Theoretical Calculations on the Acidity of the Active Site in Aspartic Proteinases†

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ABSTRACT: Semiempirical minimal neglect of differential overlap–self-consistent field calculations, corrected and modified for multiple hydrogen-bonding interactions, were applied to models of the active site of aspartic proteinases (AP). The propensities of the two active-site aspartates to ionize were compared under the influence of various neighboring residues and of water molecules. Asp-32 and Asp-215 in three aspartic proteinases (endothiapepsin, *Rhizopus* pepsin, and penicillopepsin) are found to be basically asymmetric, Asp-32 being preferentially (by 2–3 kcal) ionized with respect to Asp-215. In penicillopepsin, this asymmetry is compensated by effects of surrounding residues. In our largest model for the active site, which includes such other residues, near equality is found for the ionizing tendency of Asp-32 and Asp-215. The pK difference is rationalized in terms of first and second ionizations of the full active-site model. Its ionization enthalpies correlate well with those of other small organic diacids. This "gas-phase" approach to AP active-site interactions represents the main possible contributions to the acidity of the active site.

The aspartic acid pair in the active site of aspartic proteinases is known to be crucial for the catalytic hydrolysis of peptide substrates and is involved in the binding of both substrates and inhibitors. The two acids (numbers 32 and 215 in the pepsin numbering)[1] and their surrounding residues are conserved in this proteinase family (Sibanda et al., 1984) and form a nearly symmetric arrangement around a 2-fold axis.

Contrary to this apparent symmetry, differences in pK_a among the two acids had been noted: the low pK_a of ca. 1.0, found in pH activity profiles of pepsin (Fruton, 1976) is usually attributed to Asp-32, while Asp-215 is assigned the higher pK_a, of about 4.7 (Kitson & Knowles, 1971). DAN[2] reacts exclusively to inactivate Asp-215, while EPNP reacts mostly with Asp-32 (Foltman, 1981). The excess stabilization of one aspartate vis-à-vis the other has never been established. Their pK_a difference was compared frequently (Pearl & Blundell, 1984; James, 1980; Fruton, 1976) to the acidities of some dicarboxylic acids, but ΔpK_a, the difference between the first and second ionizations, is larger for diacids that may form intramolecular H-bonds (Jencks, 1969). Indirect evidence for the pK_a preference of Asp-32 was derived from crystallographic studies of aspartic proteinases under higher resolution (James, 1980). It dwells upon the relative inaccessability of Asp-32 and the more intricate hydrogen bonding that is assumed to be associated with this residue with respect to the other.

In penicillopepsin, H-bonding was suggested (James, 1980) to involve OH from Ser-36, NH from Gly-215, and a closely bound water molecule. In endothiapepsin (Tickle et al., 1984), hydroxyls from Ser-35 and Thr-218 and NH from Gly-34 and Gly-217 were mentioned as H-bond donors to the aspartic diad. The pK_a lowering to ca. 1.0 was attributed to hydrogen bonding from nearby residues, mostly from Ser-35 (Tang, 1979). However, in the presence of a pepstatin analogue in the active site (James & Sielecki, 1985), a possible reversal of the acidity of penicillopepsin (Asp-213$^-$–Asp-33) was noticed. The inaccessibility of Asp-32 is questionable in view of the known reactivity of this residue with EPNP (Tang, 1979), which has been studied by X-rays, too (James, 1977). This study also demonstrated the rigidity of the active-site aspartates, since very small changes in their atomic positions were observed in the EPNP complex with penicillopepsin, with respect to the free enzyme. In the higher resolution studies of penicillopepsin (James & Sielecki, 1985), a few water molecules were detected in its active site. Three of those are located close to the aspartic pair. This recent refinement of the X-ray structure for penicillopepsin raised the possibility that one of the three could be H_3O^+ or NH_4^+, occupying a "cationic site". If proved to be so, this may change present convictions about the mechanism of substrate interactions in the active site (Pearl & Blundell, 1984).

