Evidence for the Importance of Weakly Bound Water for Matrix Metalloproteinase Activity

ARTICLE *in* BIOCHEMISTRY · OCTOBER 1995

Impact Factor: 3.02 · DOI: 10.1021/bi00037a044 · Source: PubMed

5 AUTHORS, INCLUDING:

Ian R Phillips

Queen Mary, University of London

156 PUBLICATIONS **3,313** CITATIONS

Evidence for the Importance of Weakly Bound Water for Matrix Metalloproteinase Activity†

Frances Willenbrock,*,‡ C. Graham Knight,§ Gillian Murphy,§ Ian R. Phillips,‡ and Keith Brocklehurst‡

Department of Biochemistry, Queen Mary & Westfield College, University of London, Mile End Road, London E1 4NS, U.K., and Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K.

Received March 28, 1995; Revised Manuscript Received June 22, 1995⊗

ABSTRACT: The effects of organic cosolvents on the kinetic characteristics of two matrix metalloproteinases, gelatinase A and stromelysin 1, were investigated. In each case, addition of the cosolvent resulted in a decrease in the apparent k_{cat}/K_m for the catalyzed hydrolysis of fluorogenic peptide substrates. Two factors were identified as being responsible for this decrease in catalytic activity: hydrophobic partitioning of the substrate in favor of the bulk solvent and decrease in the water content of the enzyme. The former reflects the hydrophobic nature of the enzyme−substrate interaction and the effect can be corrected for by using the solvent to water partition coefficient of the substrate in the mixed solvent systems. The catalyzed hydrolysis of substrate, corrected for the effect of hydrophobic partitioning, was demonstrated to be sixth order in water for gelatinase A and third order in water for stromelysin 1. Variation in water concentration did not produce saturation even at concentrations close to 55.5 M. The results indicate that weakly bound water molecules are essential to mediate the interaction between substrate and enzyme. The sensitivity of these enzymes to water concentration could be an important mechanism for regulating catalytic activity *in vivo*.

The matrix metalloproteinases constitute a family of zinc-dependent endopeptidases which includes the stromelysins, gelatinases, collagenases, and matrilysin. The combined action of these enzymes results in complete hydrolysis of most of the components of the extracellular matrix (Docherty & Murphy, 1990; Woessner, 1991). Expression of the matrix metalloproteinases is tightly regulated and their activity is controlled by the general proteinase inhibitor α-macroglobulin and the specific tissue inhibitors of metalloproteinases, TIMPs.[1] This regulation is compromised in diseases that involve destruction of the connective tissue, such as in the arthritides, and in the degradation of basement membrane necessary for tumor invasion (Docherty *et al.*, 1992). Little is known about how these enzymes operate in physiological conditions. The extracellular matrix contains proteins and proteoglycans in high concentrations, giving an environment that is highly charged, viscous, and possibly with low water concentration. In addition, tissues such as cartilage and synovial fluid are exposed to extreme variation in environment whenever the joint is used. The effects on cellular metabolism are profound (Urban, 1994), and it is probable that the activity and specificity of extracellular enzymes would be influenced greatly by one or more of the factors mentioned above. It is of interest, therefore, to identify the roles of each of these factors in regulating activity. As part of a program aimed at studying the effect of the environment on these enzymes, we have investigated the effect of organic cosolvents on their kinetic characteristics. This relatively simple approach has revealed features of the matrix metalloproteinase mechanism that may ultimately be important in considering their regulation *in vivo*.

Solvent mixtures have been used to alter environmental properties, such as dielectric constant, viscosity, and water concentration, in a number of enzyme systems. In several cases the approach has demonstrated that a single factor has a predominant effect on enzyme activity and, therefore, has provided a fundamental insight into the mechanism of the enzyme. Examples include the use of mixed solvents to show the importance of solvent hydrophobicity for α-chymotrypsin (Maurel, 1978), viscosity and dielectric constant for carboxypeptidase B (Gavish & Werber, 1979), and the concentration of water for the zinc-dependent enzymes adenosine deaminase (Dzingeleski & Wolfenden, 1993) and carbonic anhydrase (Pocker & Janjić, 1989).

In the present work we describe the use of three different cosolvents (glycerol, methanol, and dimethyl sulfoxide) to investigate the mechanism of two matrix metalloproteinases, gelatinase A (EC 3.4.24.24, 72-kDa gelatinase, type IV collagenase) and stromelysin 1 (EC 3.4.24.17). These enzymes are likely to function *in vivo* in specifically adapted environments. Gelatinase A appears to bind to a cell surface receptor for activation and its function is likely to be confined to this location. Stromelysin 1 is highly expressed in cartilaginous tissues of the joint, where the presence of a high concentration of proteoglycans and large pressure fluctuations occur with joint loading.

EXPERIMENTAL PROCEDURES

The solvents DMSO, methanol, and glycerol were all purchased from BDH and were of AristaR grade. Buffers

† F.W. is supported by the Medical Research Council, U.K., and G.M. is supported by the Arthritis and Rheumatism Council, U.K.

* To whom correspondence should be addressed.

‡ University of London.

§ Strangeways Research Laboratory.

⊗ Abstract published in *Advance ACS Abstracts,* August 15, 1995.

[1] Abbreviations: DMSO, dimethyl sulfoxide; McaPLGLDpaAR, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropanoyl]-Ala-Arg-NH_2; McaPLANvaDpaAR, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Ala-Norval-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropanoyl]-Ala-Arg-NH_2 TIMP-1, tissue inhibitor of metalloproteinases 1.

