Electrostatic Potentials of Proteins. 2. Role of Electrostatics in a Possible Catalytic Mechanism for Carboxypeptidase A

David M. Hayes and Peter A. Kollman*

Contribution from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143. *Received December 22, 1975*

Abstract: We present the results of calculations on the electrostatic environmental effect on the scissle peptide bond of a substrate bound to carboxypeptidase A in the orientation found in x-ray studies. Although our results are directly relevant only to the earliest stages of catalysis, they do suggest an important electrostatic contribution of the groups near the substrate in facilitating peptide bond rehybridization and cleavage.

A number of different approaches have been made to examine environmental effects on the electronic structure of molecules in liquids and solids.[1-7] One procedure, which has its basis in the molecular orbital method, seeks to simulate the coulombic contribution to the intermolecular interaction by substituting appropriately chosen point charges to replace the actual neighboring molecules. The charges, which may be fractional, are placed at the positions normally occupied by the neighboring atoms. They have neither basis functions nor electrons explicitly associated with them. The effect of these charges is to simulate the polarization of the solute by the surroundings. Charge transfer and exchange are not considered within the framework of this method. Also neglected is the reverse polarization of the solvent by the solute. Almlöf and Wahlgren[8] and Tomasi[9] have used fractional point charges to study the effect of environment on the geometries and lattice energies of crystals containing H_3O^+ and $NaNO_2$. Recently, Noell and Morokuma[10] used such an approach to study the hydration of Li^+ and F^-. To our knowledge no work has been reported in which the method of fractional point charges has been applied to enzyme catalysis. In this application, the "solute molecule" is the substrate and the surroundings are the enzyme to which the substrate is bound. Important questions one would like to address include the effect of the enzyme on the electronic structure and equilibrium geometry of the substrate as well as the effect on the activation energy for the catalytic reaction.

Review of CPA–Substrate Studies. We chose for our complex the enzyme carboxypeptidase A (CPA) and the dipeptide inhibitor glycyltyrosine. Briefly, carboxypeptidase A is a zinc-containing metalloenzyme which cleaves peptide bonds at the carboxyl terminal end of the peptide chain. The structure and probable mechanism of action of this complex have been discussed by Lipscomb and co-workers[11-13] in light of their recent x-ray structural studies. Upon binding of a substrate, the enzyme appears to undergo some remarkable conformational changes involving at least three side chains of CPA: Arg 145, Tyr 248, and Glu 270. First, the guanidinium group of Arg 145 moves about 2 Å toward the terminal carboxylate of the substrate forming a salt link. Secondly, the phenolic OH of Tyr 248 moves about 12 Å to place itself roughly 2–3 Å from the nitrogen in the peptide bond. Thirdly, the carboxylate group on Glu 270 moves about 2 Å to come within 3–3.5 Å of the carbon atom of the substrate's carbonyl group. In addition, the carbonyl oxygen adjacent to the susceptible peptide bond replaces a water molecule coordinated to zinc and itself becomes a ligand to the metal atom. Based on their crystal structure data for the CPA–(Gly-Tyr) complex, Quiocho and Lipscomb[11] have suggested that the following effects are important in catalytic activity. The phenolic OH of Tyr 248 is the likely proton donor to the nitrogen of the scissile peptide bond. Its proximity to the nitrogen suggests that it can form a hydrogen bond to nitrogen and thereby decrease the conjugate stabilization of the peptide linkage. The closeness of the zinc ion to the carbonyl group suggests that the carbonyl bond may be further polarized with concomitant electron transfer to the metal. It was suggested that this would weaken the C–N linkage. Donation of charge from the nearby carboxylate of Glu 270 to the carbonyl carbon might be expected to promote rehybridization at the carbon and loss of conjugative stabilization.

As part of a continuing program[14] on the role of electrostatics in enzyme–substrate binding, we decided to use the point-charge approximation described above to study the effect of neighboring charged groups on the bonding in a prototypal substrate of CPA. In addition, we wished to test the hypothesis that the bound substrate is perturbed, either structurally or electronically or both, so as to bring it closer to the transition state of the catalytic reaction.

