Thermodynamic Criterion for the Conformation of P_1 Residues of Substrates and of Inhibitors in Complexes with Serine Proteinases[†]

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ABSTRACT: Eglin c, turkey ovomucoid third domain, and bovine pancreatic trypsin inhibitor (Kunitz) are all standard mechanism, canonical protein inhibitors of serine proteinases. Each of the three belongs to a different inhibitor family. Therefore, all three have the same canonical conformation in their combining loops but differ in their scaffoldings. Eglin c (Leu^{45} at P_1) binds to chymotrypsin much better than its Ala^{45} variant (the difference in standard free energy changes on binding is −5.00 kcal/mol). Similarly, turkey ovomucoid third domain (Leu^{18} at P_1) binds to chymotrypsin much better than its Ala^{18} variant (the difference in standard free energy changes on binding is −4.70 kcal/mol). As these two differences are within the ±400 cal/mol bandwidth (expected from the experimental error), one can conclude that the system is additive. On the basis that isoenergetic is isostructural, we expect that within both the P_1 Ala pair and the P_1 Leu pair, the conformation of the inhibitor's P_1 side chain and of the enzyme's specificity pocket will be identical. This is confirmed, within the experimental error, by the available X-ray structures of complexes of bovine chymotrypsin Aα with eglin c (lacb) and with turkey ovomucoid third domain (1cho). A comparison can also be made between the structures of P_1 $(Lys^+)^{15}$ of bovine pancreatic trypsin inhibitor (Kunitz) (1mtn and 1cbw) and of the P_1 $(Lys^+)^{18}$ variant of turkey ovomucoid third domain (1hja), both interacting with chymotrypsin. In this case, the conformation of the side chains is strikingly different. Bovine pancreatic trypsin inhibitor with $(Lys^+)^{15}$ at P_1 binds to chymotrypsin more strongly than its Ala^{15} variant (the difference in standard free energy changes on binding is −1.90 kcal/mol). In contrast, turkey ovomucoid third domain variant with $(Lys^+)^{18}$ at P_1 binds to chymotrypsin less strongly than its Ala^{18} variant (the difference in standard free energies of association is 0.95 kcal/mol). In this case, P_1 Lys^+ is neither isostructural nor isoenergetic. Thus, a thermodynamic criterion for whether the conformation of a P_1 side chain in the complex matches that of an already determined one is at hand. Such a criterion may be useful in reducing the number of required X-ray crystallographic structure determinations. More importantly, the criterion can be applied to situations where direct determination of the structure is extremely difficult. Here, we apply it to determine the conformation of the Lys^+ side chain in the transition state complex of a substrate with chymotrypsin. On the basis of k_{cat}/K_M measurements, the difference in free energies of activation for Suc-AAPX-pna when X is Lys^+ and X is Ala is 1.29 kcal/mol. This is in good agreement with the corresponding difference for turkey ovomucoid third domain variants but in sharp contrast to the bovine pancreatic trypsin inhibitor (Kunitz) data. Therefore, we expect that in the transition state complex of this substrate with chymotrypsin, the P_1 Lys^+ side chain is deeply inserted into the enzyme's specificity pocket as it is in the $(Lys^+)^{18}$ turkey ovomucoid third domain complex with chymotrypsin.

Among the elusive goals of enzymology are the high-resolution structures of transition state complexes between enzymes and substrates. However, for enzyme−substrate pairs, the half-lives of such complexes are very short, making the determination of their structures difficult, if not impossible, with technologies that are available at present. Numerous expedients have been developed to circumvent this problem. One is to determine the structures of enzyme−inhibitor complexes and to assume that they correspond to those of the transition state complexes.

The realization that complexes of standard mechanism (*1*), canonical (*2*) protein inhibitors with their cognate serine proteinases may serve as good models of proteinase−substrate (Michaelis) complexes occurred just as many scientists chose to determine such structures (*3*). Since then,

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a large number of them have been determined (*2*, *4*). The reason for the belief in the suitability of the protein inhibitor−serine proteinase complexes as models for transition state complexes stems in part from the demonstrations in our laboratory (*5*−*10*) that standard mechanism protein inhibitors serve the enzymes they inhibit as substrates, albeit very inefficient ones. The mechanisms of enzyme−inhibitor interaction and of enzyme−substrate interaction seem formally identical, differing only in the values of the various on−off rate constants such that in the enzyme−inhibitor system, the lifetime of the complex is very long. The statement of faith that the structures of enzyme−inhibitor complexes serve as excellent models for the transition state complexes has recently been made by several workers (*11*−*13*).

However, faith is needed. A greater difficulty arises when complexes of two, standard mechanism, canonical protein inhibitors belonging to two different inhibitor families but with the same P_1 residue exhibit completely different conformations of the P_1 residue.[1] This case arose quite recently. Capasso et al. (*14*) (1mtn) and Scheidig et al. (*15*) (1cbw) independently[2] reported on the structure of a complex between bovine pancreatic trypsin inhibitor (Kunitz), which has Lys^{15} at its P_1 position (see abbreviations), and bovine chymotrypsin Aα, CHYM. In this complex, the Lys^{15} side chain enters the S_1 specificity pocket but then bends out and its N^{ζ} atom forms hydrogen bonds with the Ser^{217} O of the enzyme and the Pro^{13} O of the inhibitor. In contrast, in the structure of Lys^{18}OMTKY3 complexed with CHYM (lhja) (unpublished experiments), it was found that the P_1 Lys^{18} residue is deeply embedded in the S_1 pocket of CHYM, much like Leu^{18} of the wild-type inhibitor in complex with CHYM (1cho) (*16*) (Figure 1A,B). Clearly, in the light of this result, there are at least two different expectations for the conformation of the Lys^+ side chain in the transition state complex of a substrate (we employed Suc-AAPK-pna) with CHYM. A criterion for determining which, if either, of these expectations is correct is needed. In this paper, we suggest that interscaffolding additivity (*17*) can serve as such a criterion. The criterion is applied and shows that k_{cat}/K_M for the substrate is additive to the OMTKY3 system and strongly nonadditive to the BPTI system. We, therefore, propose that the conformation of $(Lys^+)^{18}$ in the Lys^{18}OMTKY3 complex is an excellent model for the conformation of the Lys^+ P_1 residue in the transition state complex of a substrate.