Quantum mechanics cannot presently deal with whole enzymes, but it is at least intuitively understood that residues which are more remote from the active site will be less involved in the catalytic mechanism. Much understanding of mechanisms has been gained through quantum mechanical methods by isolating and modeling some parts of the active sites of enzymes. In addition to the improved description of mechanisms, such studies add energetics, charge distributions, and positions of hydrogen atoms, which cannot be determined by crystallography. The latter are extremely useful when intricate hydrogen-bonded structures are involved, as those of the active site of aspartic proteinases. Our model will thus include the effects of neighboring residues and of water molecules on the acidity of the active-site aspartic pair. In addition, we compared the first and second ionizations of the aspartic pair to those of some dicarboxylic acids to gain a better insight for future studies of the mechanism.

Experimental Procedures

Semiempirical SCF calculations by MNDO (Dewar & Thiel, 1977) have been recently corrected (Burstein & Isaev, 1984) for their inappropriate handling of hydrogen-bonding

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[1] The numbers of residues in the various AP are according to the Brookhaven protein bank files for endothiapepsin (4APE), *Rhizopus* pepsin (2APR), and penicillopepsin (2APP): 4APE D32-T33-G34-S35, D215-T216-G217-T218; 2APR D35-T36-G37-S38, D218-T219-G220-T221; 2APP D33-T34-G35-S36, D213-T214-G215-T216.

[2] Abbreviations: AP, aspartic proteinases; DAN, diazo-DL-norleucine methyl ester; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; MNDO, minimal neglect of differential overlap; NMFA, *N*-methylformamide; SCF, self-consistent field.

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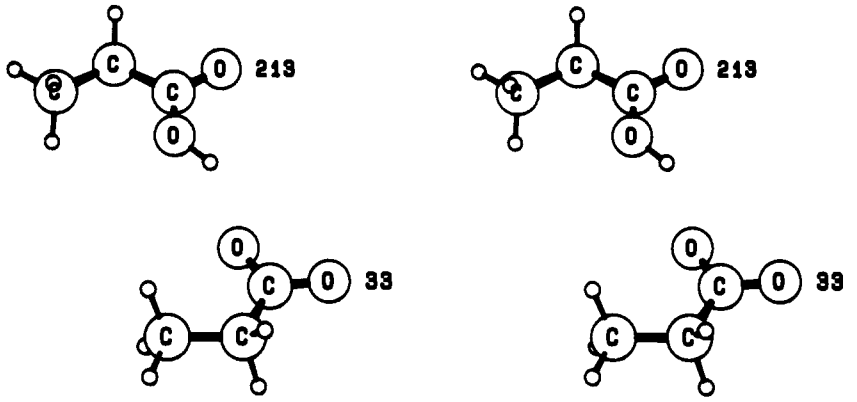

FIGURE 1: Stereo plot of the preferred ionized state for the propionic acid/propionate model of the AP in penicillopepsin, with Asp-213 as hydrogen-bond donor.

interactions and now modified to account for multiple H-bonding (Goldblum, 1987a,b), which is vital for properly describing protein–ligand interactions. Entropies were included (Flanigan et al., 1977) to account for free energy differences in some of the calculations. Following an initial comparison of the aspartic diad model of three AP (Subramanian et al., 1977; Bott et al., 1982; James & Sielecki, 1983), all subsequent modeling was based on the coordinates of penicillopepsin at 1.8 Å resolution, which were available from the Brookhaven data bank (Bernstein et al., 1977) when this study was carried out.

A model of the active site of AP was considered in a few stages: (1) The side chains of the two aspartic acids, including C_α of each, were kept in their X-ray positions and thus transformed to propionic acids. Hydrogen atom positions only were optimized, including those that replaced the backbone atoms originally bonded to C_α. Only one acidic hydrogen was optimized between the closest oxygens of the aspartic pair—once near one acid and then near the other. One, two, and three water molecules were allowed to interact with this negatively charged propionic–propionate model, and all the H_2O degrees of freedom were optimized. This was done both for Asp-33$^-$···Asp-213 and for Asp-33···Asp-213$^-$.

For the interaction of three water molecules with both options of ionization in the active site, an analysis was carried out for all the contributions to the total energy from pair interactions. We also analyzed the mutual field effects of each acid on its partner in its neutral and anionic states.