0006-2960/95/0434-12012$09.00/0

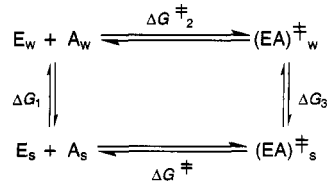
used were 50 mM HEPES, pH 7.5, containing 10 mM $CaCl_2$, 0.05% Brij 35, and the appropriate solvent. Solvent concentrations were in the range 0−30% (w/w) for DMSO and 0−45% (w/w) for methanol and glycerol. None of the solvents altered the measured pH of the buffers. The substrates (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropanoyl]-Ala-Arg-NH_2 (McaPLGLDpaAR) and (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Ala-Norval-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropanoyl]-Ala-Arg-NH_2 (McaPLANvaDpaAR) were synthesized as described previously (Knight *et al.*, 1992).

Recombinant human prostromelysin 1 was purified from the medium of C127 cells expressing this enzyme (Murphy *et al.*, 1987; Koklitis *et al.*, 1991). Recombinant progelatinase A was expressed from NSO mouse myeloma cells and purified from the conditioned medium as described previously (Murphy *et al.*, 1992). Prostromelysin 1 was activated by treatment with trypsin (10 μg/mL) for 30 min at 37 °C and progelatinase A was activated by incubation with 2 mM (4-aminophenyl)mercuric acetate for 1 h at 25 °C. Enzyme concentration was determined by titration of activated enzyme with TIMP as described previously (Willenbrock *et al.*, 1993).

Substrate Solubility Measurements. The solubilities of the substrate in the mixed solvent systems were determined by the addition of 4 μL of a concentrated solution of substrate in DMSO (approximately 50 mM) to 1 mL of the mixed solvent solution. The solutions were mixed by vortexing for 10 min at room temperature and the residual solid substrate was separated from the solution by centrifugation (650*g* for 10 min). The concentration of substrate in solution was determined by measuring the A_{410} of the supernatants (ϵ_{410} = 7500 M^{-1} cm^{-1}; Knight *et al.*, 1992).

Kinetics. Continuous fluorometric assays were performed on a Perkin-Elmer LS50B fluorometer using excitation and emission wavelengths of 328 and 393 nm, respectively. Enzyme was added to give final concentrations of 0.1−0.5 nM gelatinase A and 2−10 nM stromelysin 1 to solutions containing buffer and substrate (0.5−0.8 μM) equilibrated at 25 °C. Solutions were mixed thoroughly by repeated inversion of the cuvettes and then stirred throughout the assay. Mca-Pro-Leu was used as a standard to calibrate the fluorescence and to confirm that the presence of cosolvent did not affect the calibration. Reversibility of inhibition by the cosolvents was demonstrated by determination of the enzyme activity after the reaction mixture was diluted into buffer to give a final cosolvent concentration of <1%. The concentration of active enzyme was determined in each mixed solvent system by titration with the inhibitor TIMP-1. The substrate concentrations used were in each case considerably lower than the K_m for the reaction (which is >10 μM), allowing direct determination of k_{cat}/K_m by measurement of initial rate (v) and division by both $[E]_T$ and $[S]_0$. The specificity constant, k_{cat}/K_m (Fersht, 1985), is the apparent second-order rate constant for product formation from reaction of enzyme and substrate. It is not affected by nonproductive binding (Bender & Kezdy, 1965; Brocklehurst *et al.*, 1968), a phenomenon that can sometimes complicate mechanistic studies on reaction of proteinases with short substrates, and is a valuable parameter with which to evaluate enzyme effectiveness [see, e.g., Brocklehurst and Cornish-Bowden (1976) and Brocklehurst (1977)]. Separate values for k_{cat} and K_m could not be determined due to the low solubilities of the substrates relative to their respective K_m values.

Scheme 1



Solvent−buffer mixtures (% w/w) were made up as described above. Dielectric constants for methanol−water mixtures were interpolated from those recorded by Alerköf (1932). Values for DSMO−water and glycerol−water mixtures were from Douzou *et al.* (1975). Viscosities, cosolvent and water molarities, and freezing point depression data for methanol and glycerol mixtures were taken from published tables (Wolf *et al.*, 1989). The volumes of DMSO−water mixtures were measured in order to calculate the water and DMSO molarities in each solution.

Correction for the Effect on k_{cat}/K_m of Hydrophobic Partitioning of the Substrate in Aqueous Organic Solvents. Alteration of substrate solubility by the addition of organic cosolvents to the reaction alters k_{cat}/K_m as a result of the "solvent hydrophobic partitioning" effect (Maurel, 1978). This effect is due to the differential solvation of the substrate in the enzyme active center and the medium. Thus, when a substrate is poorly soluble in the aqueous solvent, it partitions in favor of the enzyme active center. However, if substrate solubility in the bulk solvent is increased by the addition of cosolvent, the partitioning in favor of the active center is decreased, resulting in an increase in the apparent K_m.

The effect of hydrophobic partitioning on k_{cat}/K_m can be predicted from the ratio of the solubility (i.e., equilibrium concentration) of the substrate in a given mixed solvent and that in water, which provides the value of P_S, the water to mixed solvent partition coefficient of the substrate. Scheme 1 is the thermodynamic diagram discussed by Wescott and Klibanov (1993) in connection with the prediction of the substrate specificity of subtilisin Carlsberg. E_S and A_S react in a mixed solvent system to form a transition state $(EA^{\ddagger})_S$. This transition state may be considered to be formed also from the partitioning of the free enzyme and free substrate from mixed solvent to water to produce E_W and A_W, formation of the transition state in water $(EA^{\ddagger})_W$, and finally partitioning of the transition state from water into the mixed solvent.

