Differential Effect of Halide Anions on the Hydrolysis of Different Dansyl Substrates by Thermolysin†

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ABSTRACT: The effect of sodium halide salts on the hydrolysis of three of the dansyl (Dns) peptide substrates described in the previous paper (Yang & Van Wart, 1994) by thermolysin have been studied. Increasing concentrations of sodium chloride decrease the K_M value for the hydrolysis of the tripeptides Dns-Gly-Phe-Ala and Dns-Ala-Phe-Ala but leave k_{cat} unaltered. This kinetic behavior is described by a nonessential activation mechanism in which chloride binds preferentially to the enzyme–substrate complex. Similar trends are found for the sodium bromide and fluoride salts. In contrast, sodium chloride decreases both K_M and k_{cat} almost equally for the hydrolysis of Dns-Ala-Ala-Phe-Ala, leaving k_{cat}/K_M unchanged. Thus, chloride is an uncompetitive inhibitor of this substrate. Molecular modeling studies have been carried out in order to explain the effect of chloride on the binding of these dansyl peptides. The decrease in K_M for the hydrolysis of all three substrates is attributed to an interaction of chloride with Arg-203 located in the active site to stabilize the enzyme–substrate complexes. The differential effect of chloride on the k_{cat} values for the hydrolysis of the dansyl tripeptides vs dansyl tetrapeptide is related to differences in binding on the P_n side of the substrates. The tripeptides are predicted to bind to the active site of thermolysin in a single low-energy conformation. However, there are two populations of low-energy binding modes for the tetrapeptide, one of which is believed to be a more productive binding mode. Sodium chloride may act by forcing Dns-Ala-Ala-Phe-Ala to bind in this less productive mode and lowering k_{cat}. The effect of chloride on the activity of thermolysin differs markedly from that observed for carboxypeptidase A. However, it shares several similarities with angiotensin converting enzyme in that it generally enhances substrate binding but in a manner that is strongly dependent on the identity of the substrate.

The activation or inhibition of metalloproteinases such as carboxypeptidase A (Williams & Auld, 1986) and angiotensin converting enzyme (Dorer et al., 1974; Bunning & Riordan, 1983, 1987; Riordan et al., 1986; Shapiro et al., 1987) by anions is a well-established phenomenon. It is interesting to note the remarkably different effects of anions on these enzymes, both of which are believed to share a similar catalytic mechanism. For example, chloride is a partially competitive inhibitor of carboxypeptidase A (Williams & Auld, 1986) but a potent activator of angiotensin converting enzyme toward its physiological substrate angiotensin I (Skeggs et al., 1954; Bunning & Riordan, 1983). More detailed studies of the activation of angiotensin converting enzyme by anions have revealed that the effect is strongly substrate and anion dependent (Shaprio et al., 1983). Thus, it is clear that the response of zinc proteinases toward anions is a means of detecting mechanistic differences between individual members of the family.

Although the effect of calcium ions on the stability of thermolysin has been extensively investigated (Feder et al., 1971; Roche & Voordouw, 1971), very little attention has been paid to the effects of anions on this enzyme. Holmquist and Vallee (1976) have reported that increasing concentrations of NaBr, $CaCl_2$, LiBr, NaCl, and KCl greatly increase the k_{cat}/K_M value for the hydrolysis of FA-Gly-Leu-NH_2[1] and its ester analogue FA-Gly-OLeu-NH_2. In contrast, the rate of hydrolysis of Mns-Phe-Leu-Ala by thermolysin has been reported to be relatively insensitive to NaBr (Morgan & Fruton, 1978). The most detailed investigation for thermolysin was carried out by Li (1982) who showed that chloride and bromide are uncompetitive inhibitors of the hydrolysis of Dns-Ala-Ala-Phe-Ala. Most recently, Inouye (1992) has reported that NaCl stimulates the hydrolysis of Cbz-Asp-Phe-OCH_3 by thermolysin by increasing k_{cat} and that the magnitude of the effect is altered when the cation is changed to K^+ or Li^+. In this study, the effect of the halide salts NaF, NaCl, NaBr, and NaI on the hydrolysis of three of the dansyl substrates described in the previous paper (Yang & Van Wart, 1994) by thermolysin has been investigated. It is shown that the effect for the tripeptides Dns-Gly-Phe-Ala and Dns-Ala-Phe-Ala differs markedly from that of the tetrapeptide Dns-Ala-Ala-Phe-Ala. The differences observed for these closely related substrates have been related to differences in binding that have been investigated by molecular modeling studies.

MATERIALS AND METHODS

Materials. NaF, NaCl, NaBr, and NaI were purchased as spectrographically pure salts from Sigma. All other materials

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Dns or dansyl, 5-(dimethylamino)-naphthalenyl-1-sulfonyl; Mns or mansyl, 6-(*N*-methylanilino)-2-naphthalenyl-1-sulfonyl; DE, direct excitation; ET, energy transfer; E, enzyme; S, substrate; P, product; ES, enzyme–substrate intermediate; FA, 2-furanacrylic acid; Bz, benzoyl; Cbz, benzyloxycarbonyl.

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were prepared as described in the previous paper (Yang & Van Wart, 1994).

Kinetic Measurements. The concentrations of all dansyl substrates were determined spectrophotometrically using $\epsilon_{330} = 4.1 \times 10^3\ M^{-1}\ cm^{-1}$. Assays for the hydrolysis of all dansyl substrates were carried out by continuously monitoring the fluorescence change at 550 nm on excitation at 340 nm in order to follow the conversion of substrate to product (F^{SP} studies) as described in the previous paper (Yang & Van Wart, 1994).

Computational Methods. Calculations were performed using the AMBER force field, as implemented within Discover 2.8 and 2.9. Graphical analyses were carried out using InsightII 2.1.2 and 2.2.0 (Biosym Technologies). The charges were used unscaled with a scalar dielectric of 4.0. The nonbonded cutoff radius was 15.0 Å. Minimized structures were refined to a root-mean-square derivative of less than 0.1 kcal/mol Å. Charges and force constants for the dansyl group were derived from ab initio data determined for dansyl-[SO_2-$NH(CH_3)$].[2] These data were calculated using the 3-21G*//3-21G basis sets using Gaussian 88 (Frisch et al., 1988) and Gaussian 90 (Frisch et al., 1991). Electrostatic potential-derived charges for this group were computed using the CHELPG program (Breneman & Wiberg, 1990).

