gem-Dialkyl Succinic Acids: A Novel Class of Inhibitors for Carboxypeptidases†

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ABSTRACT: *gem*-Dimethylsuccinic acid and its higher homolog, 2-methyl-2-ethylsuccinic acid (MESA) are highly potent inhibitors of both carboxypeptidase A (CPA) and B. The inhibition constant of MESA for CPA (0.11 μM for the racemic mixture) is remarkable considering the relatively simple structure of the compound. The molecular feature which is crucial for high affinity binding to both carboxypeptidases appears to be the nonpolar *gem*-dialkyl locus. The structure of the complex between MESA and CPA has been determined by X-ray crystallography to 2.0 Å resolution and shows the *R* enantiomer of the inhibitor to be bound in a generally substrate-like manner. The carboxymethyl group is coordinated to the Zn ion in the active site, and the *gem*-dialkyl locus corresponds in position to the α-carbon of the C-terminal amino acid in a peptide substrate. The methyl group of the inhibitor occupies a cavity in the enzyme which is apparently not filled upon substrate-binding. We postulate that this cavity (the α-methyl hole) is designed to allow the proximal Glu-270 residue to undergo a critical movement during catalysis. The hydrophobic nature of the above cavity may play a role in modulating the reactivity of this residue. These results suggest that similar *cenophilic* (empty-loving) inhibitors may be found for other enzymes.

Bovine pancreatic carboxypeptidase A (CPA)[1] has been the subject of extensive studies aimed at elucidating its structure and mechanism of action [for reviews, see Vallee et al. (1983) and Christianson and Lipscomb (1989)]. These studies, together with similar work on the analogous endopeptidase thermolysin [for a review, see Mathews (1988)], have produced a detailed description of the active site and some understanding of the functional role of the zinc ion in these types of proteases. In many of the above investigations, the use of specific inhibitors (Christianson & Lipscomb, 1986; Shoham et al., 1988; Kim & Lipscomb, 1990) has been valuable in determining the mode of substrate binding and the interactions which may facilitate catalysis. Potent inhibitors of zinc proteases are also of practical importance, owing to the physiological significance of enzymes such as the matrix metalloproteinases (Birkedal-Hansen et al., 1990) and peptidases involved in the regulation of hormone activity (Skeggs et al., 1956; Malfroy et al., 1978). In particular, inhibitors of the angiotensin-converting enzyme such as captopril (Cushman et al., 1977) and enalapril (Patchett et al., 1980), have proven to be clinically effective toward hypertension. Consequently, the discovery of new inhibitors for zinc proteases is of considerable interest from both theoretical and practical perspectives.

In this paper, we report a novel type of potent inhibitors for carboxypeptidases A and B. These inhibitors are unusual in that they contain a *gem*-dialkyl locus not found in the corresponding structure of substrates. To understand the physical basis for the high affinity of these inhibitors, we have determined the X-ray crystallographic structure of the complex between one of these inhibitors and CPA. Our results suggest the presence of a hydrophobic cavity in the native enzyme which may be required to accommodate a movement of the critical Glu-270 residue during catalysis (Christianson & Lipscomb, 1989). The extra methyl group in the inhibitor appears to enhance binding by exploiting potential interactions in this cavity. The deliberate introduction of such *cenophilic*[2] substituents may represent a novel approach to the design of high affinity inhibitors.

MATERIALS AND METHODS

Source of Enzymes and Reagents. Bovine pancreatic carboxypeptidase A (EC 3.4.17.1) and porcine pancreatic carboxypeptidase B (EC 3.4.17.2) were obtained from Sigma Chemical Co. (St. Louis, MO). The substrates described below for enzyme assays as well as Tris and succinic acid were also supplied by Sigma. 2,2-Dimethylsuccinic acid, racemic 2-methyl-2-ethyl succinic acid (MESA), and other inhibitors were products of Aldrich Chemical Co. (Milwaukee, WI).

