order rate constants k_{obsd}, which were divided by a_{OL} to produce the apparent second-order rate constants. The Guggenheim treatment utilized data from at least three half-lives; the constant difference between readings taken at a series of times and a series of times later was selected to be approximately two half-lives.

Determinations of the β-D KIE's utilized the same stock buffer, and the labeled and unlabeled esters were run in alternation; 6–8 H/D pairs of rate constants were obtained at each temperature. For the proton inventory, each determination of k_n was followed by determination of a k_0. Experiments for a proton inventory curve were completed within a 10-h period. The results from three inventories were averaged to give the final isotope effects.

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Transition-State Properties for the Association of α-1-Protease Inhibitor with Porcine Pancreatic Elastase

Ross L. Stein

Contribution from the Pulmonary Pharmacology Section, Biomedical Research Department, Stuart Pharmaceuticals, A Division of ICI Americas Inc., Wilmington, Delaware 19897.
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Abstract: The proton inventory (rate measurements in mixtures of H_2O and D_2O) was determined for the association of human α-1-protease inhibitor (αPI) with porcine pancreatic elastase (PPE). The overall solvent deuterium isotope effect $[^D(k_E)]$ is 0.91 ± 0.06. As simpler models of this reaction, the proton inventories were also determined for the reaction of PPE with Suc-(Ala)$_3$-pNA $[^D(k_E) = 2.02 \pm 0.09]$ and human leukocyte elastase (HLE) with MeOSuc-Ala-Ala-Pro-Val-pNA $[^D(k_E) = 1.51 \pm 0.04]$. For all three reactions, the general shape of the proton inventory was dome-shaped, and, in fact, for the association of αPI and PPE, the curve displayed a maxima. These curves could be described by the single expression

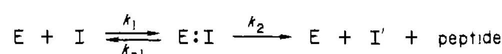
$$k_{E,n}/k_{E,0} = Z^n\left[\frac{C_1}{\Phi_1{}^n} + \frac{C_2}{(1 - n + n\phi_2)^2}\right]^{-1}$$

where n is the mole fraction solvent D_2O, Z is a composite fractionation factor corresponding to solvent reorganization and general "medium effects", C_1 and C_2 are the contributions made by each of the two partially rate-limiting transition states of k_E, a physical step (C_1), and the chemical steps of acylation (C_2), Φ_1 is a composite fractionation factor corresponding to the transition state for the physical step, and ϕ_2 is one of two identical fractionation factors corresponding to the two exchangeable protonic sites of the charge-relay system. These experiments suggest that the association of αPI and PPE proceeds through a transition state that derives its stability in part from some sort of protolytic catalysis and may therefore be at least partially rate-limited by acylation. In addition, these results point out the general importance of solvent reorganization in associative processes of enzymic reactions.

α-1-Protease inhibitor[1] is a glycoprotein of molecular weight near 53 000 that inhibits, with varying degrees of potency, all serine proteases. The minimal kinetic mechanism that can describe reactions of αPI[2] and proteases is in Scheme I. According to this view, the rapid association of protease and inhibitor ($k_1 = 10^5$–10^7 M^{-1} s^{-1}) produces a stable complex (E:I) that is resistant to both dissociation ($k_{-1} = 10^{-4}$–10^{-6} s^{-1}) and hydrolysis ($k_2 = 10^{-5}$–10^{-7} s^{-1}). Interaction of the two molecules involves the enzyme's active site and a portion of the inhibitor known as the "reactive center" and results in a single, specific cleavage at Met 358 of the inhibitor. The products of the hydrolytic decomposition of E:I are free, active enzyme, inactivated inhibitor (I′), and a small polypeptide.