RESULTS

Li (1982) has studied the effect of several salts on the hydrolysis of Dns-Ala-Ala-Phe-Ala by thermolysin in 0.1 M NaCl, 10 mM $CaCl_2$, 50 mM Mes or Tris and established several features that are the starting point for the studies reported here. He showed that there was only a small effect of pH on k_{cat}/K_M that was attributed to a small and almost equal increase in both k_{cat} and K_M between pH 5.5 and 7.5. Changes in the identity of monovalent (e.g., Na^+, K^+, $N(CH_3)_4^+$) and divalent (e.g., Ca^{++}) cations at concentrations of 0.1 and 1 M were not found to alter activity appreciably. However, the effect of certain anions at pH 6.0 was much more dramatic. Increasing the concentrations of nitrate, perchlorate, and sulfate to 0.5 M had only a small effect on the kinetic parameters k_{cat} and K_M. However, the halide anions were found to inhibit with the relative potency fluoride > chloride > bromide. The latter two halides were shown to exhibit uncompetitive inhibition, where increasing anion concentrations lowered both k_{cat} and K_M equally. In the current study, the effect of sodium halide salts on the kinetic parameters for the hydrolysis of Dns-Gly-Phe-Ala, Dns-Ala-Phe-Ala, and Dns-Ala-Ala-Phe-Ala by thermolysin has been studied at pH 7.5 by using the F^{SP} method described in the previous paper (Yang & Van Wart, 1994).

Effect of Sodium Halide Salts on the Spectral Properties of Dansyl Substrates. In order to carry out F^{SP} studies at variable salt concentrations, the effect of the salts on the maximum fluorescence change, F_o, for the reactions must be known. The F_o value is influenced by changes in the optical and emission spectra of the substrates and products. Accordingly, the effect of NaF, NaBr, NaCl, and NaI on the positions of the absorption bands of the substrates Dns-Gly-Phe-Ala, Dns-Ala-Phe-Ala, and Dns-Ala-Ala-Phe-Ala in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5, has been investigated. The shapes of the bands in the optical spectra of these three substrates are essentially the same in the presence of all four salts (data not shown) with two principal absorption bands centered near 247–249 and 330–336 nm in all cases. In contrast, the emission spectra (λ_{ex} = 340 nm) of these dansyl substrates are sensitive to the presence of these sodium halide salts (Table 1). For all four salts, the emission wavelength is red-shifted by several nanometers compared to the value observed in the absence of the salt. Moreover, the fluorescence intensity (λ_{ex} = 340 nm, λ_{em} = 576 nm) is also reduced (data not shown). Since the emission bands of these dansyl substrates are red-shifted while the optical spectra remain the same, the quenching of the fluorescence by the salts is probably due to dynamic quenching (Lakowicz, 1983). The effect of NaCl, NaBr, NaF, and NaI on the F_o value for the hydrolysis of Dns-Gly-Phe-Ala in F^{SP} studies (λ_{ex} = 340 nm, λ_{em} = 576 nm) is shown in Figure 1. These salts reduce F_o in the order NaF > NaI > NaCl > NaBr. Similar effects were observed for Dns-Ala-Phe-Ala and Dns-Ala-Ala-Phe-Ala (data not shown). These reductions have been taken into account in the F^{SP} kinetic analyses described below for these three substrates.

Effect of NaCl on Hydrolysis of Dansyl Substrates. The initial rates of hydrolysis, v_o, of Dns-Gly-Phe-Ala, Dns-Ala-Phe-Ala, and Dns-Ala-Ala-Phe-Ala have been measured as a function of [NaCl] in 10 mM $CaCl_2$, 50 mM Hepes, pH 7.5, at a substrate concentration of 0.10 mM by carrying out a series of F^{SP} experiments (λ_{ex} = 340 nm, λ_{em} = 576 nm). The results are presented in Figure 2 as a plot of $v_o/[E_o][S_o]$ vs [NaCl]. Two different types of behavior are observed where the rates of the hydrolysis of Dns-Ala-Phe-Ala and Dns-Gly-Phe-Ala *increase* with increasing [NaCl] while the rates for Dns-Ala-Ala-Phe-Ala *decrease*. Thus, there is apparent

Table 1: Effect of Sodium Halide Salts on the Emission (λ_{ex} = 340 nm) Wavelength of Three Dansyl Substrates[a]

salt	concentration (M)	λ_{em} (nm) Dns-Gly-Phe-Ala	Dns-Ala-Phe-Ala	Dns-Ala-Ala-Phe-Ala
none	0	529.2	530.0	534.0
NaF	0.5	530.2	532.1	534.0
NaCl	3	532.2	534.2	538.1
NaBr	3	533.4	534.9	538.7
NaI	3	536.0	538.1	540.6

[a] All substrates (0.1 mM) were dissolved in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5, at the indicated salt concentration.

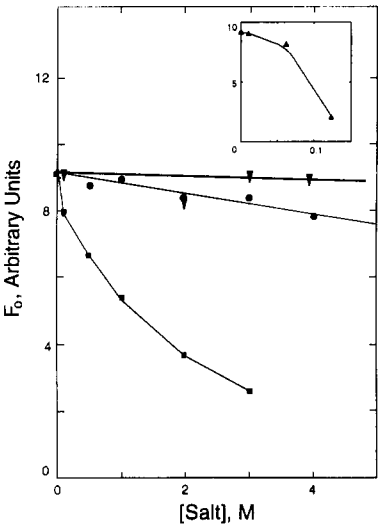

FIGURE 1: Effect of (▲) NaF, (●) NaCl, (▼) NaBr, and (■) NaI on the F_o values for the Dns fluorescence changes (λ_{ex} = 340 nm, λ_{em} = 576 nm) observed on hydrolysis of 0.10 mM Dns-Gly-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5, at 23 °C.

