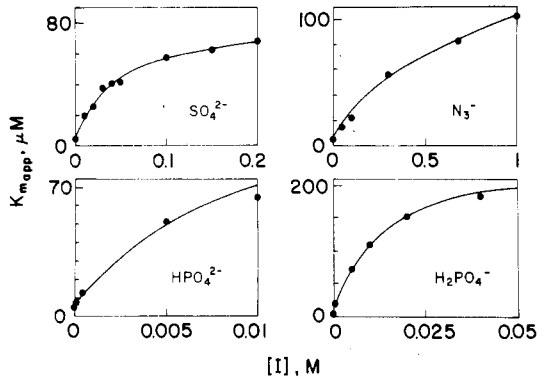

FIGURE 5: Dependence of $K_{m_{app}}$ for Dns-Ala-Ala-Phe on the concentration of sulfate, azide, and phosphate (predominate form $H_2PO_4^-$) in 5 mM Mes, pH 5.9, 25 °C, and phosphate (predominate form HPO_4^{2-}) in 5 mM Hepes, pH 7.5, 25 °C. The lines are generated from eq 1, and the values of K_m, K_m', K_I, and K_I' are given in Table I. An apparent K_m value of 123 μM at 0.1 M HPO_4^{2-} (data not shown in figure) is in excellent agreement with the calculated value, 120 μM.

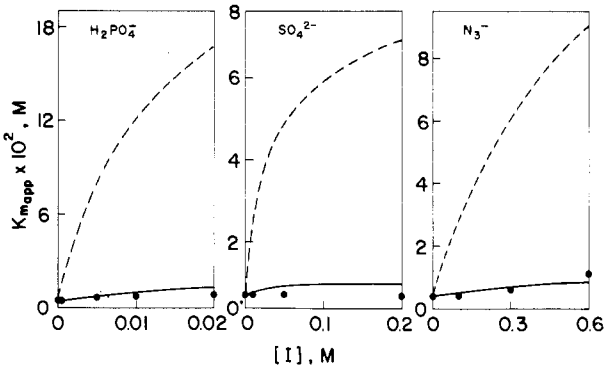

FIGURE 6: Dependence of $K_{m_{app}}$ for Dns-Ala-Ala-Phe on the azide, sulfate, and phosphate concentrations (●) determined in the presence of 1 M NaCl in 5 mM Mes, pH 5.9 at 25 °C. Theoretical curves are shown for the action of the anions at a site different from the chloride site (---) (eq 3) and at the chloride binding site (—) (eq 2).

concentration and depends strongly on the type of anion used. Figure 5 shows the anion concentration dependence for mono- and dihydro phosphates, sulfate, and azide. The data points were fit to the theoretical curves obtained by using eq 1 and the values of K_m, K_m', K_I, and K_I' given in Table I. The value for $K_{m_{app}}$ at zero anion concentration, K_m, is the same for all of the anions (at a specific pH) while the maximum value of $K_{m_{app}}$ obtained (K_m') is anion specific, and the values for K_I range from 0.2 to 51 mM (Table I).

To determine whether the anions are acting at the same site as chloride or at a different site, the anions (X) were added to the enzyme assay in the presence of a constant concentration of chloride (I). Equation 2 defines the action of anions acting at an identical site while that of anions acting at independent sites is given in 3 (Segel, 1975):

$$K_{m_{app}} = K_m \frac{1 + [I]/K_I + [X]/K_X}{1 + [I]/K_I' + [X]/K_X'} \quad (2)$$

$$K_{m_{app}} = K_m \frac{(1 + [I]/K_I)(1 + [X]/K_X)}{(1 + [I]/K_I')(1 + [X]/K_X')} \quad (3)$$

The chloride concentration is held constant at 1 M, and the second anion is added at various concentrations. Experimental values of $K_{m_{app}}$ for phosphate, sulfate, and azide are shown in Figure 6 and compared to the theoretical curves which were obtained by using eq 2 and 3 and the values for K_I, K_X, K_I', and K_X' from Table I. It is clear from these results that the anions compete with each other for an identical site on the enzyme.