Enzyme Activity Assays. Samples of CPA were diluted into 0.1 M Tris-HCl, pH 7.5, containing bovine serum albumin (0.2 mg/mL) and 0.1 mM $ZnCl_2$, and aliquots (final enzyme concentration, 0.6 nM) were added to solutions of the substrate, *N*-(3-[2-furyl]acryloyl)-Phe-Phe in 0.1 M Tris-HCl, pH 7.5, containing 0.3 M NaCl. Catalytic activity was determined by monitoring for 2 min the decrease in absorbance at 335 nm upon hydrolysis of the substrate (Petersen et al., 1982). For inhibition studies, the substrate concentration was varied between 0.04 and 0.2 mM. Carboxypeptidase B was diluted in the same way and assayed in 0.1 M

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[1] Abbreviations: CPA, carboxypeptidase A; CPB, carboxypeptidase B; MESA, 2-methyl-2-ethylsuccinic acid; LBSA, L-benzylsuccinic acid.

[2] From Greek, meaning empty-loving.

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Table 1: Crystallographic Data and Refinement Statistics

resolution range	10.0−2.0 Å
measured reflections	67 192
unique reflections	34 415
completeness	98%
Rsym[a]	8.7%
refinement resolution range	5−2.0 Å
no. of non-hydrogen protein atoms	4874
no. of inhibitor and Zn atoms	24
no. of water molecules	160
reflections (test set)	28 963 (2896)
R factor (R_{free})	20.4 (26.2)
Average *B* value	13.25 Å^2
rmsd bond lengths	0.012 Å
rmsd bond angles	3.11°
rmsd between monomers in the asymmetric unit[b]	0.37 Å

[a] Rsym = $\sum|I_{obs} - I_{ave}|/\sum I_{ave}$. [b] All C-α atoms used.

Table 2: Inhibition by Succinic Acid Derivatives

derivative	inhibition constant (K_i, mM) CPB	CPA
2,2-dimethyl	0.028 ± 0.002	0.0017 ± 0.0002
2-ethyl-2-methyl (*R*,*S*)	0.0045 ± 0.0004	0.000 11 ± 0.000 01
2-benzyl (*R*)	0.0012−0.008[a]	0.000 45[b]
2-hydroxy-2-methyl (*S*) (citramalic acid)	8 ± 1	0.26 ± 0.01
2-hydroxy-2-methyl (*R*)	17 ± 2	11 ± 2
monomethyl	4 ± 0.6	0.27 ± 0.04
2,3-dimethyl (meso)	44 ± 5	7 ± 0.6
succinic acid (unsubstituted)	28 ± 4	4 ± 0.8

[a] From the work of Zisapel and Sokolovsky (1974). [b] From the work of Byers and Wolfenden (1973).

Tris-HCl, pH 7.5, containing 0.5 M NaCl and 0.4−2 mM of the substrate *N*-(3-[2-furyl]acryloyl)-Ala-Lys (Plummer & Kimmel, 1980) at a final enzyme concentration of 12 nM. The reaction was monitored in a similar way at 25 °C by measuring the decrease in absorbance at 345 nm for 2 min.

Determination of Inhibition Constants. Following normal practice (Dixon, 1953), the K_i for all inhibitors reported in Table 2 were determined by four series of assays each with a different substrate concentration. Within each series, five assays were conducted at inhibitor concentrations which varied over a 5-fold range. The data for the initial rate (v_i) were analyzed by nonlinear regression using the computer program Enzfitter (Leatherbarrow, 1987) to obtain inhibition constants and the associated standard errors. In a few instances, we have also analyzed similar enzyme inhibition data using combination plots which have been developed recently in this laboratory (Chan, 1995). A particularly convenient version of these plots (Chan, W. W.-C., manuscript in preparation) is based on the following linear transformation of the rate equation for competitive inhibition:

$$\frac{V_{max}[S]}{v_i K_m} - 1 - \frac{[S]}{K_m} = \frac{[I]}{K_i}$$

A plot of the composite function on the left-hand side of this equation versus [I] is presented in Figure 1. It can be seen that all data points for each inhibitor could be accommodated on a single line even though the concentrations of both the inhibitor and the substrate were varied over a large range. These results confirm the competitive nature of the inhibition for the two inhibitors tested here. Once the V_{max} and K_m values have been determined, the K_i can be estimated from the slope of the above plot using only five assays. In the examples given here, linear regression of the data in Figure 1 yielded K_i values (4.5 ± 0.6 μM for MESA and 29 ± 4 μM for 2,2-dimethylsuccinic acid) essentially the same as those obtained by Dixon plots (4.5 ± 0.4 μM and 28 ± 2 μM, respectively, see Table 2), but with lower accuracy as expected from the smaller database. A more extensive comparison between combination plots and traditional ones based on a family of lines has been reported for multiple inhibitors (Asante-Appiah & Chan, 1996). An attractive feature of the above plot is that the experimental data for two or more inhibitors can be conveniently displayed together in one diagram and the slopes (=1/K_i) provide a good visual illustration of their potency (as demonstrated in Figure 1).

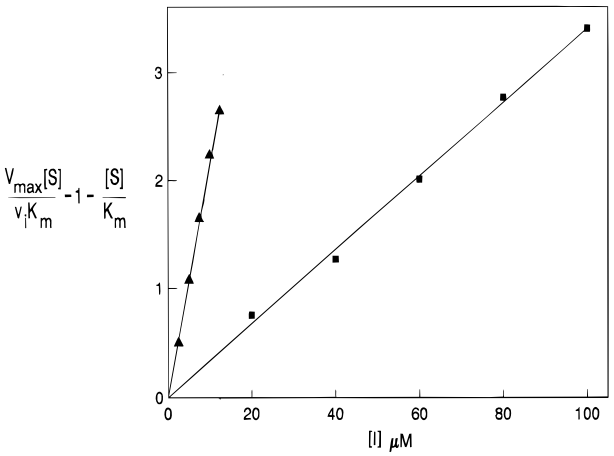

FIGURE 1: Inhibition of carboxypeptidase B by *gem*-dialkyl succinic acids. The abscissa represents the concentration of the inhibitor 2,2-dimethyl-succinic acid (■) or 2-methyl-2-ethylsuccinic acid (MESA, ▲). The substrate concentrations for these assays were varied over a 5-fold range in step with the inhibitor concentrations. A parallel series of activity measurements were made in the absence of inhibitor to provide the values for V_{max} and K_m in the calculation of the composite function used in this plot. As explained in the Materials and Methods, the linearity of the data indicates a competitive pattern of inhibition and the slope represents the reciprocal of the dissociation constant.

X-ray Structural Determination. Crystals of the enzyme−inhibitor complex were obtained by dialysis of a CPA solution (21 mg of protein/mL) containing MESA (43.7 mM) against Tris-HCl buffer (20 mM, pH 7.5) containing 1.2 M LiCl. After 6 h at room temperature, the concentration of LiCl was reduced to 0.8 M and then to 0.05 M after another 4 h. Further dialysis for 4 h yielded needle-shaped crystals. X-ray diffraction data (see Table 1) were collected at room temperature on a RAXIS-II area detector (Rigaku RU200 with a SUPER double focusing mirror) using CuKα radiation. The crystallographic parameters are a = 49.55 Å, b = 70.53 Å, c = 48.46 Å, α = 90.44°, β = 110.36°, and γ = 73.46°; space group *P*1; and two molecules per asymmetric unit. The structure was solved by molecular replacement method using the 1.54 Å uncomplexed CPA structure (Rees et al., 1983) as a search model and refined by rigid-body, simulated annealing and Powell minimization techniques using X-PLOR (Brunger, 1988).

RESULTS

Succinic Acid Derivatives as Probes for Carboxypeptidases. The discovery of inhibitors described below came

in the course of testing probes for the active site of carboxypeptidase B (CPB). This pancreatic enzyme closely resembles the more familiar CPA except that its substrate specificity shows a preference for basic rather than hydrophobic amino acids in the C-terminal position (Folk & Gladner, 1958). One type of probe which we studied (Chan & Pfuetzner, 1993) was intended to fit into the specificity pocket, which in the case of carboxypeptidase B would consist of a base such as guanidine. The other probe was designed as a strong ligand to the zinc ion in the active site but interacting at the same time with the recognition site for the C-terminal carboxyl group (Chan & Pfuetzner, 1993; Asante-Appiah & Chan, 1996). This type of bifunctional probe is exemplified by derivatives of succinic acid.