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[1] The standard mechanism of interactions between serine proteinases with their protein inhibitors is defined in ref *1* and in greater detail in refs *5*−*10*. The canonical main chain conformation of residues surrounding the reactive site (*2*) is described in the first paragraph of Results. The P_n, ..., P_1 and P_1', ..., P_n' nomenclature of Schecter and Berger (*39*) is used for residues both in substrates and in inhibitors (see Table 3). The S_n, ..., S_1 and S_1', ..., S_n' nomenclature is used for subsites on the enzyme. OMTKY3 is an abbreviation for turkey ovomucoid third domain and BPTI for bovine pancreatic trypsin inhibitor (Kunitz). For the convenience of readers, BPTI is often specified as Lys^{15}BPTI. Other abbreviations: CHYM, bovine chymotrypsin Aα; SGPA and SGPB, *Streptomyces griseus* proteinases A and B, respectively; PPE, porcine pancreatic elastase; CARL, subtilisin Carlsberg. For peptide substrates such as Suc-AAPX-pna, Suc stands for succinyl and pna for *p*-nitroanilide. Ape and Ahx represent α-aminopentanoic acid and α-aminohexanoic acid, respectively.

[2] The chronology is somewhat confused. Capasso et al. (*14*) submitted and published their paper first both in the journal and in the database. However, the relevant result of Scheidig et al. (*15*) was given at a meeting and published without details even earlier (*40*).

EXPERIMENTAL PROCEDURES

Expression and Purification of Inhibitor Variants. Variants of OMTKY3 and BPTI were expressed in *Escherichia coli* as fusion proteins with a fragment of protein A. The expression and purification of OMTKY3 variants have been described in detail elsewhere (*18*, *19*). The fusion protein for BPTI was engineered to have a CHYM cleavage site (*20*) at the junction of the protein A fragment and BPTI. Thus, after affinity separation on the IgG Sepharose column, the fusion protein was reacted with a slight molar excess of CHYM in a solution containing $\sim 1 \times 10^{-6}$ M fusion protein in Tris-HCl buffer (pH 8) for 48 h at 4 °C. The BPTI variants were purified by size exclusion column chromatography on Bio-Gel P-10 in 2.5% formic acid followed by ion exchange column chromatography on Q-Sepharose at pH 9 using a linear NaCl gradient from 0 to 0.2 M. Eglin c variants were expressed in *Bacillus subtilis* strain PGG32 and purified as reported previously (*17*).

K_a Determination. K_a measurements were performed at different pH values in the pH range of 6−10 using Bis-Tris (pH 6−7), Tris-HCl (pH 7.5−9), and Gly-NaOH (pH 9.5 and 10) buffers. All the buffers contained 0.005% Triton X-100 and 0.02 M $CaCl_2$. The procedure for K_a determination was the same as has been described in detail elsewhere (*18*, *19*). CHYM activity in each incubation mixture was determined by following the increase in absorption at 380−410 nm for the hydrolysis of appropriate tetrapeptide *p*-nitroanilide substrates on a Hewlett-Packard HP8450A diode array spectrophotometer at 21 ± 2 °C. The various substrates used in this study were Suc-AAPA-pna, Suc-AAPL-pna, Suc-AAPF-pna, and Suc-AAPK-pna. They were obtained from BACHEM. CHYM was from Worthington Biochemical Corp.

Determination of k_{cat}/K_M. The values of k_{cat} and K_M were determined by following the kinetics of enzymatic hydrolysis of substrates (Suc-AAPA-pna and Suc-AAPK-pna) at 380−410 nm on a Hewlett-Packard HP8450A diode array spectrophotometer at 21 ± 2 °C. The data were fitted to the equation $v = V_{max}[S]/(K_M + [S])$ using Enzfitter (*21*).

Determination of pK_f by NMR. Each sample of lyophilized protein or dry peptide was dissolved in 90% H_2O/10% D_2O (v/v) containing 100 mM potassium chloride to give a final sample concentration of approximately 2 mM. Each sample contained 0.4 mM 2,2-dimethylsilapentane-5-sulfonic acid (DSS) as the internal chemical shift standard. The pH of the solution was adjusted by adding concentrated HCl or KOH. The pH of the sample at room temperature was measured both before and after the NMR data were collected. The glass electrode (Biological Combination Electrode, Beckman, Fullerton, CA) was calibrated with commercial standard pH 4.0, 7.0, 10.0, and 11.0 buffers (Fisher Scientific, Fair Lawn, NJ). A degassed saturated solution of $Ca(OH)_2$ at 25 °C served as the pH 12.42 standard (*22*).

NMR data were acquired using 5 mm TXI GRASPIII triple-resonance probes on Bruker DMX500 and DMX600 spectrometers located in the National Magnetic Resonance Facility at Madison. TOCSY (total correlated spectra) (*23*) were acquired in pure absorption mode with time-proportional phase incrementation. A Watergate 3-9-19 pulse sequence (*24*) was used for solvent suppression. In all experiments, the carrier frequency was set at the middle of the spectrum. The spectral widths were 6063 Hz on the DMX-600 and 5053 Hz on the DMX-500 instrument. The mixing times were 50 ms. Each experiment consisted of eight transients; data were collected with either 2048 or 4096 time domain points and between 360 and 400 increments in the indirectly detected dimension. All experiments were performed at 25 °C with the temperature calibrated by a methanol NMR standard. All NMR spectra were processed using UXNMR software (Bruker, Billerica, MA) installed on Silicon Graphics (Mountain View, CA) workstations. Most data sets were zero filled to 2048 × 1024 data points before transformation. The data sets were multiplied by a sine-bell function with a phase shift of $\pi/2$ in both the t_1 and t_2 dimensions. The final digital resolution following Fourier transformation was 5.0 Hz/point in the F_2 dimension and 10.0 Hz/point in the F_1 dimension. The estimated uncertainties in the chemical shifts are 0.010 ppm in the F_2 dimension and 0.020 ppm in the F_1 dimension. The ^{1}H NMR chemical shifts of the $C^{\epsilon}-^1H$ peaks of lysine were used in calculating the pK_a values. The pK_a values were determined by fitting the experimental data by nonlinear least-squares analysis to a theoretical titration curve with a software package written by H. W. Anthonsen.

Superimposition. The coordinate sets of several CHYM complexes used in this study were taken from the Brookhaven Protein Data Bank (*25*). The file names are 1cho (OMTKY3; *16*), 1acb (eglin c; *26*), 1hja (Lys^{18}OMTKY3, unpublished experiments), 1mtn (Lys^{15} BPTI; *14*), and 1cbw (Lys^{15} BPTI; *15*). Insight II was used to superimpose the various structures. Root-mean-square deviations were calculated from the CCP4 program suite (*27*).

