Transition-State Characterization: A New Approach Combining Inhibitor Analogues and Variation in Enzyme Structure†

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ABSTRACT: A new strategy of potentially broad application for probing transition-state (TS) analogy in enzymatic systems is described in this paper. The degree to which a series of phosphonate inhibitors act as TS analogues of rat carboxypeptidase A1 has been determined for the wild-type enzyme, for the R127K, R127M, and R127A mutants, and for the R127A mutant in the presence of 0.5 M guanidine hydrochloride. The impact that the mutations have on the inverse second-order rate constants (K_m/k_{cat}) for substrate hydrolysis is mirrored by the effect on the inhibition constants (K_i) for the corresponding phosphonate inhibitors. These results demonstrate that the phosphonate moiety mimics some of the electronic as well as the geometric characteristics of the TS. A similar but distinctly separate correlation is observed for tripeptide analogues in comparison to analogues of the dipeptide Cbz-Gly-Phe, reflecting an anomalous mode of binding for the latter system. The selective rate increases and corresponding enhancement in inhibitor binding observed on addition of 0.5 M guanidine hydrochloride to the R127A mutant indicate that the exogenous cation can assume the role played by Arg-127 in stabilizing the TS and in providing substrate selectivity at the P_2 position.

Since the concept was first recognized by Pauling (1946) and implemented by Lienhard (1973) and Wolfenden (1976), transition-state (TS)[1] analogy has proved to be a powerful element in the design of high-affinity, reversible enzyme inhibitors. Indeed, the most tightly bound, small molecule inhibitors yet reported derive their affinity in part from mimicry of the respective TS or high-energy intermediates along the reaction pathways [for example, methionine sulfoximine phosphate, estimated $K_i < 10^{-19}$ M against glutamine synthetase (Maurizi & Ginsburg, 1982); 2-carboxyarabinitol 1,6-diphosphate, K_i = 190 fM against ribulose-1,6-diphosphate carboxylase (Schloss, 1988); and Cbz-Phe-Val^P-(O)Phe, K_i = 11 fM against bovine carboxypeptidase A (Kaplan & Bartlett, 1991)]. While usually invoked as a description of the design process, TS analogy can be confirmed by a correlation between the K_i values of a series of inhibitors and the K_m/k_{cat} values of the corresponding substrates, subject to certain qualifications (Wolfenden, 1976; Thompson, 1973; Bartlett & Marlowe, 1983). This correlation has been demonstrated most extensively for peptidases, and for the zinc peptidases in particular (Bartlett & Marlowe, 1983; Mookthiar et al., 1988; Hanson et al., 1989), because these enzymes permit considerable substrate variation and consequently a broad range of K_m/k_{cat} values.

The catalytic mechanism of the zinc peptidases involves direct addition of a water molecule to the peptide linkage, with the consequent formation of a tetrahedral species (**2**, Figure 1) that collapses to the products after proton transfer from the attacking oxygen atom to the departing nitrogen. These enzymes are effectively inhibited by tripeptide derivatives containing a tetrahedral phosphonamidate or phosphonate moiety in place of the scissile linkage [e.g., **1**, Cbz-Ala-Gly^P-(O)Phe, an inhibitor of carboxypeptidase A (CPA)] (Hanson et al., 1989; Bartlett & Marlowe, 1987; Thorsett et al., 1982; Grobelny et al., 1985; Karanewsky et al., 1988, and references therein). For both thermolysin (Bartlett & Marlowe, 1983) and CPA (Hanson et al., 1989), investigations of substituent effects at the P_2' or P_2 and P_3 positions, respectively, demonstrate that these inhibitors are TS analogues. The fact that side-chain variation at positions remote from the site of cleavage affects TS and ground-state binding differently suggests that in each case the substrate reorients in the active site as the geometry at the scissile center changes from the trigonal ground state to the tetrahedral transition state. The TS analogy so far observed for the phosphorus-containing peptide analogues seems to be a reflection of their geometric similarity to the transition-state structure.