(2) Enlargement of the first model to include the effects of neighboring residues could be achieved by transforming the aspartates to formic acid residues, Ser-36 and Thr-216 to methanol molecules, and the peptide bonds of Thr-34–Gly-35 and Thr-214–Gly-215 into formamides. All the atomic positions from X-rays were not optimized. Also, hydrogens replacing carbons were only optimized for bond length, but the crystallographic directions were fixed. The full model we used was built in stages in order to verify that the replacement by hydrogens of bonds to certain chemical groups (i.e., methyls) does not influence the results. As an example, *N*-methylacetamide was compared to formamide for the effect of the peptide bonds on the acidity. With the largest model of the active site, a single water molecule was introduced and its position fully optimized again with respect to the two ionization options.

(3) The transformation from a neutral active site to the mono- and dianion states was calculated, and the results were compared to equivalent transformations of maleic, fumaric (Jencks, 1976), and cyclobutene-1,2-dicarboxylic acid (McCoy, 1967).

Table I: Enthalpies[a] and Entropies[b] of Aspartic Diad Models from Crystallographic Positions[c]

	(A) Asp-32$^-$–Asp-215[d]	(B) Asp-32–Asp-215$^-$
Rhizopus chinensis	−208.35 (90.83)	−206.49 (91.06)
endothiapepsin	−213.55 (88.04)	−210.57 (88.25)
penicillopepsin	−210.61 (91.42)	−208.19 (91.75)

[a] In kcal/mol. [b] eu, in parentheses, at T = 298.15 K. [c] Hydrogen positions fully optimized. [d] The numbers are in the pepsin numbering system.

Table II: Total Enthalpies (kcal/mol) and Entropies[a] (eu) for the Aspartic Diad of Penicillopepsin and for Its Hydrates (1–3 Water Molecules)

no. of H_2O	(A) Asp-33$^-$–Asp-213	(B) Asp-33–Asp-213$^-$
0	−210.61 (91.42)	−208.19 (91.75)
1	−286.32 (101.61)	−283.47 (102.73)
2	−356.89 (116.88)	−354.47 (117.47)
3	−428.53 (122.86)	−426.49 (124.16)

[a] In parentheses.

All calculations were run on a CDC Cyber 170/855 (NOS operating system) in the Hebrew University computation center of Givat-Ram.

Results and Discussion

The preferences for proton binding among the two aspartic moieties were investigated for the negatively charged, hydrogen-bonded diad in the three AP that were reported with higher resolution in the data bank: *Rhizopus chinensis* proteinase[3] (1.8 Å), endothiapepsin[3] (2.1 Å), and penicillopepsin (1.8 Å). Since no optimization was done for the crystallographic reported positions, only the calculated enthalpies are meaningful (vide infra). The entropies are useful for comparative purposes. Both are reported in Table I. Results with the previous, lower resolution coordinates of *Rhizopus* pepsin (1APR) and of endothiapepsin (2APE) had larger, but similarly inclined, differences. In all three AP there is a clear preference for the proton to reside on Asp-215 and for Asp-32 to be ionized with this initial model, which is not influenced by any protein fields. Figure 1 shows the preferred state for the model of penicillopepsin. The difference in favor of ionized Asp-32 is similar in the other two pepsins (2–3 kcal/mol), and entropies are virtually identical for the alternative ionizations in each of the proteins.

The effect of water on the two ionization options in penicillopepsin was tested by introducing H_2O molecules to interact

[3] Revised coordinates for those two proteins were deposited in the protein data bank after most of this study was completed. They have not been, yet, fully reported in the literature.