RESULTS

Interscaffolding Additivity of Eglin c and OMTKY3 P_1 Variants. All the canonical (*2*) protein inhibitors of serine proteinases, with few exceptions, share the same main chain conformation for residues P_4 to P_2' (P_3'), in the combining loop. Furthermore, this conformation remains the same in the free inhibitors and in complexes with the various serine proteinases they inhibit. If two inhibitors are members of the same inhibitor family, they also share the scaffolding or overall global three-dimensional structure. However, if they are not, they have distinctly different scaffolding. At the present time, 18 different standard mechanism, canonical inhibitor families and, therefore, eighteen different scaffoldings have been characterized (*28*). OMTKY3 is a member of the Kazal family, whereas eglin c is a member of the potato I family. Leu is the P_1 residue in both inhibitors. Both are powerful inhibitors of CHYM and of many serine proteinases with hydrophobic S_1 pockets. The structures of their complexes with CHYM have been determined by X-ray crystallography (*16*, *26*). A detailed comparison (*17*) of the S_1 pockets of CHYM and of the P_1 Leu side chain of both inhibitors in their complexes with CHYM showed them to be indistinguishable within experimental error. The identity of the two structures does not mean that the K_a values for the binding of OMTKY3 and of eglin c to CHYM are identical. They are not. The reason is that the sequences surrounding the P_1 Leu, while similar, differ. Also, the noncontiguous contact residues differ strikingly.

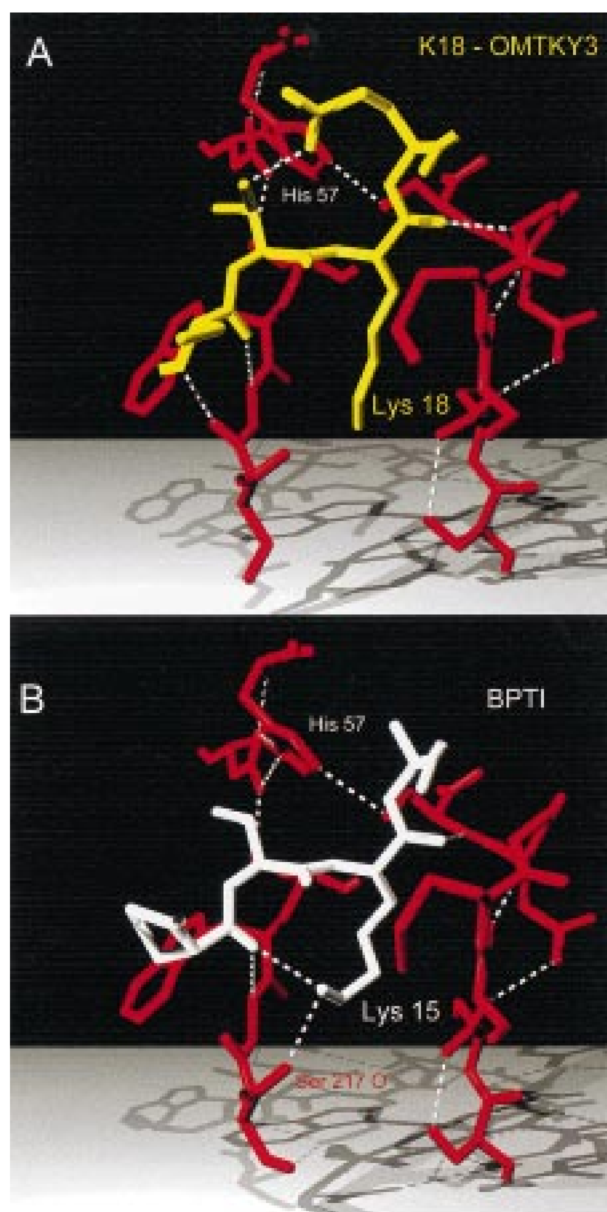


FIGURE 1: Interaction of the P_1 Lys$^+$ residue of the inhibitor with the S_1 pocket of CHYM. (A) Lys^{18}OMTKY3 (yellow) in complex with CHYM (red) (1hja, unpublished). (B) Lys^{15}BPTI (white) in complex with CHYM (red) (1cbw; *15*). Hydrogen bonds are shown as dotted white lines. Water molecules are not shown. The N^{ζ} of Lys^{15}BPTI is hydrogen bonded to the carbonyl O of Pro^{13} of the inhibitor and to the carbonyl O of Ser^{217} of the enzyme. The carbonyl O of Pro^{13} is also hydrogen bonded to the main chain N of Gly216 of the enzyme. Compare this figure to Figure 6 of Qasim et al. (*17*).

Consider now the Ala, Leu additivity cycle shown in Figure 2. If the canonical approximation outlined above is obeyed, the conformation of P_1 Ala in the two Ala inhibitor variants must be the same, as Ala side chains do not have any angles of side chain rotation. Thus, both for OMTKY3 and for eglin c, replacement of Ala with Leu involves identical side chain conformations both for the Ala forms

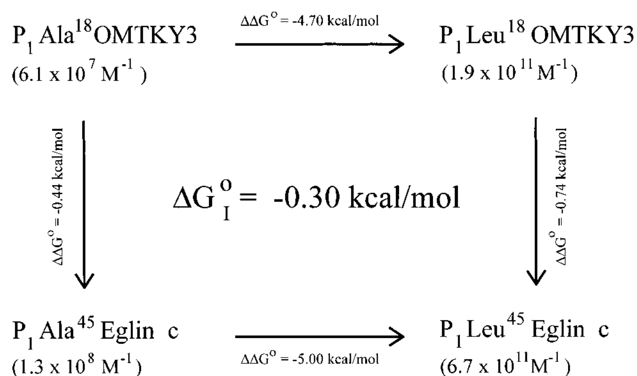


FIGURE 2: Interscaffolding additivity cycle for the interaction of P_1 Ala and Leu variants of OMTKY3 and of eglin c with CHYM. The K_a values were determined at pH 8.3 and 21 ± 2 °C.

Table 1: Matrix of $\Delta G_I°$ Values (Kilocalories per Mole) for P_1 Variants of OMTKY3 and Eglin c Interacting with CHYM[a]

	P_1 G	P_1 A	P_1 S	P_1 P	P_1 L	P_1 I	P_1 D	P_1 E	P_1 F
P_1 G		0.26	−0.01	−1.90	−0.04	−0.23	−0.18	−0.05	0.08
P_1 A			−0.27	−2.10	−0.30	−0.49[b]	−0.44[b]	−0.31	−0.18
P_1 S				−1.90	−0.03	−0.22	−0.17	−0.04	0.09
P_1 P					1.80	1.60	1.70	1.80	2.00
P_1 L						−0.19	−0.15	−0.01	0.12
P_1 I							0.05	0.18	0.31
P_1 D								0.13	0.26
P_1 E									0.13
P_1 F									

[a] This matrix is based on K_a ($\Delta G_a°$) values of Lu et al. (*19*) for the OMTKY3 variants and on the K_a ($\Delta G_a°$) values of eglin c variants of Qasim et al. (*17*) except for the data for the P_1 Ala[45] and P_1 Phe[45] variants determined in this work and given in the text. These values are then inserted into eq 1. The left-hand column indicates the X variants; the horizontal row indicates the Y variants. Eglin c variants are scaffold 2; OMTKY3 variants are scaffold 1. See Figure 2 for an illustration. [b] Slightly outside the ±400 cal/mol error range.