There are other, important differences between the ground and transition states in the zinc peptidase-catalyzed reaction, for example, in the electronic character of the carbonyl oxygen of the scissile amide. Formally uncharged in the ground state, this atom acquires a negative charge in the TS that is stabilized via coordination with the active-site zinc cation and, in the case of CPA, through electrostatic interaction with the guanidinium group of Arg-127, as shown in structure **2**. This role for Arg-127, first proposed by Lipscomb and Christianson (1989), was recently demonstrated by Phillips et al. (1990) using a series of mutants of rat CPA-1 in which Arg-127 is replaced with Lys (R127K), Met (R127M), and Ala (R127A). These mutations result in modest decreases in affinity of the enzyme for the ground-state form of typical substrates [K_m(mutant)/K_m(wild type) $\leq$ 10] but produce dramatic losses in catalytic efficiency [[K_m/k_{cat}(mutant)]/[K_m/k_{cat}(wild type)] $\approx 10^3$–10^4]. The availability of these mutants afforded an unusual opportunity to probe the TS analogy of phosphonate inhibitors, since variation in the structure of the protein could

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[1] Abbreviations: TS, transition state; CPA, carboxypeptidase A; ZFG^P(O)F = Cbz-Phe-Gly^P-(O)Phe, the phosphonate analogue of Cbz-Phe-Gly-Phe in which the Gly-Phe peptide linkage (CO–NH) is replaced with a phosphonate monoester (PO_2^--O) (related analogues are abbreviated similarly).

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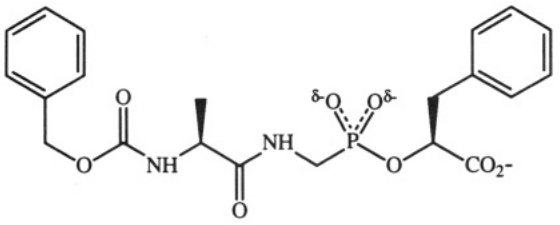
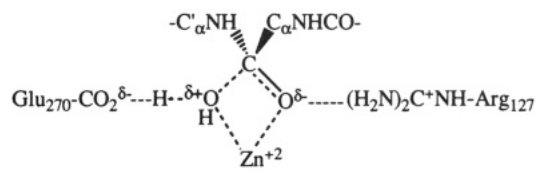


1: Cbz-Ala-Gly^{P}-(O)Phe



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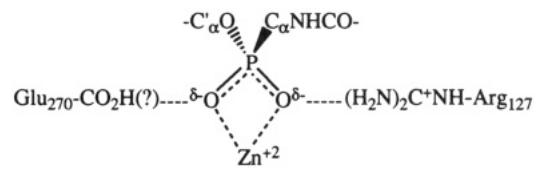

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FIGURE 1: Structures of representative phosphonate inhibitor (**1**), putative transition-state structure (**2**), and schematic of phosphonate mimicry (**3**). In **3**, protonation of the Glu_{270} carboxyl is assumed but unproven.

provide another dimension to the K_i vs K_m/k_{cat} comparison. Moreover, the effect of structural variation in the vicinity of the tetrahedral center would indicate to what extent the phosphonate moiety imitates the electronic, as opposed to simply geometric, characteristics of the TS.

Experimental Procedures

Materials. Wild-type and mutant rat CPA-1 were prepared as described previously (Phillips et al., 1990). The substrates ZGF and ZGGF were obtained commercially; ZFGF and ZAGF were prepared by standard methods; full details are presented in the supplementary material. The synthesis of the phosphonate inhibitor ZAG^{P}(O)F has been described (Hanson et al., 1989). The analogues ZFG^{P}(O)F and ZGG^{P}(O)F were prepared similarly; full details of the syntheses of these analogues and of ZG^{P}(O)F (Jacobsen & Bartlett, 1981) are presented in the supplementary material.