Effect of gem-Dialkyl Substituents on Inhibitor Potency. While screening various substituted succinic acids as probes, we found unexpectedly that the 2,2-dimethyl derivative (Table 2) was a rather potent inhibitor of CPB ($K_i = 28\ \mu$M). This potency was unusual because the compound had a relatively simple structure which lacked the basic side chain of specific substrates. We were further surprised when the higher homologue 2-methyl-2-ethylsuccinic acid (MESA, as a racemic mixture) showed an affinity for this enzyme which was more than 6-fold higher. These results strongly suggested that the same compounds might be particularly effective inhibitors of CPA because of the preference of this enzyme for hydrophobic substrates. Indeed these compounds proved to be strikingly potent inhibitors of CPA (Table 2). In the case of MESA, the dissociation constant is even lower than that of L-benzylsuccinic acid, a previously known inhibitor which was designed to mimic closely the hydrolysis products of the reaction (Byers & Wolfenden, 1973). In this connection, it should be pointed out that the dissociation constant reported here for MESA was obtained with the racemic mixture. As shown below, one of the enantiomers binds preferentially to the enzyme so that its K_i value should be significantly lower.

We have not been able to test other *gem*-dialkyl succinic acids because they are not readily available. However, strong inhibition appears to require the presence of two nonpolar substituents on the same carbon atom. Thus, dramatically lower affinities for the two carboxypeptidases were observed when one of the methyl groups in the *gem*-dimethyl derivative was replaced by a hydroxyl function (as in either of the enantiomers of citramalic acid, Table 2). Furthermore, in comparison with the monomethyl derivative, it is clear that the hydroxyl group had either no effect or a significantly adverse effect on inhibitor binding (depending on the stereoisomer). In contrast, the extra methyl group in the *gem*-position increased affinity by close to 150-fold for both enzymes. The significance of the *gem*-dialkyl locus can be fully appreciated by noting that compared to unsubstituted succinic acid the affinities of the above inhibitors are 1000- to almost 30000-fold higher. As will be discussed later, the hydrophobic nature of this binding pocket may have a special significance for the catalytic mechanism.

The location of the methyl group in the inhibitor molecule also seems to be highly specific since the 2,3-dimethyl derivative showed extremely weak binding (weaker in fact than unsubstituted succinic acid). As already indicated from previous work (Byers & Wolfenden, 1973), the distance between the two carboxyl groups is also important for favorable interaction with the carboxypeptidases. Thus, we have found that the *gem*-dimethyl substituted derivatives of both malonic and glutaric acids have little affinity for these enzymes (K_i estimated in the millimolar range and not studied in detail). The above finding of strong and specific affinity for a highly substituted nonpolar locus in the inhibitor molecule would not have been predicted from our current knowledge of the structure of the active site and prompted us to investigate the physical basis for the favorable interaction.

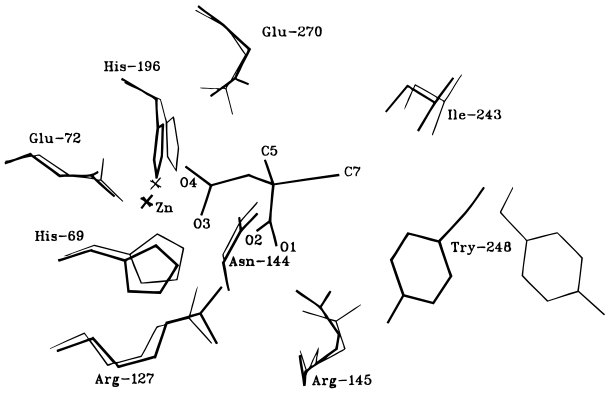

FIGURE 2: Comparison between the structure of the CPA−MESA complex and the native enzyme. The active-site residues in the native enzyme are depicted by thin lines while those in the complex and the inhibitor molecule are represented in bold. The numbering system for the atoms of the inhibitor is described in Figure 3.