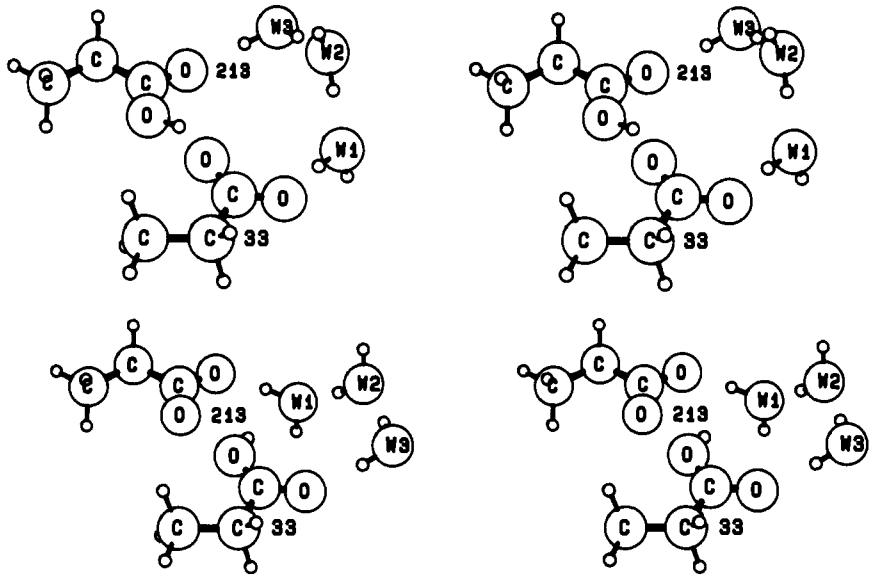

FIGURE 2: Stereo plots for the organization of three water molecules in the two optional ionized states of the AP model: (upper) D33⁻–D213; (lower) D33–D213⁻.

with both states. Three such molecules were added, one by one, fully optimizing their positions after each addition.

The results are given in Table II, and Figure 2 shows the final positions of three water molecules in the aspartic diad model for the alternative ionizations. We also calculated the entropies through their vibrational, rotational, and translational components (Flanigan et al., 1977) and found a difference of less than 1.5 entropy units among equivalent (same number of water molecules) alternative arrangements of the water molecules, always in favor of ionization B in the table. This entropy difference reduces by 0.5 kcal, at the most, the preference of Asp-33 to be ionized (column A), which was found along the addition of all water molecules. The basic free energy difference of about 2 kcal in favor of Asp-33 is maintained with the addition of 1–3 H_2O and corresponds to less than 5% of ionized Asp-213⁻ in the model at equilibrium.

Thus, with no other field present, except for water molecules, our aspartic diad crystallographic model gives a result that is in accord with the suggestions for the preference of one acid over the other to be ionized. In addition, it also predicts that a small fraction should exist in the reversed ionization state. The positions of water molecules in the crystal (James & Sielecki, 1983) were not deposited, yet, in the data bank, but the positions of their oxygen atoms are similar to those of our calculated model. Their hydrogen-bonding scheme was assumed (James & Sielecki, 1985) but found by us to be somewhat different: H_2O that hydrogen bonds to the neutral acid (213) carbonyl [061 in Figure 2a of James and Sielecki (1985)] has another H-bond to H_2O in the center of the diad (originally 039). Additional water molecules would have to hydrogen bond to three H_2O's already bound to the aspartic diad, further away from the ionized acids, or to other residues, and their influence on the relative acidity of Asp-33 and Asp-213 should be smaller than that of the investigated ones.

In this partial model, what is the source of the clear preference for ionization? In Table III we report the results of a dissection of the total energy to its single and pair contributions for the two alternatives. The contributions were calculated for the positions found in the two respective optima (last line of Table II) shown in Figure 2. From the interaction energies (given for each pair of Table III) we find equivalent contributions for both ionizations (A and B columns) from all the interactions of the aspartic pair model with water molecules

Table III: Single and Pair Contributions[a] to the Interaction of Three Water Molecules with the Two Alternative Ionizations of the Aspartic Diad Model

	(A) Asp-33⁻–Asp-213	ΔH^c int	(B) Asp-33–Asp-213⁻	ΔH^c int
	Single Contributions[b]			
Asp-33	−110.62		−91.48	
Asp-213	−87.12		−110.68	
W1	−59.53		−59.43	
W2	−60.30		−60.29	
W3	−60.58		−60.60	
	Pair Contributions[b]			
Asp-33–Asp-213	−210.59	−12.85	−208.15	−6.00
Asp-33–W1	−185.98	−15.83	−150.50	0.41
Asp-33–W2	−175.44	−4.52	−150.38	1.39
Asp-33–W3	−174.69	−3.49	−156.64	−4.56
Asp-213–W1	−146.21	0.44	−186.29	−16.18
Asp-213–W2	−146.04	1.38	−174.64	−3.67
Asp-213–W3	−152.47	−4.77	−174.30	−3.02
W1–W2	−124.14	−4.31	−124.26	−4.54
W1–W3	−120.55	−0.44	−120.55	−0.52
W2–W3	−124.76	−3.88	−125.11	−4.22
total energy	−428.53		−426.49	