Table III: Comparison of Kinetic Parameters for CPD-A-Catalyzed Hydrolysis of Peptide and Ester Substrates in 1 and 0.01 M Concentrations of Sodium Chloride[a]

substrate	[NaCl] (M)	$K_{m_{app}}$ (μM)	k_{cat} (s^{-1})	$k_{cat}/K_{m_{app}}$ (μM^{-1} s^{-1})
Dns-Ala-Ala-Phe	1.00	51	110	2.2
	0.01	5.1	110	22.0
Dns-Ala-Ala-OPhe	1.00	1.8	3.0	1.7
	0.01	1.0	4.0	4.0
Dns-Gly-Ala-Phe	1.00	67	140	2.1
	0.01	6.0	120	20.0
Dns-Gly-Ala-OPhe	1.00	6.8	13	1.9
	0.01	2.1	12	5.7
Dns-$(Gly)_3$-OPhe	1.00	110	670	6.1
	0.05	59	570	9.7
Dns-Gly-Phe	1.00	53	0.63	0.012
	0.01	8.5	0.63	0.074
FA-Phe-Phe	1.00	250	310	1.2
	0.01	14	310	22.0

[a] Assay conditions: α-CPD-A (1 μM), substrate (100 μM), 5 mM Mes, pH 5.9, 25 °C.

Table IV: Effect of NaCl Concentration on Phenylacetate Inhibition of Dns-Ala-Ala-Phe Hydrolysis[a]

[phenylacetate] (mM)	[NaCl] (M)	k_{cat} (s^{-1})	$K_{m_{app}}$ (μM)	$k_{cat}/K_{m_{app}}$ (μM^{-1} s^{-1})
0	1.0	100	35	2.9
1.0	1.0	2.0	35	0.057
0	0.01	110	4.2	26
1.0	0.01	2.6	33	0.079

[a] RET assays were performed in 5 mM Mes, pH 5.9 and 25 °C. The concentrations of Dns-Ala-Ala-Phe and enzyme were 100 and 1 μM, respectively.

Table III shows $K_{m_{app}}$, k_{cat}, and $k_{cat}/K_{m_{app}}$ values for several peptides and esters in 1 and 0.01 M salt. The furoylacryloyl and dansylated dipeptides and dansylated tripeptides all show a marked increase in $K_{m_{app}}$ at high sodium chloride concentration, but ester analogues only show a small increase. The dependence of the apparent K_m for Dns-Gly-Ala-Phe hydrolysis on chloride concentration produces the same value of K_I for chloride inhibition, 50 mM, as that obtained with Dns-Ala-Ala-Phe as substrate.

Inhibition of peptide hydrolysis by phenylacetate at low pH is dominated by a noncompetitive mode although a weaker competitive mode can be detected as the pH is elevated above 6 or at high phenylacetate concentrations (Auld et al., 1972; Auld & Holmquist, 1974). All previous studies, however, were performed in 1 M NaCl. Since phenylacetate is a carboxylate anion, this inhibition was reinvestigated at low salt concentrations. Inhibition of Dns-Ala-Ala-Phe hydrolysis at pH 5.9 and 1 M NaCl by 1 mM phenylacetate decreases k_{cat} 50-fold while leaving $K_{m_{app}}$ unchanged which is indicative of noncompetitive inhibition (Table IV). At 10 mM NaCl, k_{cat} is still decreased 43-fold, but $K_{m_{app}}$ is now increased 8-fold which is indicative of a strong competitive component to the inhibition. The presence of high chloride concentrations, therefore, decreases a competitive component of the inhibition; in the same way, it decreases the competitive inhibition by other anions (Figure 6).

Since these anions are likely to require a positive binding site on the protein, the effect of chloride on arginine modification was examined. Butanedione in borate buffer modifies 2 out of the 11 arginine residues on carboxypeptidase A and inactivates the enzyme as a peptidase (Riordan, 1973). To

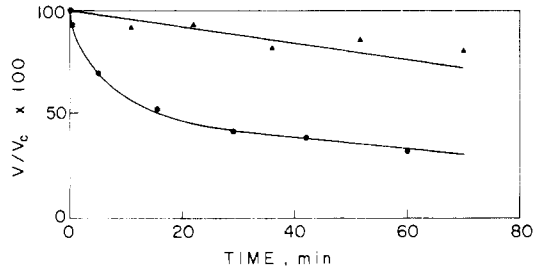

FIGURE 7: Effect of anions on the rate of inactivation of CPD-A by 0.5 mM butanedione in 50 mM borate buffer and 25 mM Hepes, pH 7.8, 20 °C. Reactions were performed in 1 (▲) and 10 mM NaCl (●). Assay conditions were 5×10^{-5} M FA-Gly-Phe in 1 M NaCl and 5 mM Hepes, pH 7.5 at 25 °C.

test whether anions bind to arginine groups, the arginine modification reaction (0.5 mM butanedione, 50 mM borate, and 25 mM Hepes, pH 7.8) was performed in the presence of 0.01 and 1 M chloride. The higher concentration of chloride decreases the rate of inactivation 10-fold (Figure 7).