The central feature of the mechanism depicted in Scheme I is E:I.[3] Considering its great stability, this complex is of considerable interest. Likely candidates for E:I are the various intermediates that normally occur during protease-catalyzed acyl-transfer reactions.[1b] These species include tetrahedral intermediates (TI),

Scheme I



resulting from attack of the active site serine on the carbonyl carbon of the targeted Met residue, acyl-enzymes, formed upon collapse of tetrahedral addition adducts, and possibly even simple noncovalent complexes, if they can be sufficiently stabilized by van der Waals interactions, hydrogen bonds, and salt bridges. A goal of protease chemistry is a structural elucidation of E:I and an understanding of the origin of this intermediate's stability. An experimental strategy for addressing the latter problem emerges if we make the assumption that interactions stabilizing E:I are first brought to bear in the transition state for its formation. Thus, studying the transition state of k_1 could lead to an understanding of how E:I is stabilized and possibly the structure of this intermediate. To this end, kinetic studies were conducted for the association[4] of αPI and the protease, porcine pancreatic elastase. Also, to serve as models of this process, kinetics were determined

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(2) Abbreviations: αPI, human plasma α-1-protease inhibitor; PPE, porcine pancreatic elastase; HLE, human leukocyte elastase; Suc, *N*-succinyl; MeOSuc, *N*-methoxysuccinyl, pNA, *p*-nitroanilide.

(3) It is likely that the interaction of α-1-protease inhibitor with serine proteases proceeds through the intermediacy of complexes other than just E:I. These would include loose Michaelis-type complexes and possibly a post-hydrolysis complex of protease and inactive inhibitor. At the present time, however, there is little evidence to support the existence of such intermediates.

(4) Throughout this paper, terms such as "association reactions" or "associative processes" refer to second-order reactions of enzyme with substrate or αPI. These processes are governed by the rate constants k_c/K_m or k_1 (see Scheme I), respectively, and reflect the energy difference between the reactants free in solution and the transition state of highest energy preceding the first irreversibly formed intermediate. Binding of substrate or inhibitor to the protease, conformational changes of initially formed encounter complexes, and enzyme acylation will all make contributions, with varying degrees of importance, to the rate determination of these associative reactions.

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for the association of PPE and HLE with low molecular weight synthetic substrates.

Experimental Section

MeOSuc-Ala-Ala-Pro-Val-pNA and Suc-$(Ala)_3$-pNA were available from previous studies.[5] Buffer salts and Me_2SO were analytical grade from several sources. D_2O (99%) was purchased from Sigma Chemical Co. Buffer solutions in H_2O and D_2O were prepared as outlined previously.[5]

PPE Solutions. Porcine pancreatic elastase was obtained from Elastin Products, Pacific, MO, as a chromatographically purified, salt-free lyophilized powder. Stock solutions were prepared in 1 mM HCl and were found to be stable for weeks if stored at 4 °C.

HLE Solutions. Human leukocyte elastase was purchased from Elastin Products, Pacific, MO. The material was purified from purulent sputum as previously described[6] and supplied as a salt-free lyophilized powder. Stock solutions of HLE (0.1 mg/mL) were prepared in 50 mM acetate, 500 mM NaCl, pH 5.5 buffer. Concentration of active enzyme was determined from established kinetic parameters for the hydrolysis of MeOSuc-Ala-Ala-Pro-Val-pNA.[5]

αPI Solutions. αPI from human plasma was obtained from Sigma Chemical Co. (α-1-antitrypsin, A6150) as a partially purified, salt-free lyophilized powder and was found to be only 10% active by titration against bovine trypsin and HLE. To purify this material further, it was dissolved to a concentration of 7 mg/mL in 50 mM Tris, pH 8.0 buffer containing 1 mM mercaptoethanol (buffer A) and 0.5 mL of the resultant solution applied to a Mono Q, anion-exchange column of an FPLC chromatography system (Pharmacia). The column was developed with 40 mL of a linear 0.1–1.0 M NaCl gradient in buffer A at a flow rate of 2 mL/min. Fractions (0.5 mL) were collected. Active αPI eluted as a single, 1.0-mL peak at a [NaCl] = 0.25 M and was followed at [NaCl] = 0.35 M by a large peak of contaminating protein. By titration with trypsin and HLE, the αPI fraction was found to be at least 90% active. This solution was used immediately in the kinetic experiments.