Enzyme Assays. The activity of wild-type and mutant rat CPA-1 in the presence and absence of inhibitors was determined in a buffer containing 0.5 M NaCl and 50 mM Tris, pH = 7.5 at 25 °C, as described previously (Phillips et al., 1990; Hilvert et al., 1986). The substrate used for the inhibition studies with wild-type enzyme was furanacryloyl-Phe-Phe, monitored at 330 nm; for the mutant enzymes, the substrate was Cbz-Gly-Phe (ZGF), monitored at 222 nm. Concentrations of substrate stock solutions were determined from absorbance: $\epsilon_{305.5}$ = 25 000 M^{-1} cm^{-1} for furanacryloyl-Phe-Phe and ϵ_{222} = 2470 M^{-1} cm^{-1} for ZGF. All assay mixtures with ZGF as substrate were allowed to stand at 25 °C for 10 min before the addition of enzyme. Data from <20% reaction were evaluated using the programs ENZFITTER (Leatherbarrow, 1987) or HYPERO (Cleland, 1979). Inhibition constants (K_i values) were determined from plots of v_0/v_i vs [I] (Segel, 1975), except for the combinations of ZFG^{P}(O)F and ZAG^{P}(O)F with the R127A mutant in the presence of guanidine, when the data were examined using a Henderson plot (Henderson, 1972). The data are presented in the supplementary material.

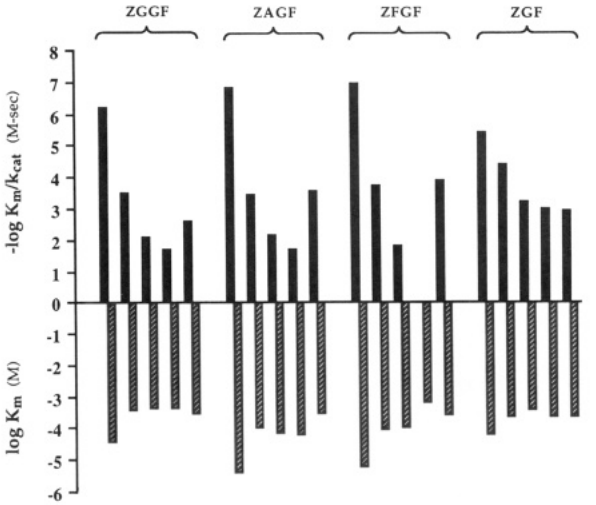

FIGURE 2: Influence of mutation at position 127 of rat CPA on kinetic parameters for hydrolysis of tripeptide substrates. Bars are clustered according to substrate, representing wild-type enzyme, R127K, R127M, and R127A mutants, and R127A mutant in presence of 0.5 M guanidine hydrochloride, respectively. Note: ZFGF is not a substrate for the R127A mutant in the absence of guanidine hydrochloride; value given for K_m corresponds to K_i.

Results and Discussion

The catalytic parameters for cleavage of a series of tripeptide substrates by wild-type and mutant rat CPA are given in Table I and displayed graphically in Figure 2. It is readily apparent that mutation of Arg-127 has a differential effect on ground-state binding, indicated by log K_m, and on TS binding, reflected in log (K_m/k_{cat}), as noted previously for similar substrates (Phillips et al., 1990).

There is a direct correlation between the inhibition constants of the phosphonate peptides and the substrate K_m/k_{cat} values with these enzyme variants (Table I, Figure 3); the comparison of K_i with K_m alone reveals no such correlation (Figure 4). The relationship in Figure 3 encompasses 5 orders of magnitude in binding affinity, reflecting differences of >7 kcal/mol. The slope of the line for the tripeptide correlation is 1.03 (lower line, Figure 3), indicating that the phosphonates, with a formal $^{1}/_{2}-$ on each oxygen, are as sensitive as the transition states to the amino acid side chains at position 127. Although the closeness of this slope to 1 is a qualitative reflection of the "degree" of TS analogy, the value of 1.03 cannot be used to estimate the charge distribution in the TS quantitatively, since van der Waals as well as electronic effects contribute to the ligand/side-chain interactions. Nevertheless, it is clear that these derivatives mimic closely not only the geometric aspects of the TS but also one of its key electronic characteristics as well.[2,3]

[2] It should be apparent that not all aspects of the TS can be mimicked by the phosphonates; in particular, the electronic nature of the oxygen in the attacking water molecule is unlikely to be reproduced by the other oxyanion of the phosphonate, as shown in **3**.