Computational Details. The coordinates for Gly-Tyr in the enzyme-bound complex are from the work of Lipscomb et al.[13] For reasons of economy we have removed the terminal ammonium and carboxyl portions of the substrate as well as the tyrosine side chain and replaced each with a hydrogen atom. Thus, our substrate in these calculations is really *N*-methylacetamide (NMAA). The susceptible peptide bond of the substrate molecule should be well insulated by adjacent methylene groups from the effects of the substitutions. We expect, therefore, that these changes will not affect our conclusions regarding the catalytic reaction.

All the calculations were performed at the ab initio LCAO–SCF–MO level with the Gaussian 70 program[15] using both STO-3G and 4-31G basis sets. We assigned point charges to the CPA residues in two ways.

(1) When a STO-3G calculation was done on NMAA, we placed "STO-3G point charges", as described in a previous paper,[14] on the three catalytically important functional groups of CPA: $-C_\zeta OH$ of Tyr 248, the protonated guanidine of Arg 145, and the carboxyl group of Glu 270. For the 4-31G calculations on NMAA, we used "4-31G point charges" [14] on the above functional groups. In addition, we also placed either STO-3G or 4-31G charges on the three zinc ligands: N_E of His 69 and His 196 and the carboxyl group of Glu 72.

(2) Integral charges (± 1) were assigned to all other (49) charged residues of the enzyme. Zinc was given a charge of +2.

The remaining residues, which carry no net charge, were ignored for the purposes of these calculations. In our previous paper[14] we showed that the major features of the electrostatic field in which the substrate sits are determined by the zinc ion

Table I. Summary of STO-3G Results on NMAA in the Presence of the Electrostatic Field of Various Residues of CPA

	NMAA	NMAA + Zn ion	NMAA + Arg 145	NMAA + Glu 270	NMAA + Tyr 248	NMAA + Zn ion and ligands	NMAA + all previous[f]	NMAA + all charges	NMAA + Mg^{2+}
Net charge on $CH_3(G)$[a]	−0.017	0.058	−0.005	−0.021	−0.015	0.050	0.061	0.025	0.100
Net charge on carbonyl C	0.327	0.362	0.324	0.338	0.328	0.361	0.372	0.373	0.402
Net charge on O	−0.260	−0.502	−0.253	−0.281	−0.256	−0.449	−0.459	−0.448	−0.384
Net charge on N	−0.363	−0.327	−0.363	−0.348	−0.367	−0.339	−0.328	−0.338	−0.307
Net charge on $CH_3(T)$[b]	0.094	0.125	0.075	0.087	0.100	0.117	0.096	0.141	0.162
Net charge on H[c]	0.220	0.283	0.221	0.226	0.209	0.260	0.258	0.247	0.299
Mulliken overlap population for C–O	0.435	0.428	0.435	0.435	0.435	0.433	0.432	0.434	0.414
Mulliken overlap population for C–N[d]	0.343	0.367	0.339	0.346	0.341	0.359	0.359	0.362	0.377
Energy, hartrees[e]	−243.759945	−243.852184	−243.758981	−243.763192	−243.758971	−243.825615	−243.827004	−243.823595	−440.432648
Interaction energy, kcal/mol	0.0	−57.9	0.6	−2.0	0.6	41.2	−42.1	−39.9	−100.9[g]
Net charge on $-NHCH_3$ fragment	−0.049	0.081	−0.067	−0.035	−0.058	0.038	0.026	0.050	0.154
Net charge on Mg									1.729

[a] Methyl group adjacent to the carbonyl group. The carbon atom is the α carbon of glycine in the Gly-Tyr substrate. [b] Methyl group attached to nitrogen. The carbon atom is the α carbon of tyrosine in the Gly-Tyr substrate. [c] Refers to the hydrogen attached to the nitrogen atom. [d] Refers to the peptide bond. [e] This is the sum of the electronic energy and the nuclear–nuclear repulsion and nuclear–point charge interaction energies. [f] "All the previous" refers to the following CPA residues: Arg 145, Gly 270, Tyr 248, Zn^{2+}, and the ligands on the zinc ion. [g] The STO-3G energy for Mg^{2+} is −196.511931.