[2] Structures and charges for the dansyl group as well as coordinates for all of the modeled structures will be provided by the authors on request.

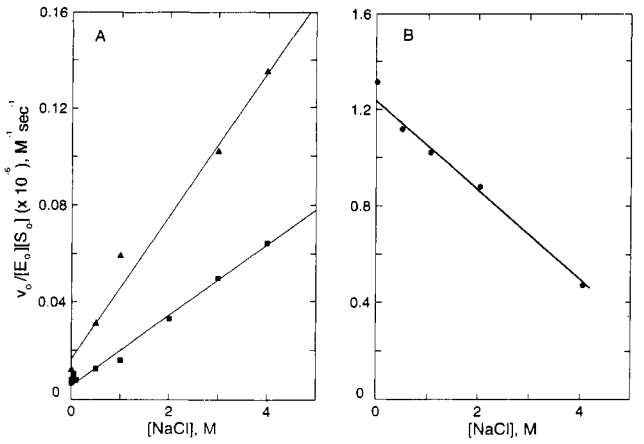

FIGURE 2: Effect of [NaCl] on the hydrolysis of 0.10 mM (▲) Dns-Gly-Phe-Ala, (■) Dns-Ala-Phe-Ala, and (●) Dns-Ala-Ala-Phe-Ala in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5, at 23 °C.

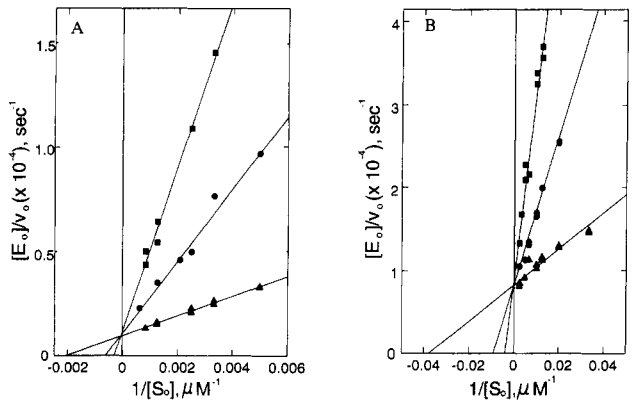

FIGURE 3: Double-reciprocal plots for the hydrolysis of (A) Dns-Ala-Phe-Ala and (B) Dns-Gly-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5, at 23 °C at [NaCl] of (■) 0, (●) 1.0, and (▲) 4.0 M.

inhibition of the hydrolysis of the tetrapeptide Dns-Ala-Ala-Phe-Ala but apparent activation of the tripeptides Dns-Ala-Phe-Ala and Dns-Gly-Phe-Ala by NaCl at this substrate concentration. These data clearly show that the effect of NaCl on the activity of thermolysin is substrate dependent.

The basis for the differential effect of NaCl on these two classes of substrates has been examined in greater detail. The kinetic parameters for the hydrolysis of Dns-Gly-Phe-Ala and Dns-Ala-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5, have been measured as a function of [NaCl] from F^{SP} experiments as described in the previous paper (Yang & Van Wart, 1994). Double-reciprocal plots for reactions carried out with Dns-Ala-Phe-Ala and Dns-Gly-Phe-Ala at three [NaCl] are shown in panels A and B, respectively, of Figure 3. For both substrates, these plots are linear, intercept at the $[E_o]/v_o$ axis, and have smaller slopes at the higher [NaCl]. Accordingly, increasing [NaCl] decreases K_M but leaves k_{cat} unaltered for both substrates. As a result, the k_{cat}/K_M values increase at higher [NaCl]. These trends are more clearly visible from the plots of these parameters vs [NaCl] illustrated for Dns-Gly-Phe-Ala in Figure 4.

These results clearly indicate that the thermolysin-catalyzed hydrolysis of these two dansyl tripeptides is enhanced by NaCl through an effect on K_M. Importantly, there is a nonzero rate in the absence of NaCl. This behavior is described by the nonessential activator mechanism shown in Figure 5, where K_X and K_S are the dissociation constants for the EX and ES complexes, respectively, α is the factor by which K_X and K_S are reduced when activator X binds to ES and E, respectively, and k_{cat} is unaffected. Application of rapid equilibrium assumptions to the scheme for nonessential activation allows one to equate K_S and K_M and evaluate the parameters in this scheme graphically. Accordingly, the data shown in Figure 3 are described by the relationship

$$\frac{[E_o]}{v_o} = \frac{K_M^{app}}{k_{cat}}\frac{1}{[S_o]} + \frac{1}{k_{cat}} \quad (1)$$

where

$$K_M^{app} = K_M\left(\frac{1 + \frac{[X]}{K_X}}{1 + \frac{[X]}{\alpha K_X}}\right) \quad (2)$$

Rearrangement of eq 2 gives the following relationship (Segel, 1975; Rohrbach et al., 1981)

$$\frac{K_M}{K_M - K_M^{app}} = \frac{\alpha K_X}{(1-\alpha)}\frac{1}{[X]} + \frac{1}{(1-\alpha)} \quad (3)$$

where K_M and K_M^{app} are obtained from the $1/[S_o]$ intercepts of double-reciprocal plots in the absence and presence of various concentrations of NaCl, respectively. According to eq 3, a plot of $K_M/(K_M - K_M^{app})$ vs $1/[X]$ should be linear with a $1/[X]$ intercept of $-1/\alpha K_X$ and a $K_M/(K_M - K_M^{app})$ intercept of $1/(\alpha - 1)$. Such a plot is shown for Dns-Gly-Phe-Ala in Figure 6 and gives α and K_X values of 0.075 and 5.3 M, respectively.