Discussion

Several anions including orthophosphate, sulfide, and cyanide as well as carboxylates like citrate, oxalate, and β-phenylpropionate have been shown to inhibit carboxypeptidase A over 30 years ago (Kaufman & Neurath, 1949; Smith & Hanson, 1948, 1949; Neurath & DeMaria, 1950). Definitive studies on the nature of anion inhibition were not readily possible at that time, however, because the available substrates were known to exhibit anomalous Michaelis–Menten kinetics. Moreover, the experiments were frequently carried out in the presence of fairly high concentrations of chloride and other anions because of the limited solubility of CPD-A in the absence of a salt.

Since that time, however, many oligopeptides and ester analogues which conform to Michaelis–Menten kinetics have been synthesized (Auld & Vallee, 1970). In addition, an analytical method has been developed which uses radiationless energy transfer between a fluorescent substrate and the intrinsic tryptophans in the enzyme to indicate the formation and decay of the enzyme–substrate complex at submicromolar concentrations of enzyme (Auld, 1977). With this method, the action of an inhibitor at a single substrate and inhibitor concentration can be determined both qualitatively and quantitatively which greatly simplifies inhibition studies.

A qualitative view of the action of the inhibitor can be obtained immediately by examining its effect on the formation and breakdown of the enzyme–substrate complex. The maximal change in fluorescence (F_{max}) that occurs upon formation of the ES complex is proportional to the steady-state concentration of ES, and the area A under the curve is inversely proportional to k_{cat}. An inhibitor may therefore be classified according to the way in which it affects the two parameters F_{max} and A (Auld et al., 1972). Sodium chloride is a competitive inhibitor (Figure 1) because in its presence F_{max} was lowered, reflecting decreased binding of peptides, while A did not change because the catalytic rate constant is not altered. Detailed analysis of these curves confirms and quantitates this inhibition (Figures 2 and 3, Table I).

That the competitive inhibition is due to the anion and not the cation is shown by the observation that variation in the size and charge of the cation had no effect on the kinetic parameters (Table II). Moreover, the effect is not due to a change in ionic strength of the reaction. Thus, an increase in the concentration of the Mes or Hepes buffer from 5 to 200 mM produced no marked variation in kinetic parameters while

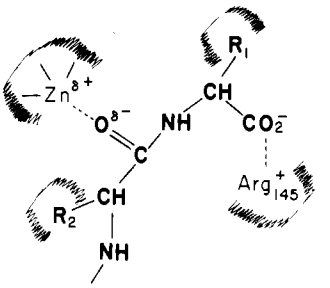

FIGURE 8: Schematic of active site of carboxypeptidase A.

several different anions inhibited over widely different concentration ranges (Figures 3 and 5) and the K_I values differed by as much as 250-fold (Table I).

The dependence of $K_{m_{app}}$ on anion concentration is not linear (Figures 3–6) as would be expected for pure competitive inhibition. The plot of $K_{m_{app}}$ vs. anion concentration curves asymptotically, indicative of partially competitive inhibition, and is described by eq 1 (Segel, 1975).

Chloride was shown to act at the same site as the other anions by experiments in which the chloride concentration was held constant and the concentration of the second anion was varied (Figure 6). The experiments further illustrated that the presence of the high concentrations of chloride anion can greatly reduce the extent of inhibition by a second anion, even an anion which binds tightly in the absence of chloride, like phosphate (K_i = 0.2 mM, Table I). As a result, examination for anion inhibition in the presence of 1 M NaCl, a typical assay condition, may be greatly hindered by the presence of the chloride ion. For example, the competitive binding mode of phenylacetate toward peptide hydrolysis is greatly decreased at a 1 M NaCl concentration while the noncompetitive binding mode remains the same (Table IV).