Structure of the CPA−MESA Complex. The structure of the enzyme−inhibitor complex was determined at a resolution of 2 Å and refined to an *R*-factor value of 0.20 and *R*-free of 0.26 in the 5−2 Å range. The overall structure (Figure 2) is well-ordered and very similar to that of the native enzyme (Rees et al., 1983) except for the region around residues 133−136 where deviations from the native structure also occur in other CPA−inhibitor complexes (Christianson & Lipscomb, 1986; Shoham et al., 1988; Kim & Lipscomb, 1990). There is also a major shift in the position of Tyr-248 upon inhibitor binding which is well documented for this enzyme (Lipscomb et al., 1968), and this conformational change is accompanied by less pronounced movements of some side chains in the active site (Figure 2).

The crystals for X-ray analysis were prepared in the presence of a racemic mixture of 2-methyl-2-ethylsuccinic acid (MESA); however, only the R-form of the inhibitor was observed in the structure of the enzyme−inhibitor complex. This enantiomer of MESA is related in stereochemistry to the L-amino acid in the C-terminal position of a typical peptide substrate (Figure 3). If the carboxymethyl group of the inhibitor is aligned in the same way as the scissile peptide bond, then the ethyl group is equivalent in position to the side chain ($R\text{-}CH_2\text{-}$) of the C-terminal amino acid (e.g., phenylalanine), in the substrate and the orientation of the methyl group in the inhibitor would correspond to that of the α-hydrogen of this amino acid.

The molecule of MESA is bound in a fairly extended conformation with the oxygen atoms of its β-carboxyl group (O3 and O4, Figure 4) coordinated to the Zn atom of the enzyme. The similar and relatively short distances between the metal ion and the above two oxygen atoms (Table 3) suggests the presence of particularly strong interactions. By comparison, the coordination distances in the complex with L-benzylsuccinic acid vary over a considerable range (Man-

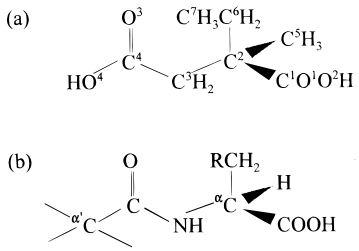

FIGURE 3: Stereochemistry of MESA in relation to that of the C-terminal residue of a peptide substrate. (a) The enantiomer of MESA illustrated here is the R-form found in the enzyme−inhibitor complex. The carbon and oxygen atoms have been given a numbering system (in superscript) which will be referred to throughout this paper. (b) A typical substrate containing an L-amino acid at the C-terminus is aligned with the scissile peptide bond in the same position as the carboxymethyl group of MESA since both these groups are coordinated to the Zn of the enzyme. The α-carbon atoms of the C-terminal and the penultimate residues are marked α and α′, respectively.

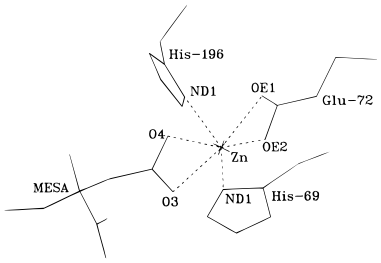

FIGURE 4: The coordination sphere of Zn^{2+} in the active site.

Table 3: Zn Coordination Distances (Å)

	inhibitor bound to CPA		
coordinating atom	none	MESA	LBSA
β-carboxylate (O3)		2.3	2.3
β-carboxylate (O4)	2.1[a]	2.1	2.6
His-69 (ND1)	2.1	2.3	2.0
His-196 (ND1)	2.1	2.3	2.0
Glu-72 (OE1)	2.2	2.2	2.0
Glu-72 (OE2)	2.3	2.2−2.3[b]	2.8

[a] The ligand in this case is a water molecule. [b] Slightly different values were obtained for the two molecules in the asymmetric unit.

gani et al., 1992) so that the interactions appear to be less than optimal. The above carboxyl oxygen atoms (O3 and O4) are also located at favorable distances to form hydrogen bonds with His-69 and His-196 as well as with Glu-72 (Figure 5 and Table 4), and these residues are themselves coordinated to the Zn (Table 3). The α-carboxyl group also forms numerous hydrogen bonds, especially with Arg-127 and Arg-145 (Figure 5). In addition, there are many van der Waals contacts, particularly between the carbon atoms of the inhibitor and various side chains in the enzyme (Table 5). Of special interest is the location of the methyl group which is within 3.6 Å from both Glu-270 and Asn-144 (Figure 5). The carbon atom of this group and the terminal carbon of the ethyl group (C5 and C7 respectively, Figure 3) are highly buried (Table 5) within the more hydrophobic area of the active site in the neighborhood of Ile-243.