ΔG_1 of Scheme 1, the free energy of transfer of enzyme and substrate from mixed solvent to water, is related to the water to mixed solvent partition coefficients of the substrate (P_S) and enzyme (P_E) by

$$\Delta G_1 = RT \ln P_S + RT \ln P_E \quad (1)$$

Similarly, ΔG_3, the free energy of transfer of $EA^{\ddagger}$ from water to mixed solvent, is related to the water to mixed solvent partition coefficient of the transition state ($P_{EA^{\ddagger}}$) by

$$\Delta G_3 = -RT \ln P_{EA^{\ddagger}} \quad (2)$$

$\Delta G^{\ddagger}$ and $\Delta G^{\ddagger}_2$ are related to k_{cat}/K_m values in the mixed solvent and water, respectively, by (Fersht, 1985)

$$\Delta G^{\ddagger} = -RT \ln [(k_{cat}/K_m)_S(h/kT)] \quad (3)$$

$$\Delta G^{\ddagger}_2 = -RT \ln [(k_{cat}/K_m)_W(h/kT)] \quad (4)$$

where R is the gas constant, T is the absolute temperature, h is the Planck constant, and k is the Boltzmann constant. The free energy changes of Scheme 1 are related by

$$\Delta G^{\ddagger} = \Delta G_1 + \Delta G^{\ddagger}_2 + \Delta G_3 \quad (5)$$

Substituting the expressions for the free energy changes given in eqs 1−4 into eq 5 and dividing through by $-RT$ gives

$$\ln [(k_{cat}/K_m)_S(h/kT)] = \ln [(k_{cat}/K_m)_W(h/kT)] + \ln P_{EA^{\ddagger}} - \ln P_S - \ln P_E \quad (6)$$

As the enzyme is considerably larger than the substrate, it can be assumed that the solubility of the enzyme is not altered significantly by combination with substrate, i.e., $P_E \approx P_{EA^{\ddagger}}$, and eq 6 can be simplified, therefore, to

$$(k_{cat}/K_m)_W = P_S(k_{cat}/K_m)_S \quad (7)$$

RESULTS

Each organic cosolvent decreased the catalytic activity (k_{cat}/K_m) of both gelatinase A and stromelysin 1 (Figure 1A,B). The effect was reversible and did not appear to affect zinc binding at the enzyme active site, as demonstrated by the lack of protection by added zinc (data not shown). Titration of both enzymes with TIMP-1 in each mixed solvent system demonstrated that the concentration of active enzyme was not altered by the addition of the cosolvent. To permit direct comparison of the effect of cosolvent on the kinetics of the catalyzed hydrolysis of the two substrates, the data were plotted as ratios of values of k_{cat}/K_m in the presence and in the absence of cosolvent. Absolute values of k_{cat}/K_m in the absence of cosolvent are given in the caption to Figure 1. When these ratios were plotted, most of the data for one substrate were found to overlay the data for the other. The only exception relates to the effect of methanol on the activity of stromelysin 1 (Figure 1B), where k_{cat}/K_m for the catalyzed hydrolysis of McaPLANvaDpaAR is more sensitive to the presence of methanol than the corresponding parameter for McaPLGLDpaAR.

Matrix metalloproteinase substrates are hydrophobic and it was anticipated that an important effect of the organic cosolvents would be to alter substrate solubility. The solubilities of both substrates were determined, therefore, in each mixed solvent system and across the range of organic cosolvent concentrations used. The effects of the three cosolvents on solubility were similar for both substrates and are exemplified in Figure 2 for McaPLGLDpaAR in the form of a semilogarithmic plot. Glycerol and methanol have little effect on the substrate solubility, whereas DMSO increases the solubility more markedly. Altered substrate solubility in the mixed solvent systems can result in "hydrophobic partitioning" (Maurel, 1978; see Experimental Procedures), which affects the value of k_{cat}/K_m as predicted by eq 7 in the Experimental Procedures section. Equation 7 shows that if hydrophobic partitioning is solely responsible for the effect of organic cosolvents on the kinetics, the value of $(k_{cat}/K_m)_W$ can be calculated from the values of $(k_{cat}/K_m)_S$ and P_S for a given mixed solvent system. Clearly the value of $(k_{cat}/K_m)_W$

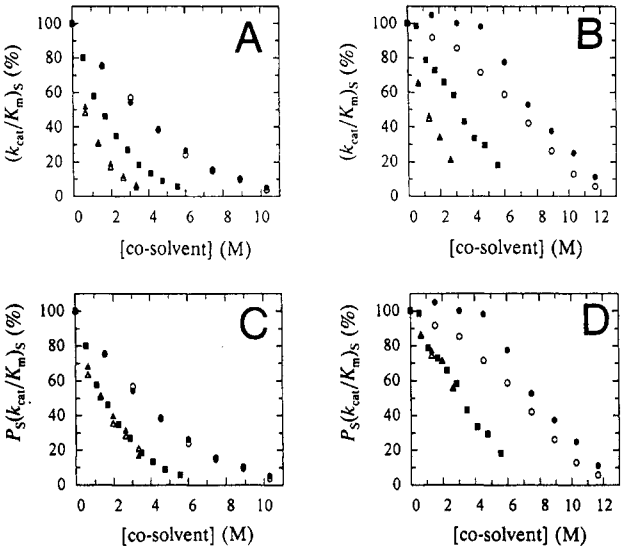


FIGURE 1: Dependence of the apparent k_{cat}/K_m in mixed solvent systems on the molarity of cosolvent for (A) gelatinase A and (B) stromelysin 1. Similar plots are given in (C) and (D) for gelatinase A and stromelysin 1, respectively, showing data that have been corrected for the hydrophobic effect. Closed and open symbols are used for data obtained using the substrates McaPLGLDpaAR and McaPLANvaDpaAR, respectively. Cosolvents were (○, ●) methanol, (■) glycerol, and (▲, △) DMSO. Values for k_{cat}/K_m are expressed as a percentage of those obtained in the absence of cosolvent, which are 6.3×10^5 M^{-1} s^{-1} and 3.5×10^5 M^{-1} s^{-1} for the gelatinase A-catalyzed hydrolysis of McaPLGLDpaAR and McaPLANvaDpaAR, respectively, and 7.4×10^3 M^{-1} s^{-1} and 2.8×10^4 M^{-1} s^{-1} for the stromelysin 1-catalyzed hydrolysis of McaPLGLDpaAR and McaPLANvaDpaAR, respectively.