The kinetic parameters for the hydrolysis of Dns-Ala-Ala-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$ have also been determined as a function of [NaCl] by using the F^{SP} initial rate method described above. The results are presented in the form of double-reciprocal plots at fixed [NaCl] in Figure 7. The data are fit by a series of parallel lines in which the $1/[S_o]$ and $[E_o]/v_o$ intercepts give k_{cat}^{app} and K_M^{app}, respectively. Increasing the [NaCl] reduces both k_{cat}^{app} and K_M^{app} equally but does not affect the ratio k_{cat}^{app}/K_M^{app}. This is shown more clearly in Figure 8 where the values of these parameters are plotted as a function of [NaCl]. Thus, when the [NaCl] is increased from 0.020 to 4 M, k_{cat}^{app} and K_M^{app} are both decreased by approximately 15-fold but k_{cat}^{app}/K_M^{app} remains approximately 1.4×10^6 M^{-1} s^{-1}.

These results are similar to those reported by Li (1982) from F^{ET} studies with this substrate at pH 6.0. The behavior shown in Figures 7 and 8 is indicative of uncompetitive inhibition. Parallel double-reciprocal plots in the presence of inhibitor can result from pure uncompetitive inhibition, in which the inhibitor binds only to ES to give an inactive EXS species, as shown in Figure 9. In this scheme, eq 1 takes the form

$$\frac{[E_o]}{v_o} = \frac{K_M^{app}}{k_{cat}^{app}}\frac{1}{[S_o]} + \frac{1}{k_{cat}^{app}} \quad (4)$$

where

$$k_{cat}^{app} = k_{cat}/(1 + [X]/K_X') \quad (5)$$

$$K_M^{app} = K_M/(1 + [X]/K_X') \quad (6)$$

Alternatively, the data in Figure 7 could be consistent with the partial uncompetitive scheme shown in Figure 10 in which

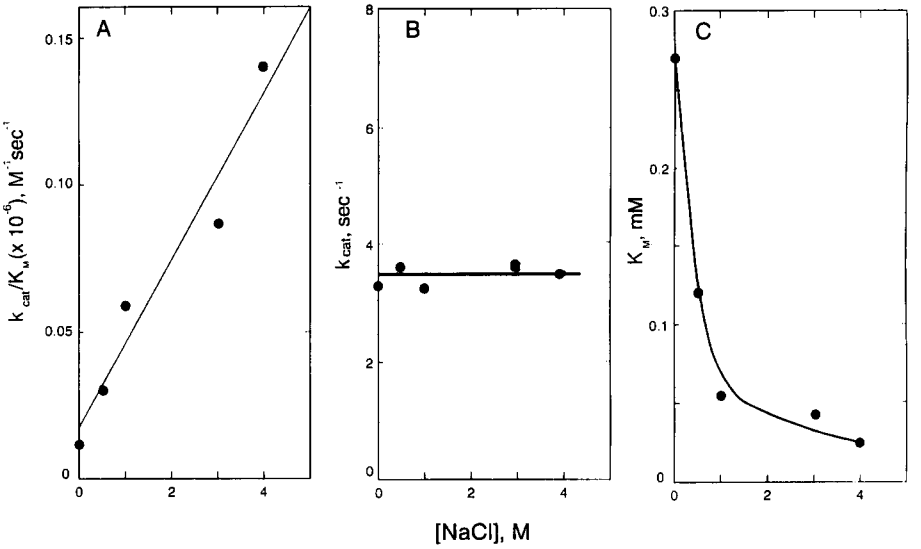

FIGURE 4: Effect of [NaCl] on the kinetic parameters for the hydrolysis of Dns-Gly-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5, at 23 °C.

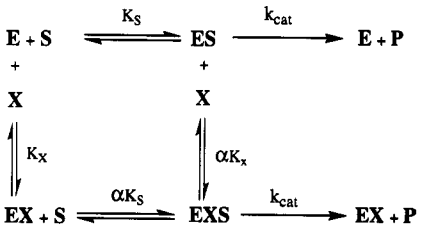

FIGURE 5: Scheme for nonessential activation of a one-substrate (S), one-product (P) reaction by activator X.

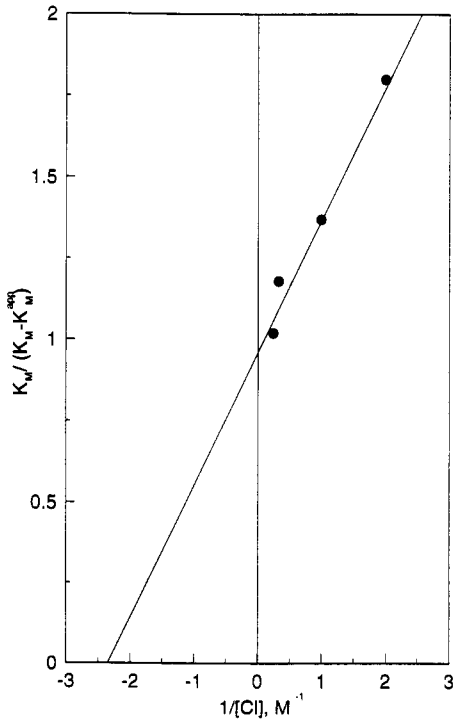

FIGURE 6: Replot of data from Figure 4 for the hydrolysis of Dns-Gly-Phe-Ala used to evaluate K_{Cl} and α.

X binds to E α-fold more weakly than to ES and in which EXS is converted to EX + P α-fold more slowly than ES. These two cases can be distinguished by plotting $1/k_{cat}^{app}$ from the $[E_o]/v_o$ intercept of the plots in Figure 7 vs [NaCl]. A hyperbolic curve is expected for a partial uncompetitive inhibitor and a linear plot for a pure uncompetitive inhibitor

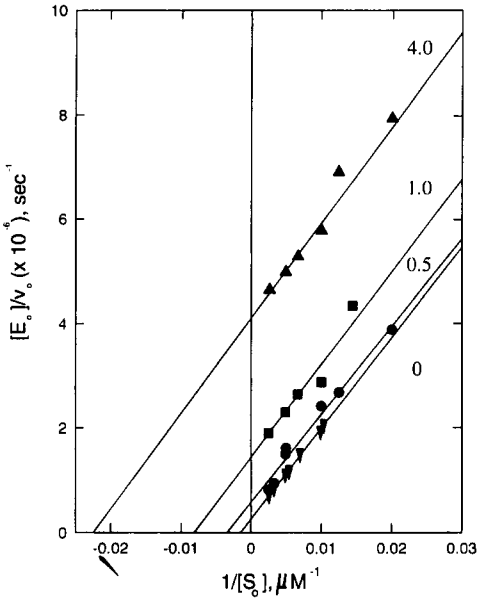

FIGURE 7: Double-reciprocal plots for the hydrolysis of Dns-Ala-Ala-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5, at [NaCl] of (▼) 0, (●) 0.50, (■) 1.0, and (▲) 4.0 M.