Comparison with Other Enzyme−Inhibitor Complexes. The overall orientation of MESA is similar to that of other substrate-analog inhibitors whose complexes with the enzyme have been studied by X-ray crystallography. The above layout corresponds generally to what may be regarded as the substrate-binding mode in so far as it can be extrapolated

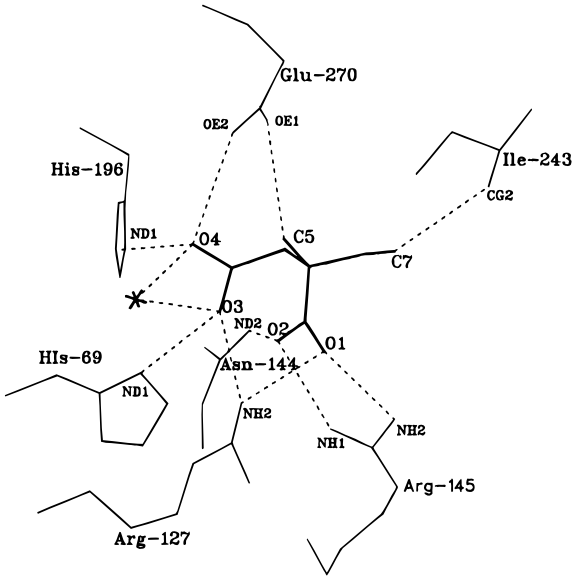

FIGURE 5: Interactions between CPA and MESA in the complex. The broken lines illustrate the coordination of O3 and O4 to Zn^{2+} and hydrogen bonds to various residues. The hydrogen bonds of the α-carboxyl group and the van der Waals interactions of C5 and C7 in MESA are similarly depicted.

Table 4: Potential Hydrogen-Bond Formation between Enzyme and Inhibitor

participating group in the inhibitor	side chain in the enzyme	distances (Å) in the complex	
		MESA−CPA[a]	LBSA−CPA
α-carboxylate			
O1	Arg-127 (NH2)	2.8−2.9	3.5
O1	Arg-145 (NH2)	2.9	2.7
O2	Arg-145 (NH1)	2.9−3.0	2.8
O2	Asn-144 (ND2)	2.7−2.8	2.8
β-carboxylate			
O3	His-69 (ND1)	2.6−2.9	3.3
O3	Arg-127 (NH2)	3.0−3.2	4.0
O4	His-196 (ND1)	2.6	3.1
O4	Glu-270 (OE2)	3.2−3.3	3.0
O4	Glu-72 (OE1)	3.3−3.6	4.0

[a] Where differences are observed in the two molecules of the asymmetric unit, the range of values is given here.

Table 5: Environment of the Carbon Atoms in MESA

atom in MESA[a]	nearest side chain of CPA (<4 Å)	total no. of van der Waals contacts	buriedness (Å^2)
C1	Arg-127, Asn-144, Arg-145	4	4.6
C2	none	0	0
C3	Glu-270	3	15.4
C4	His-69, Glu-72, Arg-127, His-196, Glu-270	6	10.5
C5	Glu-270, Asn-144	2	43
C6	none	0	20.6
C7	Ile-243	1	53

[a] For the numbering of the carbon atoms in MESA, see Figure 3.

from this type of structures. In particular, the succinate backbone of MESA is in a fairly equivalent position to that of L-benzylsuccinic acid (LBSA) with the carbon atom bearing the *gem*-dialkyl groups only 0.3 Å from the corresponding position of the α-carbon in LBSA (Figure 6). Comparison of the two structures demonstrates clearly that the site occupied by the methyl group of MESA is vacant in the LBSA complex (and in all other CPA−inhibitor com-