Kinetic Procedures. Values of k_{obsd}, the pseudo-first-order rate constant for the inactivation of PPE by αPI, were obtained by monitoring the first-order decay of *p*-nitroaniline production in reaction mixtures containing of PPE, Suc-$(Ala)_3$-pNA, and αPI according to a method similar to that of Tian and Tsou.[7] In a typical experiment, 50 μL each of substrate and αPI solution was added to a cuvette containing 2.88 mL of buffer (0.10 M phosphate, 0.50 M NaCl, pH 7.6). The cuvette was placed in a jacketed holder in the cell compartment of a Cary 210 spectrophotometer and the reaction solution allowed to reach thermal equilibrium (10–20 min). The temperature was maintained by water circulated from a Lauda K-2/RD bath. Injection of 20 μL of enzyme solution initiated the reaction. The absorbance at 410 nm, due to the release of *p*-nitroaniline, was continuously measured, digitized, averaged, and stored in an Apple II microcomputer.

First-order rate constants were determined by iterative fit of the data to the linearized exponential function

$$\ln (A_\infty - A_t) = -k_{obsd}t + \ln (A_\infty - A_0) \qquad (1)$$

where A_∞ is the absorbance at infinite time, A_t is the absorbance at time t, A_0 is the initial absorbance, and k_{obsd} is the observed first-order rate constant. The parameters optimized were A_∞, $A_\infty - A_0$, and k_{obsd}. Data were collected for no less than three half-times and frequently for more than five half-times to demonstrate the complete inactivation of PPE.

k_1, the second-order association rate constant, is related to k_{obsd} according to

$$k_{obsd} = k_1[\alpha PI]/(1 + [S]/K_m) \qquad (2)$$

In the present case, [S] was at a concentration much less than K_m ([S] = K_m/12 = 0.4 mM) and thus allowed the simplified expression of eq 3 to be used.

$$k_{obsd} = k_1[\alpha PI] \qquad (3)$$

Results

Values of k_1 for the inactivation of PPE by αPI were determined in various mixtures of H_2O and D_2O. These data, constituting a "proton inventory"[8] of k_1, are shown in Figure 1 as a plot of $k_{E,n}/k_{E,0}$ vs. n, the mole fraction of solvent deuterium. k_E[9] is used here to denote the second-order rate constant for the association of enzyme with either inhibitor or substrate, where it is equivalent to k_1 or k_c/K_m, respectively. The proton inventory of this reaction is dome-shaped ("spills water") with an overall solvent isotope effect $[^D(k_1)]$ of 0.91 ± 0.06.