(by the definition of canonical behavior) and for the Leu forms (by the determination by X-ray crystallography). Isostructural change implies isoenergetic change. It is, therefore, gratifying that the Ala versus Leu comparison is additive as the $\Delta G_I°$ term is −300 cal/mol. This is smaller than the $|\Delta G_I°|$ of 400 cal/mol which can be attributed to the experimental error (*17*). The quantity $\Delta G_I°$ is defined (*29*, *30*) as the difference between the $\Delta\Delta G°$ terms for making the same substitution in the two different scaffolds.

$$\Delta G_I° = \Delta\Delta G°(X{\rightarrow}Y)_{\text{scaffold 2}} - \Delta\Delta G°(X{\rightarrow}Y)_{\text{scaffold 1}} \quad (1)$$

Qasim et al. (*17*) examined seven P_1 variants of eglin c. They were Gly, Ser, Pro, Asp, Glu, Ile, and Leu (wild type). Ala was not among those. Also missing were any aromatic amino acid residues. For that reason, in this paper, we determined K_a values for Ala (1.3×10^8 M^{-1}) and Phe (6.9×10^{12} M^{-1}) at pH 8.3 and 21 ± 2 °C with CHYM. These are the conditions under which Lu et al. (*19*) determined the K_a values for all 20 coded P_1 variants of OMTKY3. Table 1 shows $\Delta G_I°$ values for all eglin c and OMTKY3 variants interacting with CHYM. Qasim et al. (*17*) noted with the seven available P_1 variants that all 15 pairs not involving Pro were additive in their interaction with CHYM. Now, with nine variants in hand, the additivity extends to all 28 pairs not involving Pro, whereas all eight pairs involving Pro are not additive. The failure of the Pro variant to be additive with the other P_1 residues was discussed earlier (*17*). The main conclusion is that P_1 Pro is exceptionally hard to fit into the S_1 pocket of the enzyme and that it is locally deleterious. Unfortunately, neither the structures of the free (unbound) Pro[18]OMTKY3 and Pro[45]eglin c nor of their complexes with CHYM have been determined.[3]

Behavior of Lys Residues at P_1. As many standard mechanism, canonical protein inhibitors of serine proteinases are trypsin inhibitors, P_1 Lys and Arg residues abound. Since the isolation and characterization of the classical trypsin inhibitors, it was observed that both bovine pancreatic trypsin inhibitor (Kunitz) (*31*) and soybean trypsin inhibitor (Kunitz) (*32*) inhibited CHYM "to a less marked extent" than trypsin. It is now well-known that the inhibition of trypsin and of CHYM involves the same reactive site of these inhibitors (*14*, *15*, *33*).

Insertion of a Lys^+ side chain into the hydrophobic S_1 cavity of CHYM or of a related enzyme such as an elastase or a subtilisin entails a large free energy cost of transferring a charge from a medium of a high dielectric constant in the solvated free inhibitor to one of a low dielectric constant (*34*) in the complex. The system can avoid the maximal penalty in a variety of ways. The simplest is to lower the pK of the ϵ-amino group so that at pH values higher than this pK_c, which is the pK in complex, the ϵ-amino group is deprotonated and there is no charge burial. The state of ionization of the ϵ-amino group cannot be simply determined by X-ray crystallography at the relatively low resolution of the complexes described here (1.8−2.7 Å). However, Qasim et al. (*35*) suggested a simple method for determining the pK_f (free inhibitor) and pK_c (complex) of ionizable P_1 residues in standard mechanism, canonical inhibitors. The pH dependence of the association equilibrium constant of the enzyme with two inhibitor variants is determined over a broad pH range. One of the variants has the ionizable group of interest at P_1 (e.g., Lys). The other has a nonionizable P_1 residue and serves as a control. It is assumed that the pH dependence of K_a of the ionizable variant is a product of two simple terms. The first of these arises solely from the change in the pK of the ionizable P_1 residue from pK_f to pK_c upon complex formation. The second term, common to the P_1 ionizable and nonionizable variant, arises from the pK shifts of all other ionizable residues in the inhibitor and in the enzyme upon complex formation. This simple assumption yields

$$\log K_a(P_1 \text{ ionizable}) - \log K_a(P_1 \text{ nonionizable}) = \log R = \log R° + \log[1 + 10^{(\text{pH} - \text{p}K_c)}] - \log[1 + 10^{(\text{pH} - \text{p}K_f)}] \quad (2)$$

where the two association constants, K_a, and their ratio R are pH-dependent. This is fitted by nonlinear least-squares fitting to three parameters. $R°$ is the ratio of the K_as of the protonated forms of ionizable group to that of the reference nonionizable group, pK_f the pK of P_1 in the ionizable variant in the free form, and pK_c the pK of P_1 in complex. At least three parameters are clearly required, but different parameters

[3] The structure of the Pro[18]OMTKY3 complex with *S. griseus* proteinase B was determined by K. Huang, W. Lu, S. Anderson, M. Laskowski, Jr., and M. N. G. James (unpublished).

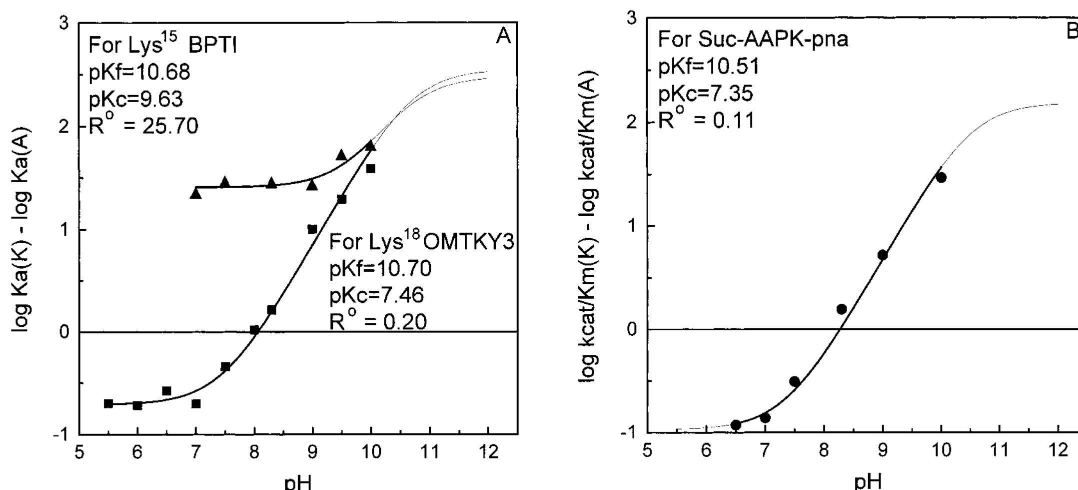


FIGURE 3: (A) pH dependence of log K_a(P_1 K) − log K_a(P_1 A) for the association of P_1 Lys^{15}BPTI (and its P_1 Ala^{15} variant) (▲) and of P_1 Lys^{18}OMTKY3 (and its P_1 Ala^{18}OMTKY3 variant) (■) with CHYM. The data were fitted to a two-parameter form of eq 2 by replacing the parameter pK_f with the NMR-determined pK_f of P_1 Lys in Lys^{18}OMTKY3 ($pK_f = 10.70$) and Lys^{15}BPTI ($pK_f = 10.68$). (B) pH dependence of log k_{cat}/K_M(K) − log k_{cat}/K_M(A) for the hydrolysis of Suc-AAPK-pna and Suc-AAPA-pna by CHYM. The results were fitted to a two-parameter form of eq 2 using the NMR-determined pK_f of Lys in Suc-AAPK-pna.

could have been chosen. The present choice includes pK_f. Since pK_f is for the inhibitor, it is independent of the cognate enzyme.