Several recent studies using both NMR and UV–vis spectroscopy suggest that a metal–anion interaction exists in carboxypeptidase A although it appears to be very weak. Navon et al. (1970) examined metal–fluoride interactions using fluoride NMR and the manganese enzyme. They postulated that there are at least five ligands in the first hydration shell. They designated three of these ligands to the protein, they found one to be water, and they suggest that the fifth is the anion. The $^{35}Cl^-$ NMR studies (Stephens et al., 1974; Stephens & Bryant 1976) have shown that there are two major contributions to NMR line broadening, indicating there are two major sites where chloride interacts with the enzyme. It was suggested that one site was a metal site and the binding in the interaction taking place at that site was strongly pH dependent and estimated to be 2 M^{-1} at low pH. Chloride and bromide are known to alter the spectral properties of cobalt carboxypeptidase A (Geoghegan et al., 1982) but only at pH values below 6 and at concentrations much greater than 1 M. Thus, if the inhibition described in the present study is due to a metal–chloride interaction, much weaker inhibition constants and a pH dependence for K_I would be expected. The results show chloride inhibition is quite strong, 45–120 mM, and is not strongly pH dependent (Table I), indicating the metal is not likely to be the site of interaction.

Figure 8 is a schematic of the active site of carboxypeptidase A which shows the two positive sites most likely to be affected by anions, the metal atom and the Arg-145 residue postulated to be the substrate carboxyl binding site (Lipscomb et al., 1968). Since the metal is probably not the site of the anion interaction observed here, the arginine site was studied by modifying arginine in the absence or presence of chloride by the method of Riordan (1973). The presence of chloride

(Figure 7) and phosphate (A. C. Williams and D. S. Auld, unpublished observations) clearly slows down the rate of inactivation of the enzyme which occurs as an essential arginine is modified. Thus, it appears that chloride may be acting at an arginine site which is postulated to be Arg-145 for the carboxyl group of substrates (Lipscomb et al., 1968) although arginine-127 and arginine-71 have also been postulated as playing a role in peptide binding (Nakagawa & Umeyama, 1978).

The difference in the extent of anion inhibition between peptides and esters may reflect the differences that have been found in their modes of binding as well. Peptides are thought to bind at Arg-145 (Rees et al., 1980) by their terminal carboxylate group and can bind in the absence of metal while esters do so only weakly [see Vallee et al. (1983) and references cited therein]. The anion inhibition of peptides is quite marked while that for ester analogues is not (Table III). If peptides bind to Arg-145, then the peptide carboxylate and chloride anions might compete for this residue. In contrast, the small effect of anions of ester hydrolysis could reflect the much weaker binding of an anion to the metal. This effect serves to underline previously found differences between peptide and ester hydrolysis by this enzyme (Riordan, 1973; Auld & Holmquist, 1974; Vallee et al., 1983; Auld et al., 1984).

Anion inhibition has been observed with other metalloenzymes such as carbonic anhydrase (Lindskog, 1983) and phospholipase C (Aakre & Little, 1982). In particular, an anion interaction site has been well documented for a closely related metalloprotease, angiotensin converting enzyme (ACE) Bünning & Riordan, 1982; Shapiro & Riordan, 1983, 1984). A critical lysine has been implicated in this interaction (Shapiro & Riordan, 1983). However, in marked contrast to what is observed here for carboxypeptidase A, the anion is a potent activator of ACE, acting by increasing substrate binding (Shapiro & Riordan, 1984). It is remarkable that these two enzymes which are believed to likely have similar mechanisms can have their activity regulated by anions in such a different manner.

The K_i value obtained for chloride inhibition, 50 mM, is particularly interesting in view of the fact that the enzyme at a concentration of 10–100 μM readily crystallizes from solution as the salt content of the solution is brought below 100 mM NaCl. The resulting crystals display a 20–1000-fold reduced enzymatic activity toward a wide range of substrates (Quiocho & Richards, 1966; Quiocho et al., 1967; Spilburg et al., 1974, 1977). In addition, the inhibition constant for β-phenylpropionate is weakened by about 2 orders of magnitude in the crystalline as compared to the solution state, a pronounced difference which could not be attributed to the cross-linking of the crystals (Quiocho et al., 1967). In a similar fashion, addition of benzoylglycine or cinnamic acid to the solution enzyme markedly alters activity, but they have essentially no effect on the crystalline enzyme (Spilburg et al., 1977). All of the above observations are consistent with weakened binding modes of substrates and inhibitors to the active center of crystalline carboxypeptidase. In view of the present anion inhibition results, it may be speculated that an intramolecular interaction between a negatively charged side chain and a positively charged site essential to substrate and inhibitor binding may be induced upon crystallization. This intramolecular interaction may be the trigger for crystallization of the enzyme and a cause of the subsequent decrease in enzymatic activity.