[a] In kcal/mol. [b] See Figure 2 for identification of molecules. [c] Calculated as the difference between the pair contribution and the relevant single contributions.

and among the water molecule pairs. Only the contribution from the aspartic diad pair is substantially different and is much smaller when Asp-213 is ionized. This interaction enthalpy overcomes the single contributions that are in favor of Asp-33–Asp-213⁻ due to the lower energy of neutral Asp-33 with respect to neutral Asp-213 in the optional ionization.

It is worth noting that the pair contributions of Asp-33–Asp-213 in their two ionization are unaffected by the water molecules, as these values (−210.59 and −208.15 kcal/mol) hardly differ from the enthalpies found in the absence of water molecules (−210.61 and −208.19 kcal/mol, respectively). This indicates that the water molecules do not alter the position of the proton that bridges the two acids. This inability has important mechanistic implications for the availability of the proton to the carbonyl of peptide substrates in this active site.

When each of the neutral and ionized acids in the two states was subjected to total geometry optimization in the absence of its partner, limiting only the C_α positions and C_α–C_β di-

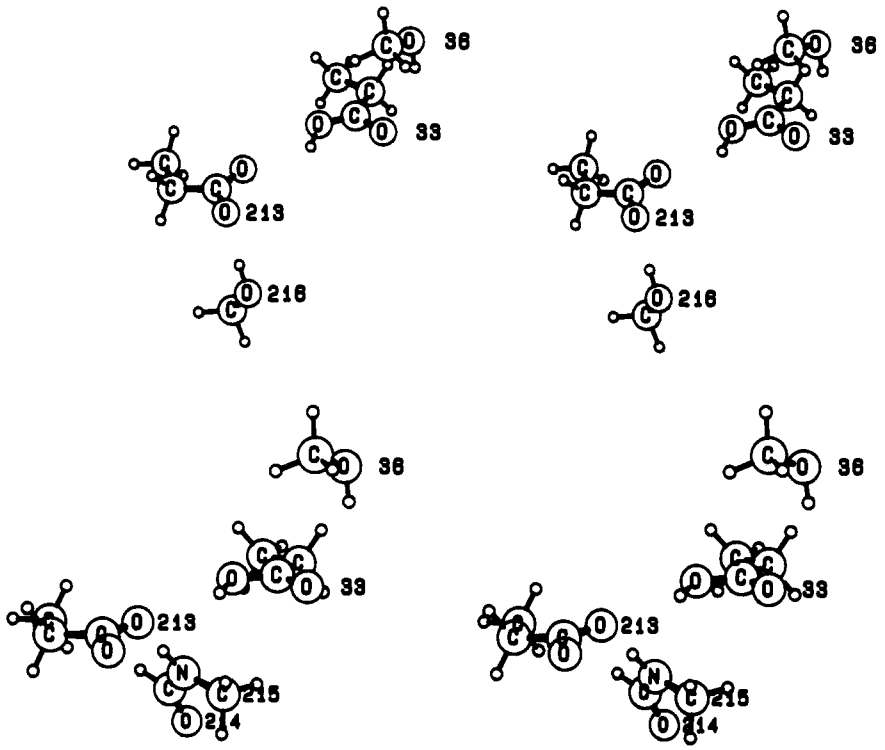

FIGURE 3: Hydrogen-bonding interactions of the AP model with neighbor residues, D33 being the preferentially protonated acid in both. (Upper) AP model + S36, T216; (lower) AP model + S36, T214–G215 peptide bond.