[Segel, p 188 (1975)]. The plot shown in Figure 11B is linear and shows that chloride can be adequately described as a pure uncompetitive inhibitor of the hydrolysis of Dns-Ala-Ala-Phe-Ala.

On the basis of eqs 5 and 6, plots of $1/k_{cat}^{app}$ vs [NaCl] and $1/K_M^{app}$ vs [NaCl] have been constructed to evaluate K_{Cl}' (Figure 11). These plots yield straight lines which intercept the [NaCl] axis at K_{Cl}' which is estimated to be 550 mM. Note that the K_{Cl}' value evaluated from data on the hydrolysis of Dns-Ala-Ala-Phe-Ala, based on the uncompetitive inhibition scheme shown in Figure 9, is comparable to the $\alpha K_{Cl}'$ value of 400 mM evaluated from data on the hydrolysis of Dns-Gly-Phe-Ala, based on the nonessential activation scheme shown in Figure 5. This indicates that chloride binds with approximately the same affinity to the thermolysin complex with both substrates. For both reactions, the binding of chloride of free thermolysin is predicted to be weak with K_{Cl} = 5.3 M for Dns-Gly-Phe-Ala and with no quantifiable binding observed for Dns-Ala-Ala-Phe-Ala.

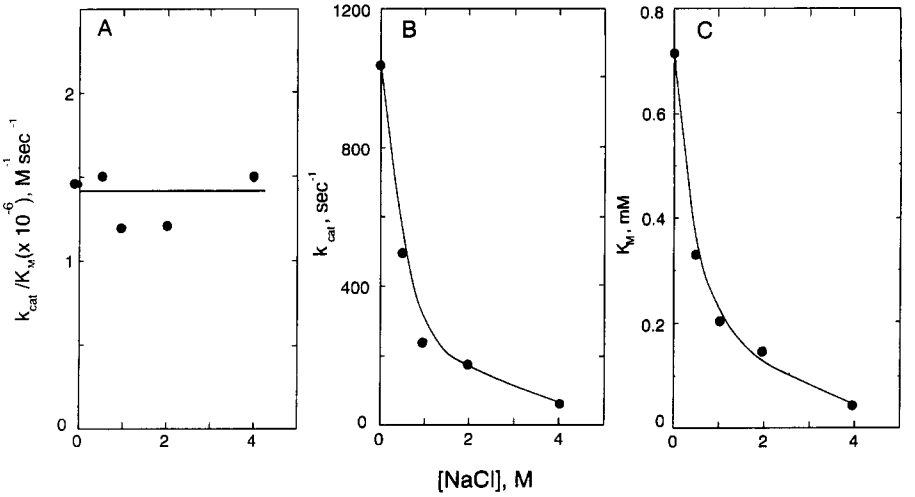

FIGURE 8: Effect of [NaCl] on the kinetic parameters for the hydrolysis of Dns-Ala-Ala-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5, at 23 °C.

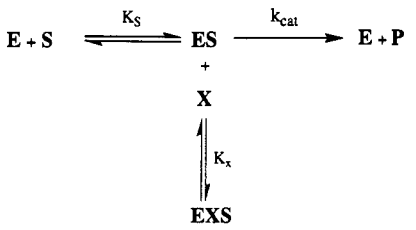

FIGURE 9: Scheme for partial uncompetitive inhibition of a one-substrate (S), one-product (P) reaction by inhibitor X.

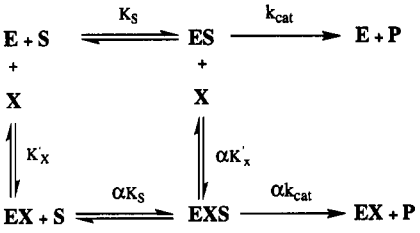

FIGURE 10: Scheme for hyperbolic mixed-type inhibition of a one-substrate (S), one-product (P) reaction by inhibitor X.

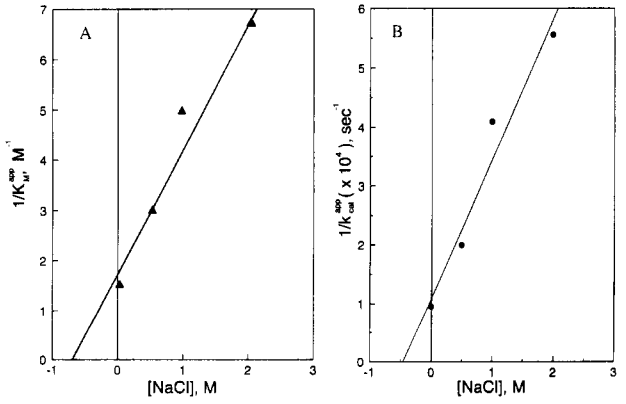

FIGURE 11: Plots of $1/K_M^{app}$ and $1/k_{cat}^{app}$ vs [NaCl] for the hydrolysis of Dns-Ala-Ala-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5, at 23 °C used to determine K_{Cl}'.