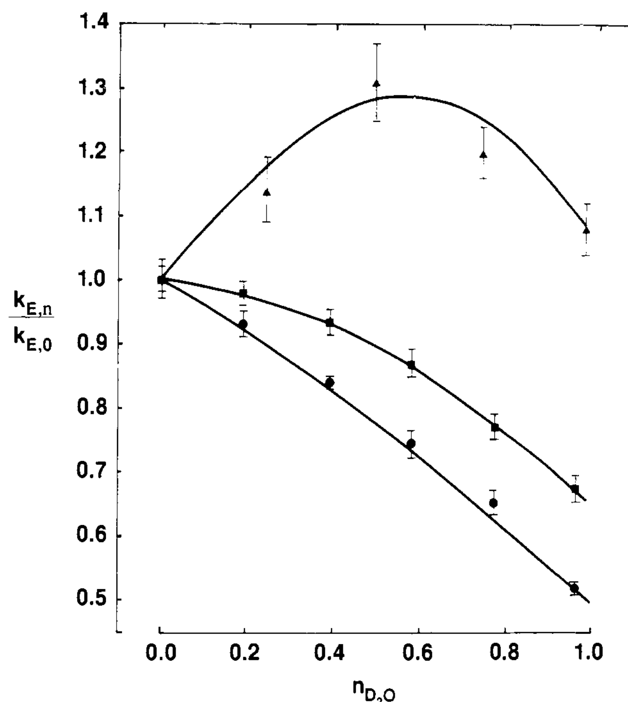

Figure 1. Proton inventories of k_E: (▲) association of α-1-protease inhibitor (αPI) with procine pancreatic elastase (PPE). $[\alpha PI]_0$ = 120 nM, $[PPE]_0$ = 12 nM; $k_{E,0}$ = 114 000 ± 2500 M^{-1} s^{-1}; (■) hydrolysis of MeOSuc-Ala-Ala-Pro-Val-pNA by human leukocyte elastase (HLE). $[S]_0$ = 2 μM = K_m/25, $[HLE]_0$ = 57 nM; $k_{E,0}$ = 178 000 ± 3500 M^{-1} s^{-1}. (●) hydrolysis of Suc-$(Ala)_3$-pNA by PPE. $[S]_0$ = 230 μM = K_m/20, $[PPE]_0$ = 69 nM; $k_{E,0}$ = 4900 ± 110 M^{-1} s^{-1}. Reactions were conducted at 25.0 ± 0.1 °C in solutions of 0.10 HEPES and 0.50 M NaCl buffered at pH 7.7 and pD equivalent. $[Me_2SO]$ = 3.3%. Solid lines drawn according to eq 6 with the parameters of Table I.

Also included in Figure 1 are proton inventories of k_E (determined as previously described[10]) for the reactions of PPE with Suc-$(Ala)_3$-pNA $[^D(k_E)$ = 2.02 ± 0.09] and HLE with MeO-Suc-Ala-Ala-Pro-Val-pNA $[^D(k_E)$ = 1.51 ± 0.04]. Both proton inventories are dome-shaped.

Development of a General Model for Proton Inventories of k_E. The general expression for the dependence of $k_{E,n}/k_{E,0}$ on n is given by the Gross–Butler equation[8]

$$k_{E,n}/k_{E,0} = \frac{\prod_i^{v_T}(1 - n + n\phi_j^T)}{\prod_i^{v_R}(1 - n + n\phi_i^R)} \qquad (4)$$

where v_T and v_R are the number of exchangeable protons in the transition state and reactant state, respectively, and ϕ_T and ϕ_R are the corresponding deuterium fraction factors for the exchangeable protonic sites relative to bulk water. In situations, such as the present, where reactant-state fractionation factors can be taken as unity[8] and the entire solvent isotope effect results from fractionation of deuterium at exchangeable protonic sites of the transition state, eq 4 simplifies to

$$k_{E,n}/k_{E,0} = \prod_j^{v_T}(1 - n + n\phi_j^T) \qquad (5)$$

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Equation 5 predicts dome-shape proton inventories for reactions in which one or both of the following conditions are met:[8] (i) there exist exchangeable protonic sites in the transition state that generate opposing normal ($\phi < 1$) and inverse ($\phi > 1$) isotope effects, or (ii) the transition state being observed is in fact a virtual transition state[9] composed of at least two serial transition states. The proton inventories for the hydrolyses of the two peptidic substrate by PPE and HLE are consistent with models in which either condition (i) or (ii) or both are met. In contrast is the proton inventory for the association of PPE with αPI which, with its extreme curvature and maxima, *requires* the existence of opposing inverse and normal isotope effect contributions.

It was hoped that a single model could be developed to account not only for the proton inventory of the association of αPI with PPE but also for the proton inventories of the other two reactions. On the surface, development of a single model for these reactions may seem inappropriate since αPI is formally an inhibitor and structurally unrelated to p-nitroanilide substrates. However, αPI is an "inhibitor" only by virtue of the extreme stability of E:I. Reactions of αPI with proteases will ultimately yield products and active enzyme and thus differ from reactions of polypeptide substrates with proteases only in that these latter reactions proceed through intermediates of much lower stability. Thus, if we take this view that reactions of proteases with both peptide anilides and αPI share several essential mechanistic features, the pursuit of a single model for these reactions is valid.