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Measurement of an Individual Rate Constant in the Presence of Multiple Exchanges: Application to Myocardial Creatine Kinase Reaction†

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ABSTRACT: Forward [creatine phosphate (CP) → adenosine 5′-triphosphate (ATP)] and reverse (ATP → CP) fluxes of myocardial creatine kinase (CK) measured by using ^{31}P nuclear magnetic resonance (NMR) and conventional saturation transfer (CST) methods are unequal; this is a paradoxical result because during steady state fluxes into and out of the CP pool must be the same. These measurements, however, treat the CK reaction as a two-site exchange problem and ignore the presence of the $ATP_\gamma \rightleftarrows P_i$ exchange involving the ATPases. We have applied a method [Uğurbil, K. (1985) *J. Magn. Reson. 64*, 207] based on the saturation of multiple resonances, by which a single unidirectional rate constant can be measured unequivocally in the presence of multiple exchanges, to the measurement of CK fluxes in isovolumic rat hearts perfused under three different conditions; two of the three perfusion conditions showed a large discrepancy in the CK fluxes determined by CST, and one did not. In contrast, when the effect of the $ATP_\gamma \rightleftarrows P_i$ exchange on the CK rate measurements was eliminated, multiple saturation transfer (MST) measurements on the same hearts yielded equal forward and reverse fluxes in all cases. The rate constant for the $ATP_\gamma \rightarrow CP$ conversion measured by MST was larger than the value obtained by the conventional methodology whereas both methods gave the same rate constant in the CP → ATP direction. These results demonstrate that the cause of the paradoxical data obtained by CST measurements of CK kinetics is the $ATP_\gamma \rightleftarrows P_i$ exchange and that CK rates when determined rigorously are consistent with the CK reaction being in equilibrium. Comparison of the MST and CST data suggests that in the myocardium, a three-site $CP \rightleftarrows ATP_\gamma \rightleftarrows P_i$ scheme adequately but not fully accounts for the phosphate exchange among these compounds. The magnitudes of the CK fluxes measured by MST and CST were ~2-fold larger in hearts perfused with glucose in the absence of insulin compared to hearts where the perfusate was supplemented with pyruvate or insulin. The higher CK rate is probably the reason why the discrepancy in CK fluxes as measured by CST is not prominent in glucose-perfused hearts; this follows from the fact that for a given rate of $ATP \rightleftarrows P_i$ interconversion, the confounding influence of this exchange on the CST measurements of CK should diminish as CK fluxes increase.

Creatine kinase is present in great abundance in the myocardium, skeletal muscle, and brain; it is believed to have an important but as yet poorly understood role in bioenergetics of these tissues. Recent studies on this enzyme have utilized ^{31}P nuclear magnetic resonance (NMR)[1] spectroscopy applied to perfused organs and whole animals. In particular, the unique capability of NMR to measure reaction rates by magnetization transfer methods (Forsen & Hoffman, 1963a,b) has been exploited to examine the CK-catalyzed exchange between CP and the γ-phosphate of ATP (Brown et al., 1978; Nunnally & Hollis, 1979; Matthews et al., 1982, 1983; Seymour et al., 1983; Ingwall et al., 1983; Micelli et al., 1983; Koretsky & Weiner, 1984; Bittle & Ingwall, 1985). However, such experiments conducted on the cardiac muscle have generated a highly significant but paradoxical result; as deter-

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; CK, creatine kinase; CP, creatine phosphate; CST, conventional saturation transfer; FID, free induction decay; LV, left ventricle; LVP, left ventricular pressure; MST, multiple saturation transfer; MVO_2, myocardial oxygen consumption rate in micromoles per minute per gram (dry weight); NMR, nuclear magnetic resonance; RPP, rate pressure product (product of heart rate and systolic pressure); SD, standard deviation; SEM, standard error of the mean; HR, heart rate; ip, intraperitoneal(ly); EDTA, ethylenediaminetetraacetic acid.

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