Effect of Other Sodium Halide Salts on the Hydrolysis of Dns-Gly-Phe-Ala. Since Li (1982) showed that chloride and bromide exhibit uncompetitive inhibition toward the hydrolysis of Dns-Ala-Ala-Phe-Ala, it was of interest to assess whether other halide anions affected the hydrolysis of the dansyl tripeptides studied here in the same manner as chloride. Thus, the effect of a fixed concentration of NaF, NaBr, and NaI

Table 2: Effect of Sodium Halide Salts on the k_{cat} and K_M Values for the Hydrolysis of Dns-Gly-Phe-Ala by Thermolysin[a]

salt	concentration (M)	k_{cat} (s^{-1})	K_M (mM)
none	0	3.3	0.27
NaF	0.1	3.3	0.43
NaCl	4	3.5	0.025
NaBr	4	3.4	0.030
NaI	3	4.3	0.20

[a] All assays were carried out in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5, at 23 °C.

on the kinetic parameters for the thermolysin-catalyzed hydrolysis of Dns-Gly-Phe-Ala has been investigated. The values of the kinetic parameters k_{cat} and K_M evaluated in the presence of 0.1 M NaF, 4 M NaBr, and 3 M NaI are listed in Table 2 together with the values for 4 M NaCl. For all four salts, the k_{cat} values are not changed appreciably. In contrast, the K_M values for these reactions are generally sensitive to the anion, where chloride and bromide lower K_M, fluoride increases K_M, and iodide has little effect.

Molecular Modeling Studies of Substrate Binding. The kinetic studies described above indicate that chloride enhances the binding of all three substrates but has a differential effect on the k_{cat} value for the hydrolysis of the dansyl tripeptides vs the dansyl-tetrapeptide. Since the crystal structures of thermolysin complexed to a variety of different inhibitors are available (Tronrud et al., 1987; Holden et al., 1987; Matthews, 1988), these structures have been used as a starting point for modeling the binding of these substrates in order to search for an explanation for the effects of chloride.

The structures of the complexes between thermolysin and the three substrates studied here were calculated starting from the most relevant X-ray crystallographic data. Specifically, Brookhaven Protein Database entry 5TMN, the structure of thermolysin complexed with the phosphinate inhibitor Cbz-Gly-PO_2-Leu-Leu, was used as a starting point for modeling Dns-Gly-Phe-Ala. This was carried out in the context of the substrate-binding model described by Hangauer and co-workers (1974) which specified the orientation of the carbonyl oxygen of the scissile peptide bond with respect to the zinc atom. In accordance with this model, His-231 was modeled in the protonated form. The zinc-bound water was removed to prevent steric repulsion with the substrate. The remainder of the substrate Dns-Gly-Phe-Ala was then constructed by adopting the crystal conformation of Cbz-Gly-PO_2-Leu-Leu

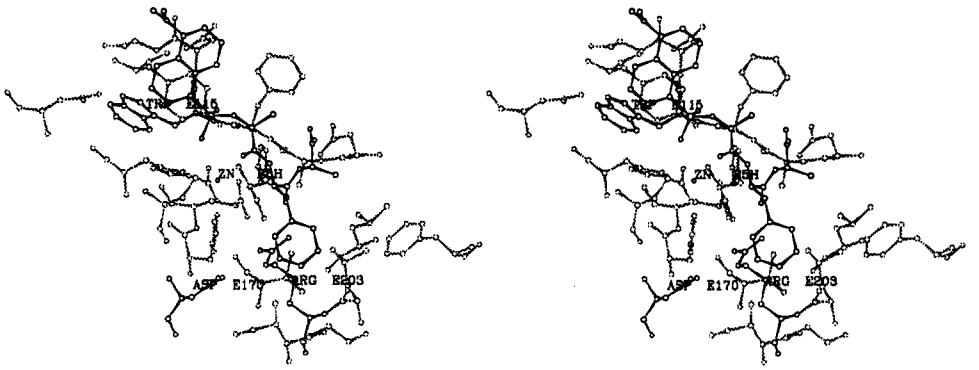

FIGURE 12: Stereoplot showing the binding of Dns-Ala-Phe-Ala (solid lines) to the active site of thermolysin (lighter lines), predicted from the modeling studies.

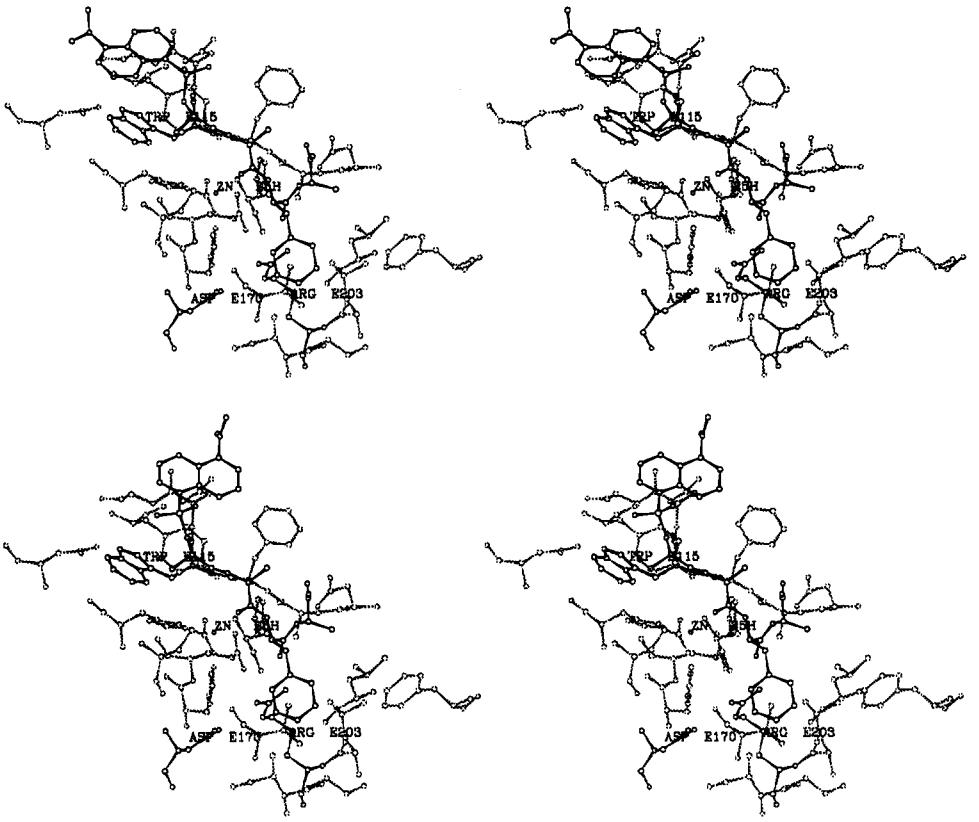

FIGURE 13: Stereoplots showing the two binding modes of Dns-Ala-Ala-Phe-Ala (solid lines) to the active site of thermolysin (lighter lines), predicted from the modeling studies.