To generate this model, the following mechanistic features were considered: (i) dissociation constants of complexes formed from serine proteases and substrates or inhibitors are frequently of lower magnitude in D_2O than H_2O[5,11-14] [i.e., ${}^{D}(K_m) \simeq {}^{D}(K_i) > 1$]; (ii) hydrolyses of specific peptide-derived substrates occurs with charge-relay catalysis and the coupled transfer of two protons;[5,15,16] and, (iii) the transition state corresponding to k_E may be virtual,[9,17] composed of a chemical transition state for acylation and the transition state for some physical step preceding the acyl-enzyme.[5,18] Equation 6 incorporates these features[17,19]

$$k_{E,n}/k_{E,0} = Z^n\left[\frac{C_1}{\Phi_1{}^n} + \frac{C_2}{(1 - n + n\phi_2)^2}\right]^{-1} \tag{6}$$

Z is the product of many small fractionation factors originating from the solvent reorganization that accompanies binding of substrate or inhibitor.[8,19] Z will be greater than 1 and similar in magnitude to dissociation constant solvent isotope effects.[5,11-14] C_1 and C_2 are the fractional contributions ($C_1 + C_2 = 1.0$) made by each of the two partially rate-limiting transition states of k_E.[17] C_1 is the transition-state contribution from the physical step, while C_2 is the contribution from the acylation transition step. Φ_1, like Z, is the product of many small fraction factors and originates from deuterium fractionation at exchangeable protonic sites in the transition state for the physical step. These physical steps may include binding of substrate or αPI, conformational changes of complexes, and release of the first product to produce the acyl-enzyme. Should any of these processes generate isotope effects, they are assumed to do so by fractionation at many sites. ϕ_2 is one of two identical fractionation factors corresponding to the two exchangeable protonic sites of the charge-relay system.

The mechanistic model implicit in eq 6, while realistic and potentially accurate in its description of serine protease catalysis, is mathematically complex. Simulations indicated that solving this equation for the four parameters, Z, Φ_1, ϕ_2, and C_1 (C_2 = $1-C_1$), by nonlinear least squares would require data of a precision and accuracy not ordinarily obtained in enzyme kinetic experiments. It therefore came as no surprise when attempts at parameter estimation were unsuccessful due to nonconvergence of the least-squares fits. This is, of course, consistent with the simulations and indicates that the data cannot be uniquely defined by eq 6.

Table I. Parameters for the Generation of Proton Inventories of k_E

reaction	${}^{D}(k_E)$	Z	C_1	Φ_1	C_2	ϕ_2
PPE + αPI	0.91 ± 0.06	1.5	0.65	3.2	0.35	0.54
HLE + MeOSuc-Ala-Ala-Pro-Val-pNA	1.51 ± 0.04	1.5	0.45	1.0	0.55	0.54
PPE + Suc-(Ala)$_3$-pNa	2.02 ± 0.09	1.5	0.00	1.0	1.00	0.54

At this point, two alternative procedures were considered: (i) fitting the data to a simpler model having fewer parameters or (ii) constraining one or more of the parameters of eq 6 to "realistic" values and then solving for the rest. The first of these alternatives was discarded on the grounds that any simplification of eq 6 would result in an expression that inaccurately reflected essential mechanistic features of serine protease catalysis. Furthermore, simpler mechanisms do not account for the extreme curvature of the proton inventory of the reaction of αPI and PPE (see below). Subsequent attempts at fitting the data involved the second alternative.

The parameters constrained were Z and ϕ_2. These two were chosen because the most is known about their magnitudes. As discussed previously in this section, Z should be similar in magnitude to solvent isotope effects on dissociation constants which generally range from 1.2 to 1.8. ϕ_2 is equal to the reciprocal of the square root of the solvent isotope effect for reactions of serine proteases involving charge-relay catalysis. Typical solvent isotope effects for these reactions range from 2.8 to 4.0 and correspond to ϕ_2 values of 0.60–0.50. With these limits in mind, Z and ϕ_2 were constrained to 1.5 and 0.54, respectively. In order to generate an internally consistent model, Z and ϕ_2 were constrained to these values for all three proton inventories.