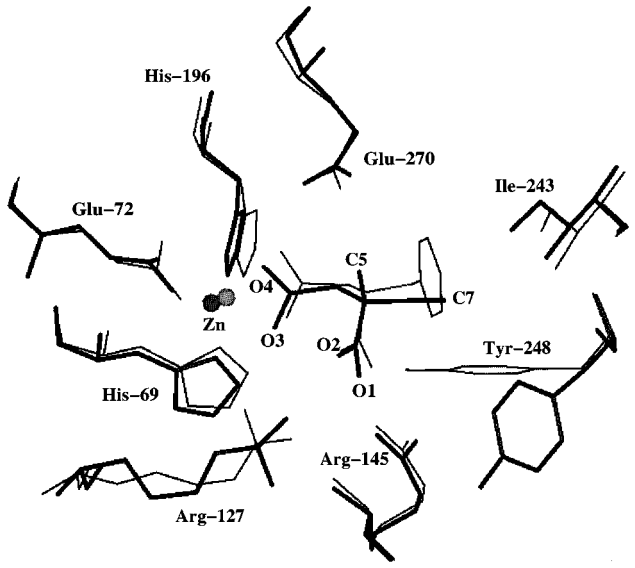

FIGURE 6: Comparison between the binding of MESA and that of L-benzylsuccinic acid (LBSA) to the active site of CPA. As in Figure 2, the active-site residues and the inhibitor in the CPA−MESA complex are represented in bold. The thin lines in this case depict the corresponding features of the CPA−LBSA complex as determined previously (Mangani et al., 1992). For the sake of clarity, the side chain of Asn-144 has been omitted in these structures. The orientation of this residue can be observed in Figure 2.

plexes available through the Protein Data Bank). The methyl substituent is, of course, also absent in all known substrates. Thus, there is apparently a small cavity in the native enzyme which remains unoccupied upon substrate binding. As will be discussed later, the existence of this putative cavity, which has not been recognized previously, may have an interesting functional significance.

The similarity between the two above enzyme−inhibitor complexes extends to the orientation of the side chains in which the ethyl group of MESA is pointed in the same direction as the benzyl moiety in LBSA. The enzyme residues lining the binding pocket are also in analogous conformations in the two complexes with the exception of Tyr-248, which has moved to a lesser extent upon the binding of MESA. This difference probably reflects the smaller size of the ethyl group which is unable to interact fully with the aromatic side chain of that residue.

DISCUSSION

Hydrophobic Cavity. In our current understanding of enzyme action, it is generally assumed that the active site will be complementary to the structure of the transition state (Pauling, 1946). As a corollary to this principle, it is expected that those parts of the substrate molecule which remain unchanged in the transition state will fit well into the binding site. Indeed, most structural models of the complex between an enzyme and its substrate-analog indicate a close fitting of the two molecules with little unoccupied space between them. In this respect, our results are surprising because they suggest the presence of a cavity distal to the reaction center which is not filled by substrate binding. Thus the X-ray crystallographic structure shows the inhibitor MESA to be bound in a substrate-like manner with the *gem*-dialkyl locus occupying the position of the α-carbon in the C-terminal residue of peptide substrates. Because of its location, we suggest that the above cavity be called the α-methyl hole. It is surprising that the enzyme should accommodate so readily the extra bulk of a methyl group in a location which contains only a hydrogen in all known substrates. The rationale for this unusual structural feature will be considered below.

As shown by the structure−activity relationships of various inhibitors tested, the above cavity appears to have a strong preference for the nonpolar methyl group. The crystal structure of the complex also indicates a distinctly hydrophobic character for this cavity except for the presence of the catalytically important Glu-270. The rather unnatural environment for this polar residue suggests a special significance which is discussed later.