Equation 2 was successfully applied to P_1 Asp, Glu, and His OMTKY3 variants interacting with SGPB (*35*). Since then, we have extended its application to CHYM, SGPA, SGPB, PPE, and CARL interacting with P_1 Asp, Glu, and Lys. In carrying out this work, we realized that K_a values had to be determined over a very large pH range, typically about 1 pH unit lower than the lowest pK and 1 pH unit higher than the highest pK. However, such determinations are nearly impossible for SGPA and CARL below pH 4.0 as these enzymes irreversibly lose activity below this pH. CHYM and PPE do not, but their enzymatic activity below pH 4.0 is very small, making K_a measurements very difficult. The pK_f of Lys in Lys^{18}OMTKY3 is 10.7. At high pH values such as 10 and above, considerable difficulties were encountered in obtaining reproducible K_a values for many protein inhibitors interacting with CHYM, PPE, and CARL.

As the inhibitors of interest to us are small, stable, and soluble over a broad pH range, the determination of pK_f in free inhibitors by NMR became attractive. The determinations have been carried out by J. Song and J. L. Markley at the University of Wisconsin. The Purdue group then used eq 2 as a two-parameter equation, since pK_f values are fixed in advance. The pK_c values for Lys^{18}OMTKY3 and Lys^{15}-BPTI complexes with CHYM were obtained in this way. In each case, the P_1 Ala variants (Ala^{18}OMTKY3 and Ala^{15}-BPTI, respectively) served as references. The results are shown in Figure 3A. In sharp contrast to the excellent agreement of pK_f values for P_1 Lys side chains in the two inhibitors, the pK_c values differ substantially. For BPTI, the pK_c of Lys^{15} is 9.63, and for Lys^{18}OMTKY3, it is 7.46. The 2.17 pK unit difference is far outside the range of experimental error.

Two three-dimensional structures of BPTI complexed with CHYM were published recently, and the coordinates were deposited in the Protein Data Bank [1mtn, pH 6.0 (*14*); 1cbw, pH 7.5 (*15*)]. In view of the very high pK_c value for P_1 Lys in Lys^{15}BPTI−CHYM, both of these structures must be for P_1 Lys^+ rather than for P_1 Lys°. The coordinates for the complex of Lys^{18}OMTKY3 with CHYM at pH 6.0 (unpublished experiments, 1hja) were also deposited in 1997. The crystallization pH is about 1.5 units lower than the pK_c measured for this complex. It seems clear that this structure is also for P_1 Lys^+.

Comparison of the Structures of Complexes of CHYM with Lys^{15}BPTI and with Lys^{18}OMTKY3. The five independent structure determinations of CHYM complexed to four different inhibitors have afforded seven structures of the CHYM molecule. Not all of these structure determinations are at the same atomic resolution (the minimum d spacings of the measured diffraction data range from 1.8 to 2.6 Å). However, the overall global comparisons (Table 2) show that the structures are exceedingly similar and the values of the rms differences (range of 0.45−0.65 Å) are only ~2 times the estimated accuracy of the atomic coordinates. This means that the structures of CHYM in these different complexes are identical within the limits of accuracy of the experiments. This conclusion is reinforced by the elements in the upper triangular matrix, given in Table 2, that shows the comparisons involving the 29 residues of the active site region of CHYM (for the backbone and C^β atoms, the range is 0.212−0.328 Å). These values are of a similar magnitude as the accuracy of the coordinate determination. Inclusion of the side chain atom coordinates in these comparisons only marginally changes the size of the rms differences, except for those comparisons involving the Leu^{18}OMTKY3 and Lys^{18}OMTKY3 with the two BPTI complexes. In these latter four comparisons, rms differences of the 196 atoms of the active site of CHYM are approximately doubled relative to those of the backbone atoms.

The amino acid sequences of the reactive site regions (P_4 to P_3') of the four inhibitor variants (Table 3, rows 1−4) crystallographically studied in complex with CHYM are all different. The most similar, of course, are the two P_1 variants of OMTKY3, differing only in the P_1 residue (Leu^{18} and Lys^{18}). Eglin c differs at four of the six residues relative to wild-type Leu^{18}OMTKY3, and BPTI differs at all six positions except for the P_1 Lys^{15} being identical to the P_1

Table 2: Comparison Matrix for Protein Inhibitors in Complex with CHYM (Root-Mean-Square Values in Angstroms)[a–d]

	1cho	1acb	1hja	1mtn	1cbw
1cho (Leu[18]OMTKY3)		0.212	0.255	0.328	0.301
		0.523	0.272	0.622	0.573
		0.463	0.206	0.403	0.434
1acb (Leu[45]eglin c)			0.271	0.325	0.301
	0.51		0.554	0.386	0.317
	0.37(8)		0.470	0.558	0.526
1hja (Lys[18]OMTKY3)				0.258	0.249
	0.45	0.51		0.587	0.550
				0.379	0.431
1mtn (Lys[15]BPTI)					0.221
	0.45	0.65	0.60		0.310
			1.47(9)		0.223
1cbw (Lys[15]BPTI)	0.57	0.49	0.52	0.59	
			1.46(9)	0.44(9)	

[a] See Experimental Procedures for file names. [b] The upper triangular part of the matrix consists of three numbers that correspond to the rms differences resulting from superimposing (1) the N, C^{α}, C^{β}, C, and O atoms of 29 residues (139 atoms) that comprise the active site of the CHYM moiety in each of the complexes, (2) all atoms of these 29 residues (196 common atoms; the C^{ϵ} atoms of the Met192 residue differed by a large amount and were omitted from the superpositions), and (3) the N, C^{α}, C^{β}, C, and O atoms of residues P_3−P_3' of the inhibitors (superpositions between the two ovomucoids and eglin c involved 30 common atoms; those between the ovomucoids and eglin c on one hand and comparisons involving BPTI on the other hand included 28 common atoms due to the presence of the P_3 Pro in BPTI having very different positions for C^{β} and N relative to those of P_3 in the other inhibitors). [c] The lower triangular part of the matrix gives the rms differences for the global superposition of the 238 common C^{α} atoms of the CHYM molecules in each of the complexes. The atoms of the P_1 residues (leucine or lysine) in these several inhibitors were also compared after the global superpositions. For eglin c and OMTKY3, there are eight common atoms; for Lys[18]OMTKY3 and BPTI, there are nine common atoms. [d] The independent determinations of 1mtn and 1cbw had two molecular complexes per asymmetric unit. The six pairwise comparisons among these four complexes yielded matrix elements ranging from 0.27 to 0.60 Å, for the superposition of 296 common C^{α} atoms (238 from CHYM and 58 from BPTI), i.e., with magnitudes similar to those in the lower triangular matrix for the 238 C^{α} atoms of CHYM. For the comparisons above, we, therefore, arbitrarily selected one of the complexes in the asymmetric unit of 1mtn and 1cbw. The values for the other gave very similar numbers and are redundant.