With these constraints on Z and ϕ_2, the data sets for the three proton inventories were fit to eq 6 and yielded the following: PI and PPE, $C_1 = 0.64 \pm 0.04$, $\Phi_1 = 3.2 \pm 0.8$; MeOSuc-Ala-Ala-Pro-Val-pNA and HLE, $C_1 = 0.43 \pm 0.03$, $\Phi_1 = 1.08 \pm 0.18$; Suc-(Ala)$_3$-pNA and PPE, $C_1 = 0.09 \pm 0.05$, $\Phi_1 = 1.14 \pm 0.22$. These values formed the basis for the parameter assignments of Table I used to draw the solid lines of Figure 1. The fits displayed in Figure 1 are quite good and illustrate the success of the general model of eq 6 in accounting for the three proton inventories.

Simpler models were tried but could not accurately describe the proton inventory data for the association of αPI with PPE. These models included versions of eq 6 in which (i) Φ_1 was set to 1.0, corresponding to a mechanism involving solvent reorganization, charge-relay catalysis, and a physical step generating an isotope effect of unity, (ii) C_1 was set to 0, corresponding to a mechanism involving solvent reorganization, charge-relay catalysis, and the absence of a partially rate-limiting physical step, and, (iii) C_1 and set to 1, corresponding to a mechanism involving solvent reorganization and a rate-limiting physical step that generates a nonunity isotope effect. However, if Z is set equal to 1, a satisfactory fit is obtained.

When Z is constrained to unity, the values $\Phi_1 = 3.3 \pm 0.6$ and $C_1 = 0.79 \pm 0.02$ are obtained by a nonlinear least-squares fit. This corresponds to a mechanism involving charge-relay catalysis and a partially rate-limiting physical step that produces an isotope effect. According to this model, the reorganization of solvent that accompanies the initial binding of αPI to PPE does not generate an isotope effect. If we compare this model to the original (Table I), we see that the reduction of Z from 1.5 to 1.0 is balanced by increases in both the transition-state fractionation factor for the physical step and the contribution of this step to the virtual transition state. This indicates that Z and Φ_1 are dependent parameters and, without independent knowledge of C_1, cannot be extracted from the proton inventory data. Choice of a nonunity value for Z rests on analogy with the other systems presented here

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and previously sited literature.[5,11-14,19]

Discussion

A general model was developed in this paper to account for the proton inventories of associative reactions of serine proteases. The proton inventories determined in this work were of k_E for the reactions of αPI with PPE, MeOSuc-Ala-Ala-Pro-Val-pNA with HLE, and Suc-Ala-Ala-Ala-pNA with PPE and can be described by similar mechanisms. For reactions of peptide p-nitroanilides with proteases, it appears that as the reactants approach each other in solution, extensive solvent reorganization must occur to allow initial contacts to be established. At least for the reactions reported herein, this process generates inverse solvent isotope effects, suggesting an overall strengthening of hydrogen bonds and ordering of solvent structure. This view is supported by the large negative entropy of activation of −36 eu observed for the association of MeOSuc-Ala-Ala-Pro-Val-pNA with HLE.[20]

These initial reaction steps ultimately lead to the formation of an encounter complex of enzyme and substrate. Reaction from this intermediate proceeds to the acyl-enzyme through a virtual transition state composed of transition states for the chemical steps of acylation and the transition state for a physical step preceding the acyl-enzyme.[18] Although the identity of the physical step and its transition state are unknown, likely candidates include substrate binding, conformation changes of enzyme-bound intermediates, and release of the first product. The acylation transition state corresponds to either formation or decomposition of the tetrahedral addition adduct.