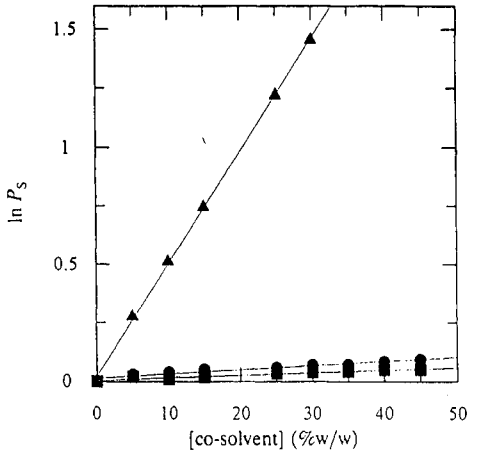


FIGURE 2: Solubility of the substrate McaPLGLDpaAR in mixed solvent systems containing (●) methanol, (■) glycerol, and (▲) DMSO. Solubility is expressed as the natural logarithm of P_S, the ratio of the solubility in the mixed solvent system to that in the aqueous buffer alone.

is independent of the organic cosolvent content. For eq 7 to be in accord with this requirement, therefore, changes in P_S and $(k_{cat}/K_m)_S$ must compensate exactly as the organic cosolvent content is varied. The marked decreases in the values of $P_S(k_{cat}/K_m)_S$ with increases in organic cosolvent molarity shown in panels C and D of Figure 1, for gelatinase A and stromelysin 1, respectively, are not predicted by eq 7. This demonstrates that hydrophobic partitioning alone cannot account for the observed solvent effects.

In an attempt to ascertain whether a common factor, either alone or in combination with hydrophobic partitioning, is responsible for the effect of the three cosolvents on the

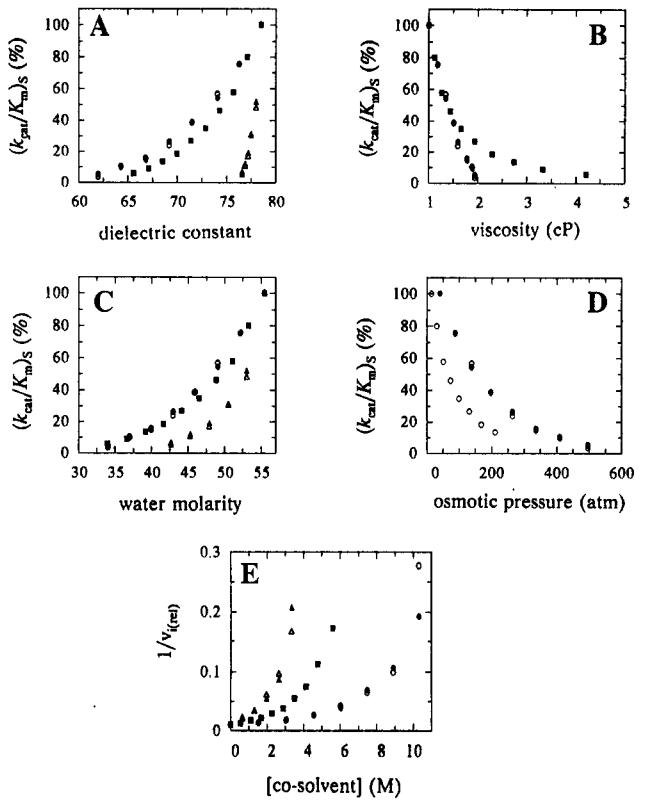


FIGURE 3: Dependence of the apparent k_{cat}/K_m for the hydrolysis of McaPLGLDpaAR (closed symbols) and McaPLANvaDpaAR (open symbols) catalyzed by gelatinase A in mixed solvent systems on (A) dielectric constant, (B) viscosity, (C) water molarity, and (D) osmotic pressure. The dependence of $1/v_{i(rel)}$ on solvent molarity is given in (E). Cosolvents were (○, ●) methanol, (■) glycerol, and (▲, △) DMSO.