with conversion of the Leu side chain in subsite P_1' to Phe and the Leu side chain in subsite P_2' to Ala. This structure was refined holding the heavy-atom positions of the backbone, the crystallographic waters, and the metal ions fixed. The initial structures of the complexes of Dns-Ala-Phe-Ala and Dns-Ala-Ala-Phe-Ala were created serially from this refined structure. Crystallographic waters were retained unless precluded by steric interactions with the substrates. Torsional angles were adjusted manually to produce several different starting points for each substrate. An effort was made to align hydrogen-bonding partners as observed in the crystallographic studies. These initial structures were subjected to 3–5 ps of low-temperature (100 K) molecular dynamics refinement and reminimized.

The two dansyl tripeptides were found to adopt a single and very similar family of low-energy conformations, as illustrated in Figure 12 for Dns-Ala-Phe-Ala. In particular, the backbone conformation and interactions of this substrate with thermolysin are very similar to those of the phosphinate inhibitor Cbz-Phe-PO_2-Leu-Leu. The major difference is that the bulkier dansyl group in both tripeptide substrates occupies a greater portion of the active site in the vicinity of subsites S_2 and S_3. Figure 12 also shows a number of potentially important residues that comprise the active site and that will be discussed later. Of immediate interest, however, is the location of Trp-115, the only Trp residue that is near the active site. Since the indole side chain of this Trp residue is very close (approximately 4.5 Å) to the dansyl group of the substrate, it is undoubtedly responsible for the energy transfer observed in the previous paper (Yang & Van Wart, 1994).

In contrast to the two dansyl tripeptides, Dns-Ala-Ala-Phe-Ala was found to bind in two distinct families of configurations, as shown in Figure 13. Both of these binding modes are extremely similar to those of Dns-Gly-Phe-Ala and Dns-Ala-Phe-Ala on the P_1'–P_2' side of the substrates. However, the additional Ala residue in subsite P_2 forces the dansyl group further out of the active site. Importantly, this extension of the substrate allows more flexibility in its

interactions with the enzyme with the result that the two families of binding modes illustrated in Figure 13 are allowed. In keeping with the observation described in the previous paper (Yang & Van Wart, 1994) and the efficiency of energy transfer is greater for the dansyl tripeptide than for Dns-Ala-Ala-Phe-Ala, the dansyl group in the tripeptides is closer to Trp-115 than it is in the tetrapeptide.

DISCUSSION

The effect of anions on the kinetics of hydrolysis of substrates by metalloproteinases has been a topic of intense interest, particularly for angiotensin converting enzyme (Dorer et al., 1974; Bunning & Riordan, 1983, 1987; Riordan et al., 1986; Shapiro et al., 1987; Hook, 1990) and carboxypeptidase A (Williams & Auld, 1986). While many aspects of the mechanism of thermolysin have been well characterized and a few reports have touched on the effect of salts on catalysis for individual substrates (Holmquist & Vallee, 1976; Morgan & Fruton, 1978; Li, 1982; Inouye, 1992), few detailed studies have been carried out. This has been due in part to the lack of a series of substrates with appropriately low K_M values that permitted the study of the effect of salts on the individual kinetic parameters for the reactions. The studies presented here were carried out to investigate the effect of sodium halide salts on the hydrolysis of three of the dansyl peptide substrates identified in the previous paper (Yang & Van Wart, 1994).

The effect of chloride on the thermolysin-catalyzed hydrolysis of the two tripeptides Dns-Gly-Phe-Ala and Dns-Ala-Phe-Ala shows both similarities to and differences from the effect on the tetrapeptide Dns-Ala-Ala-Phe-Ala. For all three reactions, chloride exerts its influence by binding preferentially to the ES complex and lowering K_M. The effect of this binding on the catalytic competency of the resultant ESX complex, however, is markedly different for the tripeptides vs the tetrapeptide. For Dns-Ala-Phe-Ala and Dns-Gly-Phe-Ala, k_{cat} is unaffected, leading to nonessential activation. In contrast, for Dns-Ala-Ala-Phe-Ala, k_{cat} is lowered by approximately the same factor as K_M, leading to uncompetitive inhibition. This behavior is rare in a one-substrate, one-product reaction.

The results presented here agree with many of the trends reported in earlier studies but more clearly establish the importance of the identity of the substrate in interpreting anion effects on catalysis. Holmquist and Vallee (1976) were the first to show that halide salts significantly increased the k_{cat}/K_M values for the hydrolysis of several Cbz- and FA-blocked peptides and esters. They were the first to point out that the magnitude of the effect was markedly dependent on the identity of the substrate. Thus, while there was a large effect of NaBr for substrates such as FA-Gly-Leu-NH_2, the effect was much smaller for Cbz-Gly-Phe-Ala. In agreement with the latter observation, Morgan and Fruton (1978) reported a small effect of NaBr on the rate of hydrolysis of Mns-Phe-Leu-Ala. Li (1982) subsequently studied the effect of chloride on the k_{cat} and K_M values for the hydrolysis of Dns-Ala-Ala-Phe-Ala at pH 6.0 and showed that chloride was an uncompetitive inhibitor. The results of the current study also show uncompetitive inhibition by chloride toward Dns-Ala-Ala-Phe-Ala at pH 7.5 while showing that the effect on two closely related tripeptide substrates is different. Thus, there appear to be different binding modes for different substrates. Interestingly, Inouye (1992) has recently reported very different results for the effect of chloride salts on the hydrolysis of Cbz-Asp-Phe-OCH_3. For NaCl, there is activation brought about by an increase in k_{cat} with no effect on K_M. The effect was shown to be attributable to the cation rather than to the anion. Thus, it seems likely that these different results are related to the fact that the Asp residue of the substrate has a negatively charged side chain and that its interactions with the enzyme are modulated by the cation.