[3] It is interesting to note that the TS correlation appears to hold even for the most rapidly cleaved substrates with the wild-type enzyme (the lowest two points in Figure 3). We have suggested previously (Bartlett & Marlowe, 1987) for substrates with $k_{cat}/K_m > 5 \times 10^6$ M^{-1} s^{-1} that substrate association may be partially rate limiting (cf. Brouwer & Kirsch, 1984) and that the assumptions underlying TS analogy, namely, that $K_m = K_s$, may break down.

Table I: Effect of Arg-127 Mutation on Kinetic and Inhibition Parameters[a]

enzyme	substrate (RCO-Gly-Phe) or inhibitor [RCO-Gly^P-(O)Phe]			
	RCO = Cbz-Gly	RCO = Cbz-Ala	RCO = Cbz-Phe	RCO = Cbz
K_m (μM)				
WT	36 ± 4	3.9 ± 0.5	6.1 ± 1.0	61 ± 6
R127K	360 ± 30	96 ± 16	92 ± 12	230 ± 20
R127M	400 ± 100	71 ± 8	99 ± 34	390 ± 40
R127A	400 ± 84	64 ± 20	630 ± 250[b]	220 ± 25
R127A-g[c]	274 ± 26	280 ± 57	266 ± 110	210 ± 40
k_{cat} (s^{-1})				
WT	53 ● 3	24 ± 0.6	51.7 ± 1.5	15 ± 1
R127K	1.1 ± 0.05	0.25 ± 0.02	0.5 ± 0.02	5.70 ● 0.03
R127M	0.049 ± 0.01	0.01 ± 0.0004	0.0065 ± 0.0010	0.64 ± 0.01
R127A	0.02 ± 0.003	0.003 ± 0.0003	–[b]	0.20 ● 0.01
R127A-g[c]	0.11 ± 0.007	1.0 ± 0.11	1.9 ± 0.5	0.17 ± 0.002
K_m/k_{cat} (μM s^{-1})				
WT	0.68 ● 0.08	0.159 ± 0.021	0.118 ● 0.019	4.1 ± 0.5
R127K	327 ± 30	384 ± 73	184 ± 25	40 ± 0.36
R127M	8160 ± 2600	7100 ± 830	15200 ± 5600	609 ± 61
R127A	20000 ± 5000	21300 ± 6800	–[b]	1100 ± 130
R127A-g[c]	2490 ± 274	280 ± 61	140 ± 67	1250 ± 200
K_i (nM)				
WT	4.19 ± 0.09	0.156 ● 0.003	0.081 ± 0.004	30.9 ± 0.39
R127K	1240 ± 43	1560 ± 31	576 ± 14	739 ± 14
R127M	26900 ± 890	35200 ± 1200	31400 ± 1100	7500 ± 280
R127A	22500 ● 950	28900 ± 720	12000 ± 520	12300 ± 210
R127A-g[c]	10000 ± 220	1130 ± 48	126 ± 10	8030 ± 220

[a] Kinetic and inhibition constants were determined at 25 °C, pH 7.5 (50 mM Tris and 0.5 M NaCl buffer); inhibitors were assayed using Cbz-Gly-Phe as substrate, except wild-type CPA, which was assayed using furanacryloyl-Phe-Phe as substrate. [b] Cbz-Phe-Gly-Phe binds nonproductively to the R127A mutant in the absence of guanidine; value given for K_m corresponds to K_i value. [c] R127A mutant in the presence of 0.5 M guanidine hydrochloride.