Table IV: Hydrogen-Bonding Characteristics for Two Ionization Options

	33^-–213	33–213^-
R (H–033)	2.022	0.958
D[a] (H–033)	0.007	0.434
R (H–0213)	0.975	2.407
D (H–0213)	0.428	0.001
O–H–O[b]	145.7°	110.1°
Q[c] (H)	0.391	0.355
Q (033)	−0.689	−0.363
Q (0213)	−0.406	−0.661

[a] Mulliken bond density. [b] The O–O distance is 2.880 Å. [c] Mulliken charges.

rection in space (to model the backbone limitations), both ionized acids reached a similar enthalpy, and so did the neutral ones. The increased stabilization of Asp-33^-–Asp-213 with respect to Asp-33–Asp-213^- in the model is found to be a result of the change in the hydrogen-bond energy between the two, due to the acids' crystallographic positions. Table IV presents distances, angles, mulliken bond densities, and charges for the three atoms that participate in the hydrogen bond. The change in the O–H–O angle, due to the rigid positions of the acids' carboxyls, dictates a change in the participation of the proton's orbital in the molecular orbitals, which is reflected in the charges and in the bond densities and lengths. When 33^- is ionized, the hydrogen bond is much stronger due to its shorter distances and the larger charges on all the atoms involved. Within the concepts of protein crystallography (Baker & Hubbard, 1984) a distance of 2.88 Å between the closest oxygens of the two aspartic residues is sufficient for suggesting the existence of an H-bond among them. The energy difference due to the proton's position on the two acids may be suggested only by theoretical models. Here, quantum mechanical theory helps crystallographers to distinguish between two H-bonding options that may otherwise seem to be equivalent. Thus, we may conclude that the basic electrostatic nature of H-bonding is clearly manifested in the difference between the two options for ionization. It imposes the requirement for a microscopic description of the acidity in the active site, for which the use of "continuum" methods should become obsolete, as already discussed before (Warshel, 1978).

Table V: Effects of Other Residues on the Acidity of the Aspartic Diad Model

model of	modeling molecules[a]	$D33^-$–D213	D33–$D213^-$	ΔH^b
(1) S36	EtOH	−276.94	−271.88	−5.06
(2) S36	MeOH	−272.12	−267.16	−4.96
(3) S36, T214–G215	MeOH, FAM	−311.73	−314.36	2.63
(4) S36, T216	MeOH, MeOH	−330.92	−331.78	0.86
(5) S36, T214–G215	MeOH, NMFA	−307.57	−309.92	2.35
(6) (D33–D213)$^-$	PRAC, PRAC	−210.61	−208.19	−2.42
(7) (D33–D213)$^-$	FAC, FAC	−186.53	−183.66	−2.87
(8) full model[c]		−393.48	−393.10	−0.38
(9) full model + H_2O		−466.48	−465.96	−0.52

[a] FAM, formamide; PRAC, propionic acid; FAC, formic acid. [b] Difference in enthalpy (kcal/mol) between the two ionizations. Negative values favor $D33^-$. [c] Including entry 7 + S36, T216, T214–G215, and T34–G35.

Neighbor Residues Effect on Active-Site Acidity. Various single and pairs of residues among those that were suggested as hydrogen-bond donors to the aspartic diad were modeled and added, in our routine procedure (X-ray positions for non-hydrogens, optimization of hydrogen positions, except the angles, for hydrogens replacing carbons), to the initial active-site model. The enthalpies of the two ionization states with those perturbations are depicted in Table V. The hydrogen bond from Ser-36 increases the propensity of D33 to be ionized by an additional 2 kcal/mol. This tendency is efficiently opposed by Thr-216, H-bonded to D213, and reverses the ionization trend. An even stronger effect in favor of ionized D213 is contributed by the peptide bond of T214–G215. It is nearly similar for both models of the peptide bond: *N*-methylformamide or just formamide. Some of the hydrogen-bonding patterns are shown in Figure 3. In addition to