Table 3: Amino Acid Sequences at the Reactive Sites[a]

	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'
Leu[18]OMTKY3	A	C	T	L	E	Y	R
Lys[18]OMTKY3	A	C	T	K	E	Y	R
Leu[45]eglin c	P	V	T	L	D	L	R
Lys[15]BPTI	G	P	C	K	A	R	I
Ala[18]OMTKY3	A	C	T	A	E	Y	R
Ala[45]eglin c	P	V	T	A	D	L	R
Ala[15]BPTI	G	P	C	A	A	R	I
Ala[13]Lys[15]BPTI	G	A	C	K	A	R	I

[a] The top four lines are for sequences of inhibitor variants whose complexes with CHYM were determined by X-ray crystallography and analyzed in detail (Table 2 and Figure 1A,B). These and the four variants in the lower part of the table had the pH dependence of their K_a values determined and were used in the various additivity cycles described here. The residues are numbered according to Schechter and Berger (*39*). The P_1−P_1' peptide bond is the reactive site peptide bond. The P_1 side chain is inserted into the S_1 pocket of the enzyme upon complex formation.

Lys[18] of the Lys[18]OMTKY3 variant. Despite the tremendous variation in sequence among these four inhibitors, they all bind to the active site of CHYM essentially in an identical mode for the main chain. The major difference between the two P_1 Lys inhibitors (Lys[18]OMTKY3 and Lys[15]BPTI) is

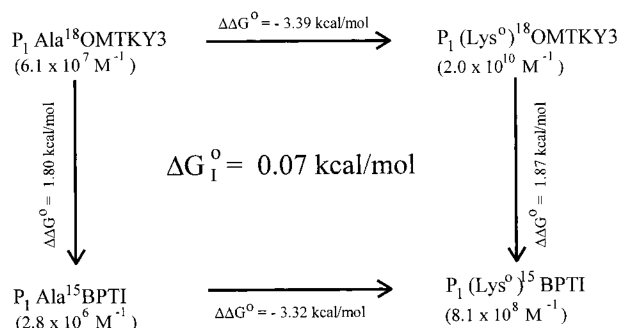
Table 4: χ_n Values for the P_1 Residue in Leu[18]OMTKY3 (lcho), Leu[45]Eglin c (lacb), Lys[18]OMTKY3 (lhja), and Lys[15]BPTI (lmtn and lcbw)

	χ_1 (deg)	χ_2 (deg)	χ_3 (deg)	χ_4 (deg)
1cho	−48	157		
1acb	−61	179		
1hja	−67	154	171	179
1mtn	−45	−77	−177	−114
1cbw	−43	−106	144	−82

the conformation of the lysine side chains (Tables 2 and 4 and Figure 1A,B). The Lys^+ side chain of Lys[18]OMTKY3 inserts into the S_1 pocket in the classical "DOWN" position similar to the P_1 Leu residues in the OMTKY3 and eglin c complexes with CHYM (*17*). In contrast, the Lys^+ side chain in Lys[15]BPTI binds to CHYM with a very different side chain conformation for χ_2 and χ_4 [both gauche (+)] so that the side chain curls up and nestles against the body of the inhibitor and forms hydrogen bonds with the main chain carbonyl oxygens of Ser[217] of the enzyme and of P_3 Pro[13] of the inhibitor (Figure 1B). This is the newly described "UP" position of lysine (*15*). It is clear that the dielectric constant surrounding the ϵ-ammonium group of P_1 Lys^+ in CHYM-bound BPTI is much higher than that surrounding this group in $(Lys^+)^{18}$OMTKY3. This observation qualitatively explains the high pK_c in Lys[15]BPTI and the much lower one in Lys[18]-OMTKY3 (Figure 3A).

P_1 Ala and P_1 Lys^+ Variants of BPTI and of OMTKY3 Are Strongly Nonadditive in Their Interaction with CHYM. Previously, we reported (*19*, *36*) that at pH 8.3, Lys[18]-OMTKY3 binds to CHYM only 1.5 times better than Ala[18]-OMTKY3. This is a very small difference, and we have referred to Lys[18] (P_1) and Ala[18] (P_1) as isofunctional with respect to CHYM (*36*). If interscaffolding additivity held, it would be expected that the same ratio of 1.5 would be observed for K_as of Lys[15]BPTI to Ala[15]BPTI. Castro and Anderson (*20*) measured this ratio. They report it as 25 rather than 1.5 for these two variants. We agree. This system is not interscaffolding additive (*17*).

The much earlier work from Tschesche's laboratory, who made several semisynthetic replacements at P_1 in BPTI (*37*, *38*), shows that P_1 Ape, Ahx, and Met are interscaffolding additive for CHYM between BPTI and OMTKY3 (*19*, *36*). On the other hand, P_1 Lys is not. The deviation from additivity in these cycles is 30−50-fold, in fair quantitative agreement with the discord in the P_1 Lys, Ala cycle discussed above. Since the three hydrophobic, aliphatic residues (Ape, Ahx, and Met) are interscaffolding additive, it seems clear that interscaffolding nonadditivity between BPTI and OMT-KY3 is due to P_1 Lys and not to the P_1 Ala residue. This conclusion could also have been reached a priori. As long as the main chains of both inhibitors remain in their canonical conformations, the position of P_1 Ala in the S_1 pocket is fixed. On the other hand, P_1 Lys has χ_1−χ_4 angles to vary. As can be seen in Figure 3A, comparing the data at pH 8.3 or 8.2 is not ideal. Because of its much lower pK_c, the Lys[18]-OMTKY3 variant shows a much greater pH dependence. It is best to compare the values of the equilibrium constant for Lys°, a neutral Lys side chain, both in the free inhibitor and in the complex and the values for the equilibrium constant for the Lys side chain that is protonated (Lys^+) both in the free inhibitor and in complex. Such values were calculated, and the resultant additivity cycles are shown in Figure 4. It