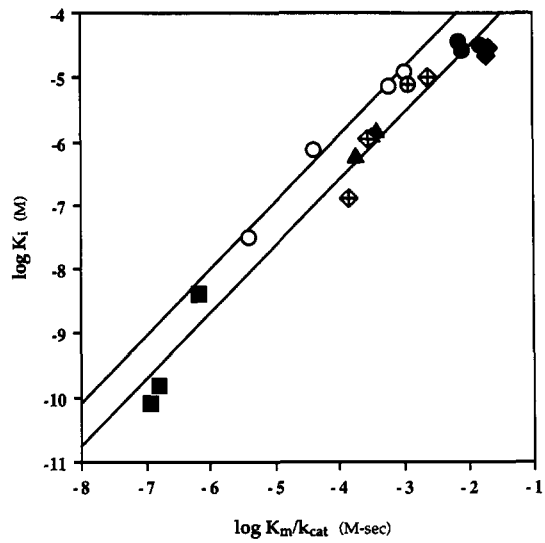

FIGURE 3: Correlation of K_i values for phosphonate inhibitors with K_m/k_{cat} values for peptide substrates for rat CPA (tripeptide analogues: ■ = wild-type enzyme, ▲ = R127K mutant, ● = R127M mutant, ◆ = R127A mutant, ◈ = R127A mutant in the presence of 0.5 M guanidine hydrochloride; ○ = Cbz-Gly-Phe analogues with all mutants; ⊕ = Cbz-Gly-Phe analogues with R127A mutant in the presence of 0.5 M guanidine hydrochloride). Line for tripeptide analogues represents least squares fit to solid symbols (slope = 1.03, r^2 = 0.980); line for Cbz-Gly-Phe analogues is least squares fit to open circles (slope = 1.05, r^2 = 0.985).

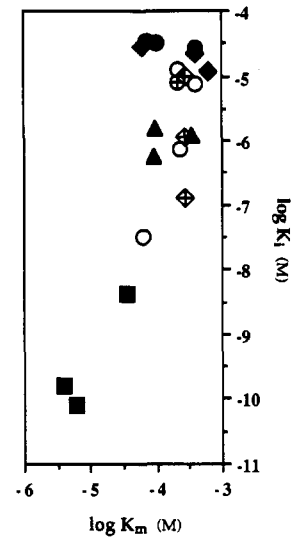

FIGURE 4: Comparison of K_i values for phosphonate inhibitors and K_m values for peptide substrates for rat CPA; symbols are as in Figure 3.

Some of the catalytic handicap from loss of the side chain in the R127A mutant can be relieved by 0.5 M guanidine hydrochloride (Phillips et al., 1991). Experiments with varying amounts of guanidine hydrochloride established that a concentration of 0.5 M is sufficient to saturate the synergistic effects on substrate hydrolysis and inhibitor binding by the R127A mutant without denaturing the enzyme. Methyl- and ethylguanidine, but not primary aliphatic amines, are also effective in rescuing the R127A mutant. Although guanidine does not affect substrate binding to a significant extent, it increases k_{cat}/K_m by up to 400-fold. Presumably, this cation occupies the pocket vacated by the Arg-127 side chain and assumes the role that it plays as part of the wild-type enzyme. The recruitment of a detached functional group in this manner has been observed previously for the K258A mutant of aspartate aminotransferase in the presence of exogenous amines (Toney & Kirsch, 1989). Importantly, guanidine hydrochloride increases the binding of the phosphonate inhibitors to the same extent that it accelerates hydrolysis of the substrates (Figure 3); this indicates that the structural and electronic correspondence of the inhibitors and transition states is maintained in the presence of this auxiliary functional group.

In addition, it is apparent from the data in Figure 3 that CPA discriminates between Gly, Ala, and Phe in the P_2 position only in the TS and only when there is a guanidinium moiety present, either as part of the wild-type enzyme or as