For the association of αPI with PPE, the transition state for the physical step not only makes a major contribution to determining the structure of the virtual transition state but also is accompanied by a large solvent isotope effect. That this step might correspond to a conformation change is supported by a recent X-ray crystallographic study[21] of αPI in which a major structural rearrangement was observed upon bond cleavage of inhibitor at its reactive center. The large inverse isotope effect on this step would then correspond to tightening of many hydrogen bonds within the structure of the enzyme–inhibitor complex as the transition state for the conformational change is reached.

The other major contribution to the virtual transition state for association comes from acylation. This suggests that the stable complex, E:I, following the virtual transition state of k_1 is either a tetrahedral intermediate or the acyl-enzyme itself.

More generally, the results of this paper alert us to the importance of solvent reorganization in associative processes and suggest that dome-shaped proton inventories of k_E will frequently have their origins in the solvent reorganization that accompanies binding processes. Finally, these results provide an alternative view to the complex interpretations of previously reported proton inventories.[22-25]

Acknowledgment. I gratefully acknowledge Prof. Michael S. Matta (Southern Illinois University) for having first suggested to me the importance of solvent reorganization in interpretation of solvent isotope effects and proton inventories. I also thank Barbara R. Viscarello (Yale University) for preparing α-1-protease inhibitor.

Registry No. αPI, 9041-92-3; PPE, 9004-06-2; Suc-(Ala)$_3$-pNA, 52299-14-6; MeOSuc-Ala-Ala-Pro-Val-pNA, 70967-90-7; deuterium, 7782-39-0.

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Binding of Calcium to Amino Acids: The Crystal Structure of Pentaaquobis(hydroxy-L-prolinato)calcium, $Ca(C_5H_8O_3N)_2 \cdot 5H_2O$

Eunice E. Kim, Andrew Sicignano, and Klaas Eriks*

Contribution from the Department of Chemistry, Boston University, Boston, Massachusetts 02215. *Received May 24, 1984*

Abstract: Crystals of pentaaquobis(hydroxy-L-prolinato)calcium were obtained from aqueous solution at pH 11.0. They are monoclinic, space group $P2_1$, with a = 6.213 (1) Å, b = 14.905 (4) Å, c = 9.483 (3) Å, β = 96.41 (2)°, Z = 2, D(calcd) = 1.486 g cm^{-3}, and D(measd) = 1.49 g cm^{-3}. The structure was determined by using least-squares and difference Fourier calculations. The final values of R and R_w were 0.033 and 0.042, respectively, for 1222 reflections with $I_o > 3\sigma(I_o)$. Positional and thermal parameters were refined for the non-hydrogen atoms. Hydrogen atoms were included but not refined. The calcium atom is seven-coordinated by a carboxyl oxygen atom and a nitrogen atom from each of the two hydroxy-L-proline molecules and three water molecules. The hydroxy-L-proline molecules act as bidentate ligands. The coordination geometry around calcium is that of a distorted pentagonal bipyramid. Ca–O distances range from 2.330 to 2.452 Å; Ca–N distances are 2.595 and 2.613 Å. The conformation of the two crystallographically independent hydroxy-L-proline molecules is almost identical with that in the free amino acid. The structure consists of single, monomeric units of composition $Ca(Hyp)_2 \cdot 3H_2O$ that are tied together by hydrogen bonding involving the two additional water molecules. The extensive hydrogen-bonding network is discussed in detail, and structural comparisons are made with 11 other calcium amino acid complexes. It appears that binding of calcium to nitrogen will only occur in crystals grown from solutions of pH >10.

The structure determination described below was motivated by our interest in the manner in which calcium is capable of binding to amino acids and organic bases. Knowledge of the structure of small calcium amino acid complexes has a direct bearing on the understanding of the calcium-binding ability of proteins such as calmodulin and possibly of the mechanism of calcification in living tissue. The hydroxy-L-proline complex of calcium was found to be comparatively easy to obtain in single-

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