Possible Significance for the Catalytic Mechanism. In the mechanism proposed for CPA which has been fairly broadly accepted (Christianson & Lipscomb, 1989), the Glu-270 residue has been assigned the critical role of removing a proton from the Zn-bound water molecule and enhancing its nucleophilic character. The above mechanism also postulates that this proton is subsequently delivered to the nitrogen of the scissile peptide bond in order to facilitate the breakdown of the tetrahedral intermediate. This dual role assigned to Glu-270 requires the carboxyl group of this residue to undergo a significant movement. It is therefore tempting to suggest that the cavity found in this work in fact serves to accommodate the above movement. Since this movement of Glu-270 must occur rapidly (and possibly a second time to generate the ionized forms of the products) within the catalytic cycle, a more extensive conformational change of the protein might be kinetically undesirable. Thus, in the evolution of proteins, it may often be expedient to provide active-site cavities wherever such movements of side chains become integral parts of the mechanism. If the above cavity is indeed essential for catalysis, then our results would imply that peptides which incorporate a *gem*-dialkyl locus at the α-carbon of the C-terminal amino acid would not undergo hydrolysis although they might bind well to the enzyme.

The hydrophobic nature of the cavity may also be important for the role of Glu-270 in the catalytic mechanism. In order to act as a general base, the γ-carboxyl group of this side chain must initially exist in the deprotonated state and must therefore have a pK_a (in the native enzyme) significantly lower than the pH of the reaction medium. However, once the substrate is bound and water is excluded from active site, it would be catalytically advantageous to raise the effective pK_a so that the carboxylate group becomes a stronger base. This, of course, can be accomplished by providing a hydrophobic environment for Glu-270 upon binding of the substrate. We have previously postulated on the basis of the synergistic binding of inhibitors to zinc proteases that such *xerophilic shifts* might occur in these enzymes (Chan & Pfuetzner, 1993). In the case of Glu-270, the shift in pK_a might occur simply as the residue alternates between its two possible positions. Thus, the results reported here are consistent with our earlier hypothesis and suggest specifically one residue that might be involved.

Implications for Inhibitor Design. Although MESA is an excellent inhibitor of CPA, it is likely that further work will lead to even more potent *gem*-dialkyl inhibitors for this enzyme. For example, it remains to be seen whether the cavity discussed above can accommodate a larger entity than the methyl group. It should also be worthwhile to replace

the ethyl group with a substituent (e.g., benzyl) which resembles more closely the side chain of optimal substrates. In the case of CPB, it is clear from the substrate specificity that a basic functional group instead of the ethyl substituent might increase the potency of the inhibitor considerably. In this connection, it would be useful to develop a convenient synthetic route for *gem*-dialkyl derivatives in order to facilitate such structure and function studies.

In addition to derivatives of succinic acids, many kinds of inhibitors for carboxypeptidases have been described in the literature (Ondetti et al., 1979; Sugimoto & Kaiser, 1978; Kam et al., 1979). In general, the various types of inhibitors differ in those functional groups which act as zinc ligands but otherwise retain the essential structural features of specific substrates. It may therefore be highly interesting to investigate whether the introduction of a methyl group in a similar location would increase the potency of these inhibitors. In theory, at least, it would appear that *gem*-dialkyl thiols, phosphonates, and hydroxamates might prove as effective as the corresponding succinic acid derivatives. Because the synthesis of these inhibitors lies outside the scope of our laboratory, it is hoped that other workers may find it worthwhile to test inhibitors of this type. Apart from the practical benefits of obtaining new inhibitors, such studies may provide further information concerning the putative α-methyl hole discussed above.

It has been shown previously that principles which operate in the design of inhibitors for carboxypeptidases also apply to other zinc proteases (Ondetti et al., 1979; Maycock et al., 1981). It may therefore be beneficial to investigate whether the introduction of a *gem*-dialkyl locus would increase the potency of inhibitors for the other zinc proteases such as thermolysin and the angiotensin-converting enzyme. These studies might eventually reveal whether the same type of cavities exist generally in this class of enzymes. Furthermore, if our hypothesis is correct, there may be other enzymes in which the movement of an amino acid side chain in the catalytic mechanism also requires the provision of a cavity. It would be of considerable interest to see whether similar cenophilic[2] inhibitors could be devised in these cases.

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