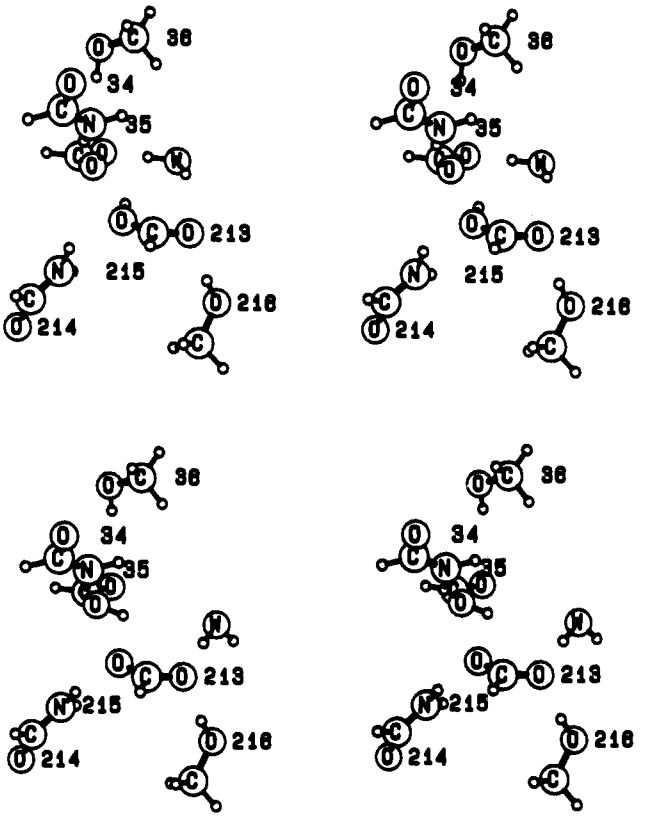

FIGURE 4: Full model of the main interactions in the active site, including a water molecule: (upper) D33 ionized; (lower) D213 ionized (stereo plots).

the modeling of other residues, we also found that a formic acid–formate model of the aspartic diad keeps the same characteristics as the propionic–propionate one but enables the inclusion of more neighbor effects, which was subsequently tested.

Due to computer system limitations, only one water molecule could be included in the "full" model for the active site. This model is shown in Figure 4 for both ionizations. $D33^-$–D213 in this model was found to have a lower enthalpy, of 0.4 kcal, than D33–$D213^-$. Inclusion of the water molecule did not affect this difference.

For evaluating the free energy (and pK_a) difference between the two alternatives in the full model, the entropy difference from the singly hydrated propionic–propionate model (Table II) was assumed. However, entropy calculations in this and in similar studies are limited in application as long as they do not include all the contributing interactions, in their correct geometries, which are indispensable for evaluating, primarily, the sensitive vibrational entropy components (Kollman, 1984).

The final free energy difference, of less than 0.1 kcal in favor of D33 ionized, corresponds to nearly equal ionization of both acids, under equilibrium conditions and at room temperature. Additional water molecules will contribute some fluctuation to this value, as they did in the partial model (Table II). Had it been possible to continue with the propionic acids models of the aspartic diad, a contribution of some 0.4 kcal in favor of D213 ionization was expected.

The quantum mechanical model of the active site does not show, therefore, an absolute preference of one acid to be ionized over the other. It predicts a large proportion of $D33^-$ over $D213^-$ in the partial model and near equality in the large model. It may be that residues which are further away from the diad could add small increments in favor of one ionization state. Such contributions should stay quite small. The largest

Table VI: Enthalpies (kcal/mol) and pK Values for the Two Ionizations of Diacids

	ΔH_1[a]	ΔH_2[b]	pK_1[c]	pK_2[c]
diacid				
cyclobutene-1,2-dicarboxylic	329.17	441.70	1.12	7.63
maleic	329.19	446.96	1.83	6.07
fumaric	344.61	421.91	3.03	4.44
o-hydroxybenzoic	339.61	460.74	2.97	13.40
aspartic diad model				
FAC/FAC[d]	337.34	439.13	~1.0	~4.7
PRAC/PRAC[e]	338.57	434.57	~1.0	~4.7
full model	315.66	399.93	~1.0	~4.7
full model + H_2O	314.80	395.04	~1.0	~4.7

[a] Value according to eq 1. [b] Equation 2. [c] pK_a values are from Weast (1973) (maleic, fumaric, *o*-hydroxybenzoic) and McCoy (1967) (cyclobutene-1,2-dicarboxylic acid). [d] FAC, formic acid. [e] PRAC, propionic acid.

effect we found was the reversal of acidity by adding the peptide bond of T214–G215 to the diad model + S36, 7.5 kcal. The N–H of this bond interacts strongly due to its coulombic contributions. Residues that do not have a direct H-bond with the diad will add much less stabilization in any direction and are not mandatory for the quantum mechanical modeling. The model accounts for the possible contributions of water molecules, since no more than three of those were found near the diad by X-rays of penicillopepsin, and the other eight H_2O in the active site (James & Sielecki, 1983) are more remote and may not be regarded as "solvent". Thus, in this case at least and possibly for other AP, the gas-phase approach of quantum mechanics is appropriate and accounts for all the main contributions to acidity.