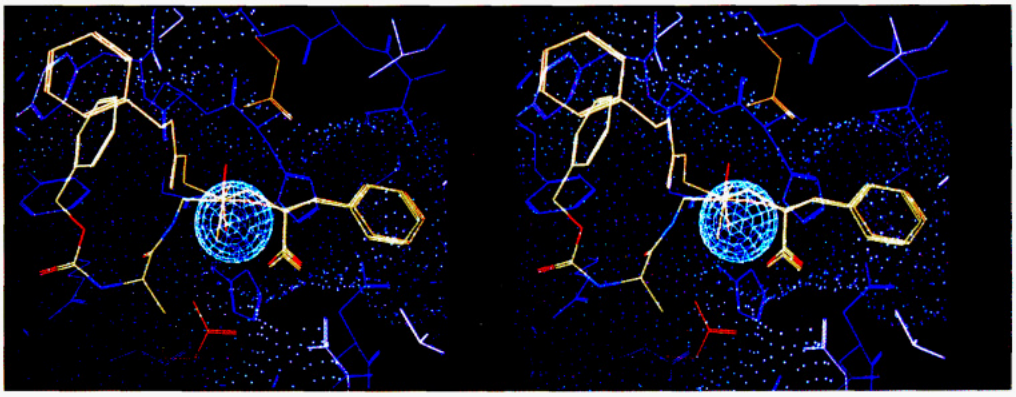

FIGURE 5: Stereoview of the active site of bovine CPA, showing the relationship between the zinc cation (cyan sphere), the Glu-270 and Arg-127 side chains (yellow and orange, respectively), and the bound orientations of Cbz-Ala-Gly^P-(O)Phe (colored by atom) (Kim & Lipscomb, 1990, 1991) and Cbz-Gly^P-Phe (yellow) (Christianson & Lipscomb, 1988). The dotted surface represents the solvent-accessible surface (Connolly, 1983a,b) for the enzyme in its complex with Cbz-Ala-Gly^P-(O)Phe. The side chains and surface of Ile-247 and Tyr-248, which cover the active site in this view, have been cut away to expose the zinc atom and P_1' residue.

an auxiliary to the R127A mutant. For the mutant enzymes, variation at this position produces less than a 3-fold difference in either k_{cat}/K_m for substrate hydrolysis or K_i for inhibitor binding, whereas differences up to 80-fold are found for the wild-type enzyme or the R127A/guanidine hydrochloride combination.[4] The X-ray crystal structures of complexes of bovine CPA with Cbz-Ala-Gly^P-(O)Phe and other phosphonate tripeptide inhibitors (Kim & Lipscomb, 1990, 1991) reveal that the guanidinium group of Arg-127 forms the floor of the S_2 side-chain binding pocket (Figure 5). The function of the Arg-127 side chain in catalysis is therefore not limited to its electrostatic role in stabilizing the oxyanion; its interaction with the P_2 side chain, specifically the change in this interaction from ground state to TS, is the basis for substrate selectivity at this position.

The importance of the interaction between the P_2 residue and the side chain at position 127 is also underscored by the observation that Cbz-Phe-Gly-Phe is an inhibitor, and not a substrate, for the R127A mutant. The P_2 side chain of Cbz-Phe-Gly-Phe may occupy the pocket created by removal of the arginine side chain, resulting in nonproductive alignment of the peptide in the active site. In restoring the ability of the R127A mutant to hydrolyze this substrate, the guanidinium ion may play an additional role by displacing the errant P_2 side chain from the abnormal orientation.

The behavior of Cbz-Gly-Phe as a substrate of the various CPA mutants is anomalous, in that alteration of residue 127 does not have as great an impact on the rate of hydrolysis of this dipeptide as it does on the tripeptide substrates. For example, whereas k_{cat}/K_m for the tripeptides is reduced by 10^4–10^5 in the R127A mutant in comparison with wild-type CPA, that for Cbz-Gly-Phe is reduced only 270-fold. In addition, guanidine hydrochloride does not improve the ability of the R127A mutant to hydrolyze this substrate, in contrast to its effect on tripeptide cleavage. Nevertheless, although the behavior of Cbz-Gly-Phe as a substrate is peculiar, the TS analogy of the corresponding phosphonate analogue is maintained, with the correlation of K_i vs K_m/k_{cat} generating a line of slope 1.05 (upper line in Figure 3). While the structure of the transition states for Cbz-Gly-Phe and the tripeptides must be similar, since they are both equally well mimicked by the corresponding phosphonates, there must also be a fundamental difference in the way that they interact with the enzyme and in particular with the side chain of residue 127.