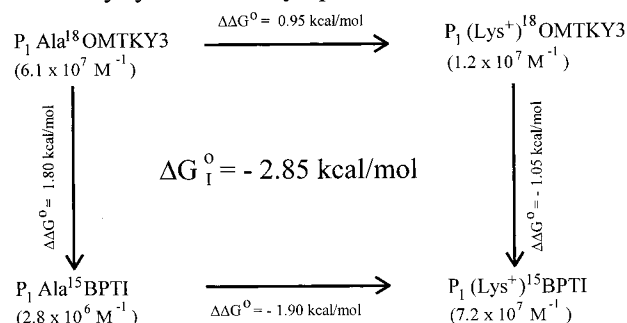


FIGURE 4: Interscaffolding additivity at P_1 between Lys and Ala variants of OMTKY3 and BPTI. Lys° and Lys^+ refer to the Lys state at high pH (>pK_f + 1) and low pH (<pK_c − 1) values, respectively.

is seen there that the interscaffolding comparison is additive for the P_1 Ala, Lys° residue pair as $\Delta G_I°$ is only 0.07 kcal/mol. In contrast, the Ala, Lys^+ cycle is nonadditive with a $\Delta G_I°$ of −2.85 kcal/mol. This dramatic nonadditivity is in agreement with the large discord seen in the three-dimensional structures of the two P_1 Lys^+ side chains. Comparisons could be made with X-ray work in two cases only. In the case of eglin c and OMTKY3, it led to an isostructural is isoenergetic conclusion. In the present case of Lys^{15}BPTI and Lys^{18}OMTKY3, the P_1 Lys^+ side chains are neither isostructural nor isoenergetic. It appears that a thermodynamic criterion for the identity of a side chain conformation to an already determined one is at hand. Thus, thermodynamic data are not just valuable in themselves, but useful to practitioners of other fields such as structural biology. Prior to the determination of an X-ray structure of the second member of a pair such as P_1 Leu^{18}OMTKY3 and P_1 Leu^{45}eglin c in complexes with CHYM or of P_1 $(Lys^+)^{18}$-OMTKY3 and P_1 $(Lys^+)^{15}$BPTI complexes with CHYM, an additivity cycle such as the one we have done should be performed. If it proves additive, as it did for the OMTKY3 and eglin c complexes, one of the two structures would be redundant. Of course, both structures were greatly needed to confirm the validity of the method on which the judgment of redundance is made. In contrast, the additivity cycle of Lys^{18}OMTKY3 and Lys^{15}BPTI complexes with CHYM was so grossly nonadditive that it should encourage the workers, if encouragement were needed, to obtain the second of the two structures. As pointed out here, these are very different.

Inferring the Structure of Transition State Complexes with Substrates. While saving labor in structure determinations is worthwhile, it is even more worthwhile to infer structures that cannot be determined directly. Such is the case for transition state complexes. As pointed out in the introductory section, they can be inferred by assuming that they are the same as those of complexes of standard mechanism, canonical protein inhibitors of serine proteinases in complex with their cognate enzymes. This implies an act of faith. Such faith further leads one to believe that the identical components of all standard mechanism, canonical inhibitors have the same structure. However, it has been shown (refs *14* and *15* and unpublished experiments) that the P_1 Lys^+ side chain of Lys^{15}BPTI and the P_1 Lys^+ side chain of Lys^{18}OMTKY3 have very different conformations in their complexes with CHYM. Which of these, if either, is the conformation of Lys^+ in the transition state complex of Suc-AAPK^+-pna with CHYM? To probe this question, pH-dependent measurements of k_{cat}/K_M of Suc-AAPK-pna and of Suc-AAPA-pna were carried out. Taking the ratio of these values at each pH eliminates the pH dependence of the overall reaction and leaves only the effects due to the P_1 Lys^+ side chain. It is clearly seen in Figure 3B that the difference pH dependence between Lys and Ala is very similar to that observed for OMTKY3 and very different from that of BPTI. This is seen more quantitatively from the pK values obtained from the fit. If the conformations of the three Lys^+ side chains in the three complexes were the same, one might expect identical pK_c values. This is clearly not the case; they are 7.35 for the substrate, 7.46 for Lys^{18}OMTKY3, and 9.63 for Lys^{15}-BPTI. We already know that P_1 Lys^+ side chains in Lys^{18}-OMTKY3 and in Lys^{15}BPTI differ in conformation. The pK_f value of Lys in Suc-AAPK-pna was found to be 10.51. This value agrees well with the pK_f (10.7) values of Lys in Lys^{18}-OMTKY3 and Lys^{15}BPTI.

We are now ready for interscaffolding additivity cycles involving substrate (Figure 5). Comparing P_1 Ala^{18} and P_1 $(Lys°)^{18}$ in OMTKY3, we find that the Lys° variant binds to CHYM 330 times [$\Delta\Delta G°$(A18K°) = −3.39 kcal/mol] more strongly than the Ala variant. The P_1 Lys° substrate is hydrolyzed 148 times [$\Delta\Delta G°$(AP$_1$K°) = −2.93 kcal/mol] more rapidly than the P_1 Ala substrate. The difference between these free energy changes, $\Delta G_I°$, is 0.46 kcal/mol, and the cycle is effectively additive (upper cycle in Figure 5). The additivity cycle comparing Ala to Lys^+ substitution in OMTKY3 and the substrate is also additive (lower cycle in Figure 5). In this case, the $\Delta G_I°$ value is 0.34 kcal/mol.

We conclude, therefore, that the conformation of Lys^+ in the transition state complex of Suc-AAPK-pna is the same as that found in Lys^{18}OMTKY3 (Figure 1A). Since the conformation of the P_1 Lys^+ side chain in the BPTI complex with CHYM (Figure 1B) is clearly different, we should expect that the Ala, Lys^+ additivity cycle for BPTI and the substrate should be nonadditive. This is very much the case as the value of $\Delta G_I°$ equals −3.19 kcal/mol, far out of the range acceptable for additivity (cycle not shown). On the other hand, the Ala, Lys° cycle is additive for BPTI and substrate ($\Delta G_I°$ = 0.30 kcal/mol). This should have been anticipated from transitivity and from Figures 4 and 5. In the absence of a three-dimensional structure of a complex between CHYM and any inhibitor with P_1 Lys°, we can only guess at the common conformation. We assume that it is the "DOWN" position of Scheidig et al. (*15*) and Capasso et al. (*14*) and the position of P_1 $(Lys^+)^{18}$ in the Lys^{18}-