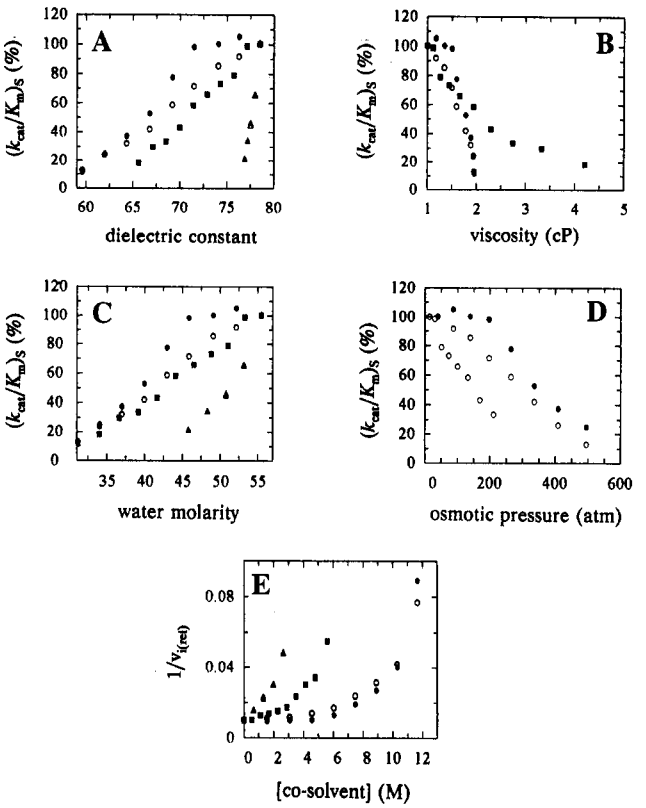


FIGURE 4: Dependence of the apparent k_{cat}/K_m for the hydrolysis of McaPLGLDpaAR (closed symbols) and McaPLANvaDpaAR (open symbols) catalyzed by stromelysin 1 in mixed solvent systems on (A) dielectric constant, (B) viscosity, (C) water molarity, and (D) osmotic pressure. The dependence of $1/v_{i(rel)}$ on solvent molarity is given in (E). Cosolvents were (○, ●) methanol, (■) glycerol, and (▲, △) DMSO.

kinetic behavior of the two metalloproteinases, various potential correlations were assessed by the plots shown in Figures 3–6. Figures 3 and 4 show plots of $(k_{cat}/K_m)_S$ against (A) dielectric constant, (B) viscosity, (C) water concentration, and (D) osmotic pressure for gelatinase A and stromelysin 1, respectively. The data sets do not cluster for all three organic cosolvents in any of these plots, demonstrating that when hydrophobic partitioning is not corrected for, no single factor is responsible for the decrease in catalytic activity of the two enzymes. Figures 3E and 4E are plots of $1/v_{i(rel)}$ against [cosolvent] for gelatinase A and stromelysin 1, respectively, and are equivalent to Dixon plots of $1/v_i$ against [inhibitor]. Such plots are linear for simple reversible inhibition. The plots in Figures 3E and 4E are markedly curved, showing that simple linear reversible inhibition does not explain the effects of the added cosolvent on the kinetics.

In Figures 5 and 6 plots of $P_S(k_{cat}/K_m)_S$ for the two enzymes were used to investigate the various effects referred to above when correction for hydrophobic partitioning is applied. Curvature in the plots of $1/P_S v_{i(rel)}$ against [cosolvent] in Figures 5E and 6E again demonstrates that the cosolvents do not behave as simple reversible inhibitors. Of the various plots shown in Figures 5 and 6, it is only those in Figures 5C and 6C, of $P_S(k_{cat}/K_m)_S$ against water concentration, in which the data points cluster for all three organic cosolvents. Thus, both the solvent hydrophobicity and the concentration of water are responsible for the effect of the cosolvents. The only exception is the data set for the stromelysin 1-catalyzed hydrolysis of McaPLGLDpaAR in the presence of methanol (Figure 6C), where activity is independent of water concentration down to a concentration of 45 M. At water concentrations below 45 M, the activity decreases sharply until the values cluster with those of the other data sets at concentrations below 35 M. Possible explanations for this observation are discussed in the Discussion section.

Analysis of the Effect of Water Molarity on k_{cat}/K_m. The results in Figures 5C and 6C for gelatinase A and stromelysin 1 suggest that the extent of hydration of these enzymes is important for their catalytic competence. The data sets, particularly for gelatinase A (Figure 5C), show upward curvature in the plot of $P_S(k_{cat}/K_m)_S$ against water molarity. This demonstrates the need for more than one water molecule per molecule of enzyme for optimal catalytic activity. Separate analysis of K_m and k_{cat} was prevented by insolubility of the substrates. Thus it is not possible on the basis of this data to determine whether the water is essential for catalysis or substrate binding. The values of $P_S(k_{cat}/K_m)_S$ used to provide the relative values used in Figure 5C (and in Figure 6C) were computed as follows. The initial rate $v_{i(S)}$ measured in a given mixed solvent system was shown to increase linearly with enezyme concentration $[E]_T$ and with substrate concentration $[A]_0$. Thus the range of values of $[A]_0$ used were much less than the values of $K_{m(A)}$, and therefore $(k_{cat}/K_m)_S$ could be calculated by using eq 8 and corrected for hydrophobic partitioning by using eq 9:

$$v_{i(S)} = (k_{cat}/K_m)_S[E]_T[A]_0 \quad (8)$$

$$(k_{cat}/K_m)_{obs(corr)} = P_S(k_{cat}/K_m)_S \quad (9)$$

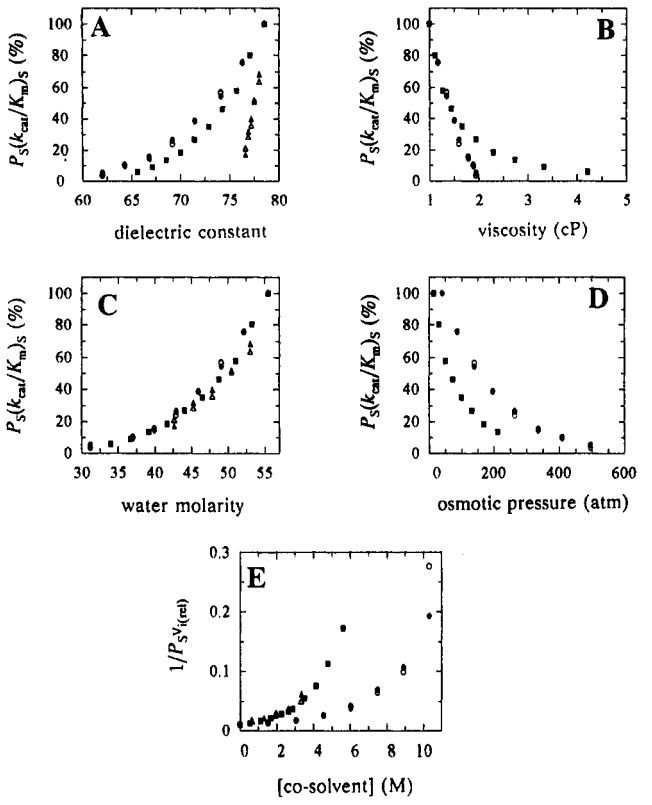


FIGURE 5: Dependence of the apparent k_{cat}/K_m corrected for the hydrophobic effect, $P_S(k_{cat}/K_m)_S$, for the gelatinase A-catalyzed hydrolysis of McaPLGLDpaAR (closed symbols) and McaPLANvaDpaAR (open symbols) on (A) dielectric constant, (B) viscosity, (C) water molarity, and (D) osmotic pressure. The dependence of $1/P_S v_{i(rel)}$ on solvent molarity is given in (E). Cosolvents were (○, ●) methanol, (■) glycerol, and (▲, △) DMSO.

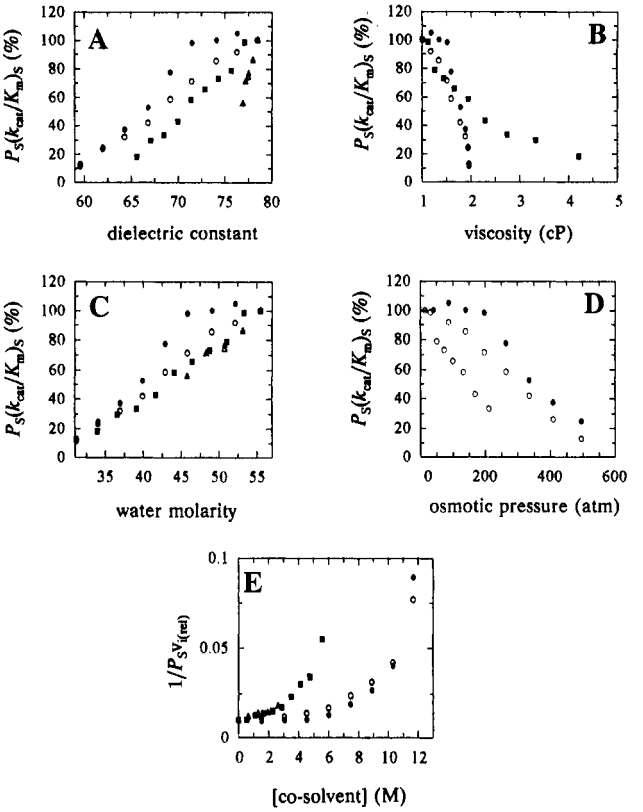


FIGURE 6: Dependence of the apparent k_{cat}/K_m corrected for the hydrophobic effect, $P_S(k_{cat}/K_m)_S$, for the stromelysin 1-catalyzed hydrolysis of McaPLGLDpaAR (closed symbols) and McaPLANvaDpaAR (open symbols) on (A) dielectric constant, (B) viscosity, (C) water molarity, and (D) osmotic pressure. The dependence of $1/P_S v_{i(rel)}$ on solvent molarity is given in (E). Cosolvents were (○, ●) methanol, (■) glycerol and (▲, △) DMSO.

Values of initial rate corrected for hydrophobic partitioning ($v_{i(corr)}$) may be calculated by multiplying $v_{i(S)}$ by P_S and related to reactant concentrations by eq 10 or, with water shown explicitly, by eq 11:

$$v_{i(corr)} = (k_{cat}/K_m)_{obs(corr)}[E]_T[A]_0 \quad (10)$$

$$v_{i(corr)} = k[E]_T[A]_0[H_2O]^n \quad (11)$$

Equation 11 assumes that $[H_2O] \ll K_{m(H_2O)}$, which appears to be a reasonable assumption in view of the lack of evidence for saturation in Figure 5 even at values of $[H_2O]$ close to 55.5 M. To determine the order of reaction in H_2O, $v_{i(corr)}/[E]_T[A]_0$ (i.e., $(k_{cat}/K_m)_{obs(corr)}$) is plotted against (water molarity)^n (Figure 7) for various values of n to determine the value that provides a linear relationship. Panels A and B of Figure 7 show upward curvature in the plots with n = 1, 3, or 5, downward curvature for n = 7 and 12, and a linear relationship for n = 6. Analogous plots for stromelysin 1 give a linear relationship when n = 3. Thus six water molecules are required for optimal catalytic activity for gelatinase A and three water molecules are required for stromelysin 1. These water molecules all have a low affinity for the enzyme with K_m values of >55.5 M. The free enzymes, therefore, exist in a partially dehydrated form in aqueous solution, a phenomenon discussed by Dzingelski and Wolfenden (1993) in connection with their studies on adenosine deaminase.

DISCUSSION

As an initial stage in our investigation of the influence of the molecular environment on matrix metalloproteinase activity, the effects of three different organic cosolvents on k_{cat}/K_m for the hydrolysis of two synthetic fluorescent substrates catalyzed by stromelysin 1 and gelatinase A were studied. The criteria used in the choice of cosolvents in the present work were (i) the kinetic effects of the cosolvent must be fully reversible, (ii) the cosolvent must not act as chelator for zinc or calcium, as both are essential for activity, and (iii) the different cosolvents should have different physicochemical characteristics to provide opportunities to discover the origins of the solvent effects on the kinetics, in particular, whether there is a multiplicity of effects and/or an effect common to different cosolvent types.