Christianson and Lipscomb have shown that the [(carbobenzoxy)amino]methyl group of the phosphonamidate Cbz-Gly^P-Phe occupies the S_2 side chain pocket in the active site of bovine CPA (Christianson & Lipscomb, 1988) rather than extending toward the S_2 site as is observed for tripeptide phosphonates (Figure 5); in addition, the phosphonate–zinc coordination geometry is quite different for the Cbz-Gly^P-Phe complex in comparison to the tripeptide phosphonate complexes (Kim & Lipscomb, 1990, 1991). The previous rationalization (Hanson et al., 1989) of the anomalous behavior of glycine-containing dipeptide substrates of CPA appears to be confirmed: both the substrate and the phosphonate inhibitors adopt this unusual orientation in the active site, such that the nature of both ground-state and TS interactions with the enzyme is different than for extended or α-substituted substrates.

While the term "transition-state analogue" can be useful as a conceptual descriptor, in many cases it is important to demonstrate rigorously that an inhibitor mimics, in some manner, the TS in its interactions with the target enzyme. For example, interpretation of the structure of an enzyme–inhibitor complex is enhanced if there is confidence that the inhibitor is indeed a TS analogue (Lolis & Petsko, 1990). As noted previously (Bartlett & Marlowe, 1983; Wolfenden, 1976), a single point of comparison between inhibitor and substrate is not sufficient to demonstrate TS analogy. Absolute affinity cannot be the criterion, because the dissociation constant expected for a "perfect" TS analogue can only be estimated in most cases and because it is similarity to the TS, not perfect mimicry, that is usually at issue. These problems are circumvented by comparison of the binding and kinetic properties across a series of inhibitors and their corresponding substrates, through the correlation of K_i and K_m/k_{cat} values, respectively. Unfortunately, this approach cannot be applied to enzymes with stringent substrate specificity, and few alternative approaches have been explored. In one instance, the disparate influence of pH on substrate hydrolysis and inhibitor binding for β-glucosidase has been used to argue against TS analogy for 1-deoxynojirimicin and D-glucono-1,5-lactam (Dale et al., 1985). "Slow-binding" inhibition has been suggested as a characteristic of TS analogue inhibitors of nucleoside de-

[4] Selectivity at the P_2 position is manifested differently for wild-type enzyme and the R127A–guanidine combination. For the former, the effect arises primarily in K_m, whereas for the latter it appears in k_{cat}. However, this distinction is blurred by the recognition that $K_m \neq K_S$ for hydrolysis of Cbz-Phe-Gly-Phe and Cbz-Ala-Gly-Phe by wild-type CPA; i.e., the measured value for K_m reflects more than ground-state binding.

aminases (Frieden et al., 1980; Ashley & Bartlett, 1984); however, this subjective designation is not readily quantifiable, nor does it appear to be generally applicable [see, for example, Bartlett and Marlowe (1987)]. An approach is required that can reveal differences in the way the enzyme interacts with the substrate in the ground and transition states. Site-specific mutagenesis allows these interactions to be varied for a single substrate, thus enabling the correlation between inhibitor K_i and substrate K_m/k_{cat} values to be probed for a single substrate/inhibitor combination.

In conclusion, the binding affinity of phosphonate di- and tripeptide analogues to CPA correlates closely with that of the transition states for the corresponding substrates. This correlation is maintained for a series of mutant enzymes, in the presence of auxiliary prosthetic groups, and in the face of different modes of substrate binding. The results demonstrate extensive electronic and geometric similarities between a class of TS analogues and the transition states they mimic. These inhibitors therefore provide a valuable structural model for the elusive tetrahedral TS species, as well as a probe of the electronic nature of the TS itself. In a more general sense, mutagenesis of enzyme structure may prove to be a valuable approach for evaluating TS analogy in other enzyme–inhibitor systems, particularly when only a limited range of substrates is accepted by the enzyme or when only a single inhibitor is available.

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SUPPLEMENTARY MATERIAL AVAILABLE

Details of synthesis of ZFGF, ZAGF, ZFG^P(O)F, ZGG^P-(O)F, and ZG^P(O)F and 23 figures showing assays of all inhibitors (19 pages). Ordering information is given on any current masthead page. This material also appears in the microfilm edition of the journal, which is available in many libraries.

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