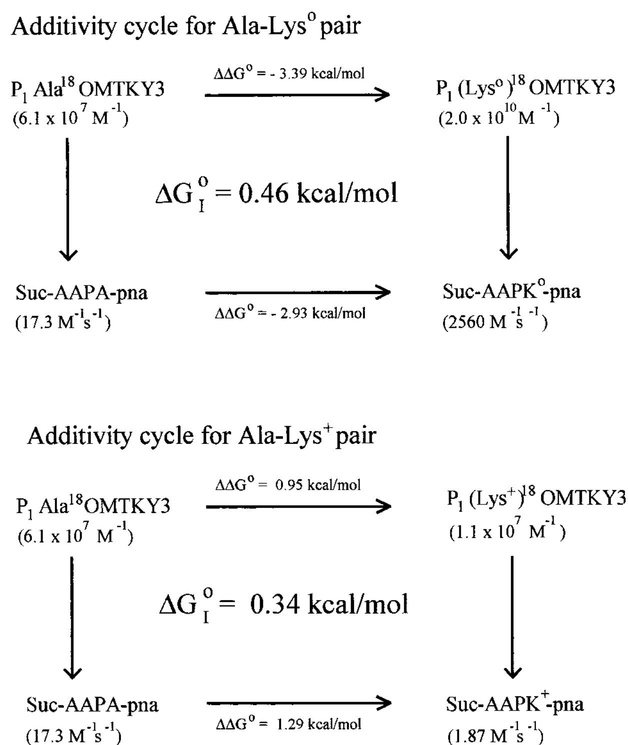


FIGURE 5: Additivity involving association of P_1 Lys^{18} and Ala^{18} variants of OMTKY3 with CHYM and the k_{cat}/K_M for the hydrolysis of Suc-AAPK-pna and Suc-AAPA-pna by CHYM. For other details, see the legend of Figure 4.

OMTKY3−CHYM complex. Therefore, the protonation of Lys^{15} in the BPTI−CHYM complex leads to a "DOWN" to "UP" position change. In the Lys^{18}OMTKY3−CHYM complex, Lys remains in the "DOWN" position in both Lys° and Lys^+ forms.

DISCUSSION

The interaction of standard mechanism, canonical protein inhibitors of serine proteinases attracted a great deal of attention of structural biologists. We are aware of at least 70 (not all of them have been deposited in the Protein Data Bank) completed structures of inhibitor−enzyme complexes. It seems like a large number for making structural comparisons. However, to place our additivity data on a firm structural basis, we need structures of complexes with the two different inhibitors interacting with the same enzyme. Among the 70 structures, 15 different enzymes are represented. Furthermore, we need inhibitor pairs with the same P_1 residue, but since inhibitors are hypervariable, many different P_1 residues are represented in the data set.

Nonetheless, a few pairs of structures are available for comparison. Qasim et al. (*17*) compared the CHYM complexes with OMTKY3 (*16*) and eglin c (*26*). Both have P_1 Leu, and their P_1 Leu residues in the complexes are isostructural. Additivity cycles were constructed (Figure 2). For seven reference pairs, they were additive, but for the Pro pair, they were not (Table 1). We concluded that the replacement of Ala (or any of the other seven normal residues) with Leu is isoenergetic in the OMTKY3 and in the eglin c structural frames.

Recently, a new pair of structures became available when structures of Lys^{15}BPTI (*14*, *15*) and Lys^{18}OMTKY3 (unpublished experiments) in complex with CHYM were determined. The conformation of the P_1 Lys^+ side chains is strikingly different in the two complexes. The Ala, Lys^+ cycle for these two inhibitors is dramatically nonadditive (Figure 4, lower cycle). These findings complete the following statement. "The P_1 residue conformation in complexes of two different standard mechanism, canonical, protein inhibitors with the same P_1 residue will be the same (isostructural) if and only if the additivity cycle involving these two inhibitors and another appropriate reference pair of P_1 variants of the inhibitors is additive". The word appropriate was inserted to avoid using Lys^+ (Lys^{18}-OMTKY3 and Lys^{15}BPTI) or Pro (Pro^{18}OMTKY3 and Pro^{45}-eglin c) variants as reference pairs.

An immediate question comes to mind. Why are P_1 Pro for OMTKY3 and eglin c and P_1 Lys^+ for BPTI and Lys^{18}-OMTKY3 not isostructural? We do not have any three-dimensional structures of CHYM complexes dealing with Pro, but on the basis of the structure of Pro^{18}OMTKY3 in complex[3] with SGPB and on the basis of K_a values for P_1 Pro variants interacting with six different enzymes, we can conclude that (1) insertion of a P_1 Pro into the S_1 pocket of most serine proteinases is highly deleterious, (2) it may be accompanied by changes in main chain−main chain interactions in positions other than P_1, and (3) these changes may differ as different inhibitors accommodate the deleterious binding of P_1 Pro.

The situation is much better for the Lys^+ comparison of $(Lys^+)^{18}$OMTKY3 and $(Lys^+)^{15}$BPTI complexes because three-dimensional structures of both are available (Figure 1). It is clear that $(Lys^+)^{15}$BPTI lowers its free energy considerably by adopting the "UP" position of Capasso et al. (*14*) and Scheidig et al. (*15*). Why does $(Lys^+)^{18}$OMTKY3 not do the same thing? Most standard mechanism, canonical protein inhibitors make two main chain to main chain hydrogen bonds between their P_3 residues and the enzyme. However, BPTI has P_3 Pro and, thus, can make only one. The resultant structure leaves more room for the N^{ζ} of P_1 Lys^+ in the "UP" position and allows it to form the two favorable hydrogen bonds. While this explanation initially looked attractive, it proved to be wrong. Recently, we acquired the P_3 Ala variant of Lys^{15}BPTI and subjected it to K_a determinations with CHYM as a function of pH. The behavior of this variant was virtually identical to that of Lys^{15}BPTI.

The most important aspect of the "if and only if" statement above is that it provides us with a technique for predicting whether the structure of a P_1 side chain in another inhibitor−enzyme complex will match the structure of an already determined one. Before this new method of saving the time of the beleaguered structural biologist becomes a reality, a number of related systems must be examined since the proposed technique is based on a yes or no decision; if the cycle is additive, isostructural behavior is expected, and if it is not additive, the structures are expected to differ. Our criterion at the moment is that $|\Delta G_I°|$ be <0.4 kcal/mol. This is probably too stringent. However, if it is relaxed too greatly, it will probably be robbed of its predictive value. We plead with our colleagues for a comparison of several more additive and nonadditive pairs.

Even though saving labor for structural biologists is highly laudable, it is obviously less attractive than measuring something that cannot be measured directly. As was pointed

out in the introductory section, there was a long-standing belief that the structures of enzyme−inhibitor complexes are good models of enzyme−substrate complexes. However, an act of faith was required. We have shown that an additivity test can reduce the need for faith in this matter considerably.

This paper provides a much stronger example. If all enzyme−inhibitor complexes are good enzyme−substrate transition state models, then all enzyme−inhibitor complexes should have the same structure. This is not the case for P_1 Lys^+. An additivity test provides an unequivocal answer that in this case the $(Lys^+)^{18}$OMTKY3 complex with CHYM provides a better model for the enzyme−substrate transition state than does the $(Lys^+)^{15}$BPTI complex with CHYM.

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