Since chloride affects the thermolysin-catalyzed hydrolysis of substrates of different structure, including electrostatically neutral substrates, it is clear that its effect is the result of a direct interaction with the enzyme. Thus, the X-ray structure of thermolysin has been examined in order to search for a site of interaction for chloride. All of the side chains bearing a formal positive charge were located in the protein. Of the 11 Lys and 10 Arg residues, only Arg-203 is within 15 Å of contact with the closest portion of any of the substrates shown in Figures 12 and 13. Thus, the effects of chloride on thermolysin centered on interaction with this residue. The side chain of Arg-203 is salt-bridged to Asp-170. This latter residue is hydrogen-bonded to His-142, one of the ligands to the active-site zinc. If it is postulated that the salt bridge between Arg-203 and Asp-170 must be weakened for optimal binding of the P_n' portion of the substrate, then the effect of NaCl on the K_M values for both the dansyl tri- and tetrapeptides is easily explained. The effect of chloride is to compete with Asp-170 for the salt bridge to Arg-203 and to facilitate substrate binding, particularly on the P_n' side where all three substrates have identical structures. An inspection of the enzyme structure shows that this could be accomplished if the chloride ion were to replace water molecule 404 that is adjacent to the salt bridge. Thus, the ESX complex is more stable than either the ES or EX complexes, accounting for the reduction in K_M.

It is not possible to use the available crystallographic data to assess whether the postulated interaction of chloride ion with Arg-203 exists, since no structures have been elucidated at the concentrations ($[Cl] \gg K_{Cl}$) predicted from the kinetic studies to observe this binding. It is, however, possible to compare the crystallographic data on thermolysin in the presence and absence of inhibitors to evaluate the suggestion that substrate binding weakens the salt bridge between Asp-170 and Arg-203. In native thermolysin, the distances between the nearby guanidino nitrogen atoms of Arg-203 and the oxygen atoms of Asp-170 are 2.93 and 2.81 Å. In the crystal structures of thermolysin complexed to Cbz-Phe-PO_2-Leu-Ala, these distances are lengthened to 3.08 and 2.92 Å, respectively, while in the complex with Cbz-Gly-PO_2-Leu-Leu, the distances are 3.07 and 2.97 Å, respectively. Thus, making the reasonable assumption that these transition-state analogue inhibitors bind like substrates, the lengthening of these bonds supports the view that substrate binding weakens this salt bridge.

The differential effect of chloride on the k_{cat} values for the dansyl tripeptides vs the tetrapeptide must be related to differences in binding on the P_n side of the enzyme. The results of the molecular modeling calculations, in fact, suggest that Dns-Ala-Ala-Phe-Ala can bind in two distinct modes. It is reasonable to assume that one of the two binding modes is more productive and results in more efficient turnover of substrate, as reflected in a higher value of k_{cat}. Thus, one interpretation that is consistent with the data is that the conformation of thermolysin that is stabilized at high [NaCl] selects the less productive of the two substrate binding modes, thus lowering k_{cat}. This would not affect the k_{cat} values for the tripeptides, since they bind in a single mode.

It is of interest to compare the results discussed here for thermolysin with those for the more extensively studied

metalloproteinases. Williams and Auld (1986, 1991) have investigated the inhibitory effect of several salts on the hydrolysis of Dns-Ala-Ala-Phe by carboxypeptidase A. Changes in the identity of the cation were unimportant, but the identity of the anion markedly affected inhibition. NaCl was found to inhibit the reaction by a partially competitive mechanism throughout the pH range of 6–10. Similar effects were seen with other di- and tripeptide substrates. The inhibitory effect of chloride was attributed at least in part to its interaction with Arg-145, the recognition site for the C-terminal carboxylate anion of the substrate. Competition by chloride for binding at this site would explain the inhibition toward the substrate. In support of this proposal, chloride was shown to decrease the rate of inactivation of the enzyme by butanedione, a reagent known to modify Arg-145. Since there is no similar recognition feature for the C-terminal carboxylate anion of the substrate in thermolysin, which is an endopeptidase, the differences in anion inhibition between carboxypeptidase A and thermolysin are not surprising.

The effects of anions on angiotensin converting enzyme are more similar to those reported here for thermolysin. The activation of angiotensin converting enzyme by anions has been studied with 23 FA- and Bz-blocked tripeptides (Shapiro et al., 1983). Chloride stimulates the hydrolysis of all of these substrates at least 24-fold; however, the mechanism of stimulation, the amount of chloride required, the effect of pH on the activation, and the relative potency of various anions are all strongly dependent on the substrate employed. Three classes of substrates have been identified where class I substrates all contain ultimate (subsite P_2') or penultimate (P_1') residues with neutral or negatively charged side chains, such as FA-Phe-Gly-Gly. The hydrolysis of these substrates follows an ordered bireactant mechanism in which chloride binds before substrate. In this case, K_M is reduced and k_{cat} is unaffected. Class II substrates, such as FA-Phe-Phe-Arg, all have positively charged side chains in subsites P_1' and P_2'. For these substrates, chloride is a nonessential activator that both raises k_{cat} and lowers K_M. The class III substrates, such as FA-Lys-Ala-Phe, are similar to class I substrates, except that they have an Ala residue in subsite P_1'. Both k_{cat} and K_M are altered by chloride, but the kinetic plots are complex and do not fit a simple mechanism. Thus, while the effects of chloride are strongly substrate dependent, it is clear that chloride functions mainly to enhance substrate binding (Bunning & Riordan, 1987). This is believed to arise as the result of an interaction of chloride with a critical Lys residue on the enzyme. The results reported here for thermolysin are somewhat similar in that the effect of chloride is substrate dependent, is believed to arise from the interaction of the anion with a positively charged side chain of the enzyme, and results in enhanced substrate binding. Thus, although the bacterial and mammalian metalloproteinases share many important mechanistic features, there are very clear differences in the response of individual enzymes to specific anions.

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