The rigidity of the atoms involved in the acidity of the diad was shown by introducing an inhibitor into the active site (James & Sielecki, 1985). This is the crucial evidence for not using geometry optimization of crystallographic positions in this study, after one reviewer questioned the usefulness of such previous optimizations in our work.

pK_a at the Active Site of Penicillopepsin. The difference in acidity found for the aspartic diad corresponds to the stabilizing energy of the monoanion and of the dianion, i.e., to pK_1 (~1.0) and pK_2 (~4.7), respectively. There is no experimental method known to us that can determine the relative acidity of each of a pair of acids in the same molecule. Analogies to other systems may be used whenever the dicarboxylic acid (or other diacid) is nonsymmetric. As yet, only quantum mechanical methods or analogous theoretical approaches may be used to solve such a problem. The acquired information about the aspartic diad in AP urged comparisons of its acidity constants to those of rigid dicarboxylic systems and prompted us to calculate the gas-phase acidity of those in relation to our active-site model. In those calculations, stable neutral molecules were transformed to mono- and dianions according to the equations

$$AH_2 \rightarrow H^+ + AH^{-1} \quad (1)$$

$$\Delta H_r = \Delta H_f(H^+) + \Delta H_f(AH^{-1}) - \Delta H_f(AH_2)$$

$$AH^{-1} \rightarrow H^+ + A^{-2} \quad (2)$$

$$\Delta H_r = \Delta H_f(H^+) + \Delta H_f(A^{-2}) - \Delta H_f(AH^{-1})$$

where AH_2 is a neutral diacid. No water molecules were added to the organic acids while their neutral and anionic structures underwent full geometry optimization. In Table VI we report ΔH_r values (gas phase) and corresponding experimental pK. All the values reported are very large due to the nature of the simulated transfer of H^+ from acid to gas

phase and not to water or another base. Lower values of ΔH_r reflect higher stability of the ions. It is clear from the table that the pK values are not linearly related to the energies, but the lowest and highest pK_1 go along with the extreme ΔH_r values for AH^{-1}, and such a relationship generally holds for pK_2 values as well. In comparison, we added to Table VI a monocarboxylic acid (salicylic, line 4) and report that the ΔH_r for monomeric propionic acid (ionizing to monoanion) is 357.3 kcal/mol, so its anion is less stabilized than any of the diacids in the table. Such examples raise the reliability of the method, since those results correlate clearly with experiments in solution. It is also evident that the partial models of the active site cannot account for its acidity since their ΔH_r values (lines 5 and 6 in the table) are too large. On the other hand, the values for the full model fit nicely into the overall relation (line 7). The stabilization by water is larger on the second ionization (4.9 kcal) than on the first (0.9 kcal) of the full model.

In conclusion, the stepwise examination of interactions in the active site of penicillopepsin enables us to draw the following conclusions: (1) There is a structural difference between the two "bare" aspartates in the active site, and their H-bonding to each other is not symmetric. (2) Water molecules in the active site interact strongly with the ionized aspartate but do not alter the acidity trend. (3) Hydrogen bonding to the aspartic diad by neighboring residues affects the ionization considerably. The peptide bonds interact more strongly than the hydroxyls with the diad. (4) A full model was presented and accounts for all the crucial interactions to the acidity. (5) With all the main interactions present, there is little preference, if at all, of D33 to be ionized over D213. (6) The propensity for first and second ionizations correlates successfully with the ionization trends in other diacids.

Thus, this model, which could be described as a gas-phase one, properly reflects the main contributions to the acidities of the active site of penicillopepsin, and recent improvements in the resolution of other AP shows that the present analysis would hold for them, too.

Registry No. A, 56-84-8; AP, 78169-47-8; endothiapepsin, 37205-60-0; *Rhizopus* pepsin, 9074-09-3; penicillopepsin, 9074-08-2.

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