In the present work the use of cosolvents has permitted the identification of two factors that are important in determining the catalytic activity of both gelatinase A and stromelysin 1, namely, solvent hydrophobicity and the water concentration. The basis of the solvent hydrophobicity effect was described by Maurel (1978), who demonstrated that for both α-chymotrypsin and trypsin the changes observed in K_m/k_{cat} reflected those in K_s, the dissociation constant for the enzyme−substrate complex. The effect occurs because when the substrate is nonpolar and thus poorly soluble in aqueous medium, it partitions in favor of a hydrophobic enzyme center. However, when the addition of cosolvent increases substrate solvation in the bulk solvent, the parti-

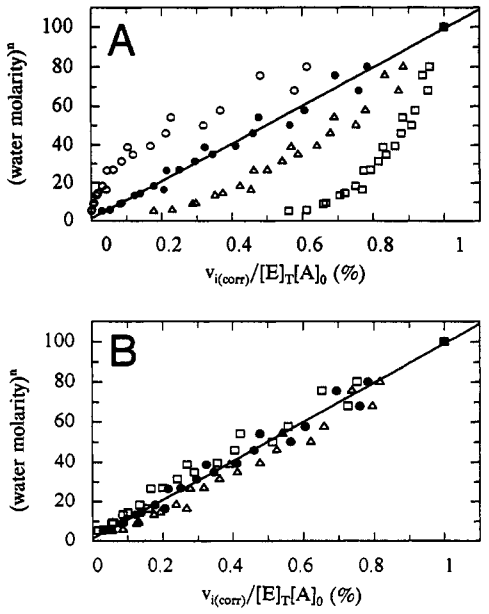


FIGURE 7: Determination of the order of reaction in water for the gelatinase A-catalyzed hydrolysis of McaPLGLDpaAR and McaPLANvaDpaAR. Data from each solvent system and for both substrates are included. Values for $v_{i(corr)}/[E]_T[A]_0$ are expressed as a percentage of the value in the absence of cosolvent. Values for (water molarity)^n are expressed as a fraction of the value in the absence of cosolvent, i.e., $(55.5)^n$. (A) Data sets are for $n = 1$ (□), $n = 3$ (△), $n = 6$ (●), and $n = 12$ (○), and the straight line is fitted to the data for $n = 6$. (B) Data sets are for $n = 5$ (△), $n = 6$ (●), and $n = 7$ (□), and the straight line is fitted to the data for $n = 6$.

tioning in favor of the active center is decreased. It seems reasonable to view the hydrophobic partitioning observed in the present work as having the same basis although this cannot be demonstrated because the low solubilities of the substrates did not permit K_m and k_{cat} to be determined separately.

With the exception of the stromelysin 1-catalyzed hydrolysis of McaPLGLDpaAR in methanol−buffer mixtures, the catalytic activity of both gelatinase A and stromelysin 1 is very sensitive to the concentration of water in the assay, with no indication of saturation of catalytic activity at the highest concentration of 55.5 M. By plotting $v_{i(corr)}/[E]_T[A]_0$ against (water molarity)^n for several values of n, it was possible to establish that the reaction is sixth order in water for gelatinase A and third order in water for stromelysin 1. Although these weakly bound water molecules could occupy sites anywhere on the enzyme molecule, the simplest view is that they may be bound within the active center. Definition of the role of this water is obscured because water not only is important for solvation of the enzyme and substrate but also is involved in the hydrolysis reaction. It has generally been considered that the catalytic water in zinc metalloproteinases, as typified by the bacterial enzyme thermolysin, is bound tightly to the zinc atom and remains in place throughout the catalytic cycle. However, a recently proposed mechanism for thermolysin demands that the zinc-bound water molecules must be displaced before catalysis can occur and is not involved in the reaction (Mock & Aksamawati, 1994). In addition, solvent isotope partitioning studies on thermolysin suggest that the catalytic water molecule is not tightly associated with the enzyme during catalysis (Angeles *et al.*, 1992). Thus it is conceivable that the binding of the catalytic water is sensitive to changes in the water concentration, although this would account for only one of the required water molecules unless the water molecule directly involved in the catalytic act needs to be associated with a network of additional water molecules. Another explanation is that the water molecules are required to mediate the binding of substrate to enzyme. Such a dependence on correctly positioned low-affinity solvent water for ligand binding has been suggested previously for enzyme reactions, such as adenosine deaminase (Dzingeleski & Wolfenden, 1993) and carbonic anhydrase (Pocker & Janjić, 1989), and for antibody−antigen binding, as in the case of the association of the Fv fragment of an anti-lysozyme antibody with lysozyme (Bhat *et al.*, 1994).

The lack of a solvent effect when methanol is used as cosolvent at concentrations up to approximately 5 M in the stromelysin 1-catalyzed hydrolysis of McaPLGLDpaAR suggests that the methanol can substitute for at least one of the water molecules. The observation that the lack of cosolvent effect is observed only with the substrate McaPLGLDpaAR suggests that the water may be involved in binding either the Gly or the Leu residue, which bind to the P_1 and P_1' sites, respectively, as these are the two positions in which the substrates differ. The poorer substrate (McaPLGLDpaAR) has an additional methyl group in the P_1' position (leucine instead of norvaline) but lacks a methyl group in the P_1 position (glycine instead of alanine). Thus it seems probable that the methanol in the mixed solvent could bind in the P_1 subsite to fill the pocket occupied by the alanine side chain in the McaPLANvaDpaAR substrate.

The sensitivity of gelatinase A and stromelysin 1 to water concentration suggests that water may play an important physiological role in regulating matrix metalloproteinase activity *in vivo*. The enzymes have a broad specificity and are capable of accommodating a variety of residues in their extended substrate binding sites. Thus it is likely that the recruitment of water at the interface of the enzyme and substrate observed in the present work for synthetic peptide substrates may be essential also for the hydrolysis of physiological protein substrates. The concentration of water in the extracellular matrix is unknown but is probably considerably lower than 55.5 M (Carney & Muir, 1988; Hardingham & Fosang, 1992), and therefore, enzyme activity would be substantially below maximum and thus sensitive to small changes in water concentration. It is noteworthy that the catalytic activity correlates with water concentration rather than with the activity of the water (which is related to osmotic pressure, panels D in Figures 3−6). Such an effect has been observed previously (Dzingeleski and Wolfenden, 1993; Pocker and Janjić, 1989; Bell and Critchlow, 1971) and it was proposed that the activity coefficients of the critical water molecules remain the same throughout the reaction. In the present study small peptide substrates were used to probe an open and extended enzyme active center which is readily accessible to the low M_r organic cosolvents present. This situation differs, therefore, from that in many osmotic stress experiments, where a protein−protein interface can be regarded as a semipermeable membrane (Rand and Parsegian, 1989; Kornblatt *et al.*, 1993). The catalysis of the hydrolysis of larger protein substrates by matrix metalloproteinases would also be expected to result in formation of a protein−protein interface which would effectively alter the activity of the water in the surrounding medium. In this case it might be expected that the requirement for water at

the interface would be reflected in a dependence on the osmotic pressure of the system. Future experiments are planned which will use the osmotic stress technique (Parsegian *et al.*, 1986) to assess both the role of osmotic pressure and the numbers of water molecules involved in the optimal processing of protein substrates.

REFERENCES

Alerköf, G. (1932) *J. Am. Chem. Soc. 54*, 4125−4139.

Angeles, T. S., Roberts, G. A., Carr, S. A., & Meek, T. D. (1992) *Biochemistry 31*, 11778−11784.

Bell, R. P., & Critchlow, J. E. (1971) *Proc. R. Soc. London A325*, 35−45.

Bender, M. L., & Kezdy, F. J. (1965) *Annu. Rev. Biochem. 34*, 49−76.

Bhat, T. N., Bentley, G. A., Boulot, G., Greene, M. I., Tello, D., Dall'Aqua, W., Souchon, H., Schwarz, F. P., Mariuzza, R. A., & Poljak, R. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1089−1093.

Brocklehurst, K. (1977) *Biochem. J. 163*, 111−116.

Brocklehurst, K., & Cornish-Bowden, A. (1976) *Biochem. J. 159*, 165−166.

Brocklehurst, K., Crook, E. M., & Wharton, C. W. (1968) *FEBS Lett. 2*, 69−73.

Carney, S. L., & Muir, H. (1988) *Physiol. Rev. 68*, 858−910.

Docherty, A. J. P., & Murphy, G. (1990) *Ann. Rheum. Dis. 49*, 469−479.

Docherty, A. J. P., O'Connell, J. P., Crabbe, T., Angal, S., & Murphy, G. (1992) *Trends Biotechnol. 10*, 200−207.

Douzou, P., Hui Bon Hoa, G., Maurel, P., & Travers, F. (1975) in *CRC Handbook of Biochemistry & Molecular Biology* (Fasman, G. D., Ed.) Vol. I, pp 520−528, CRC Press, Cleveland, OH.

Dzingeleski, G. D., & Wolfenden, R. (1993) *Biochemistry 32*, 9143−9147.

Fersht, A. (1985) in *Enzyme Structure and Mechanism*, 2nd ed., Chapter 10, Freeman, New York.

Gavish, B., & Werber, M. M. (1979) *Biochemistry 18*, 1269−1275.

Hardingham, T. E., & Fosang, A. J. (1992) *FASEB J. 6*, 861−870.

Knight, C. G., Willenbrock, F., & Murphy, G. (1992) *FEBS Lett. 296*, 263−266.

Koklitis, P., Murphy, G., Sutton, C. S., & Angal, S. (1991) *Biochem. J. 276*, 217−221.

Kornblatt, J. A., Kornblatt, J. M., Hui Bon Hoa, G., & Mauk, A. G. (1993) *Biophys. J. 65*, 1059−1065.

Maurel, P. (1978) *J. Biol. Chem. 253*, 1677−1683.

Mock, W. L., & Aksamawati, M. (1994) *Biochem. J. 302*, 57−68.

Murphy, G., Cockett, M. I., Stephens, P. E., Smith, B. J., & Docherty, A. J. P. (1987) *Biochem. J. 248*, 265−268.

Murphy, G., Willenbrock, F., Ward, R. V., Cockett, M. I., Eaton, D., & Docherty, A. J. P. (1992) *Biochem. J. 283*, 637−641.

Parsegian, V. A., Rand, R. P., Fuller, N. L. & Rau, D. C. (1986) *Methods Enzymol. 127*, 400−416.

Pocker, Y., & Janjić, N. (1989) *J. Am. Chem. Soc. 111*, 731−733.

Rand, R. P., & Parsegian, V. A. (1989) *Biochim. Biophys. Acta 988*, 351−376.

Urban, J. P. G. (1994) *Br. J. Rheumatol. 33*, 901−908.

Wescott, C. R., & Klibanov, A. M. (1993) *J. Am. Chem. Soc. 115*, 1629−1631.

Willenbrock, F., Crabbe, T., Slocombe, P. M., Sutton, C. W., Docherty, A. J. P., Cockett, M. I., O'Shea, M., Brocklehurst, K., Phillips, I. R., & Murphy, G. (1993) *Biochemistry 32*, 4330−4337.

Woessner, J. F. (1991) *FASEB J. 5*, 2145−2154.

Wolf, A. V., Brown, M. G., & Prentiss, P. C. (1989) in *CRC Handbook of Chemistry and Physics* (Weast, R. T., Ed.) pp D221−D271, CRC Press, Boca Raton, FL.

BI950706J