Table II. Summary of 4-31G Results on NMAA in the Presence of the Electrostatic Field of Various Residues of CPA

	NMAA	NMAA + Zn ion	NMAA + Arg 145	NMAA + Glu 270	NMAA + Tyr 248	NMAA + Zn ion and ligands	NMAA + all previous[f]	NMAA + all charges
Net charge on $CH_3(G)$[a]	0.020	0.154	0.046	0.014	0.030	0.115	0.144	0.082
Net charge on carbonyl C	0.792	0.851	0.785	0.805	0.803	0.854	0.874	0.883
Net charge on O	−0.618	−0.991	−0.603	−0.648	−0.605	−0.889	−0.892	−0.877
Net charge on N	−0.871	−0.797	−0.871	−0.844	−0.901	0.838	−0.845	−0.865
Net charge on $CH_3(T)$[b]	0.269	0.309	0.234	0.257	0.294	0.320	0.299	0.368
Net charge on H[c]	0.407	0.474	0.410	0.415	0.379	0.438	0.421	0.409
Mulliken overlap population for C–O	0.441	0.289	0.440	0.437	0.443	0.360	0.361	0.381
Mulliken overlap population for C–N[d]	0.149	0.279	0.126	0.172	0.128	0.238	0.226	0.239
Energy, hartrees[e]	−246.546748	−246.700501	−246.545083	−246.552228	−246.544742	−246.645590	−246.644100	−246.639879
Interaction energy, kcal/mol	0	−96.5	1.0	−3.4	1.3	−62.0	−61.1	−58.4
Net charge on $NHCH_3$ fragment	−0.195	−0.014	−0.227	−0.172	−0.228	−0.080	−0.125	−0.088

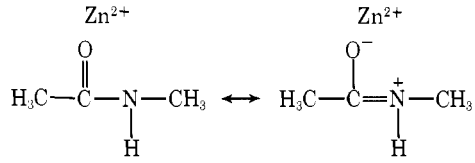
[a]–[f] See corresponding footnotes in Table I.

and the residues with net positive or negative charge. Thus, the small fractional charges on each atom of CPA due to the polarization of the individual bonds have only a relatively small modulating effect on the overall electrostatic field of the charged groups.

Results and Discussion

Our initial calculations on NMAA were carried out using the relevant Gly-Tyr coordinates reported by Lipscomb et al.[13] Although these coordinates present some rather long bond lengths, e.g., the peptide and tyrosine N–C_α bonds are roughly 1.49 and 1.67 Å in length, respectively, we nevertheless decided to use them as a basis for our preliminary calculations. The results of these calculations at both the STO-3G and 4-31G levels are summarized in Tables I and II. The first column refers to *N*-methylacetamide in the absence of the enzyme. As expected, the charge distribution is what would be predicted based on the electronegativities of the atoms but the polarization of the various bonds is greater in the 4-31G than the STO-3G calculations. Column 2 shows the effects of including

in the calculation a +2 point charge at the position occupied by the zinc ion in CPA. Since the zinc lies close to the carbonyl oxygen of NMAA, we would anticipate increased polarization of this bond. This is, in fact, reflected in the calculations with both basis sets. Although the oxygen substantially increases its net negative charge, it does so at the expense of the remaining atoms of NMAA. The carbonyl C becomes more electrophilic and the amide N less nucleophilic. The effect of adding a +2 charge on the strengths of the C=O and C—N bonds is also included in the tables. Thus, changes in the Mulliken overlap populations predict a weakening of the carbonyl bond and strengthening of the peptide bond. This is interesting since it implies that the presence of the zinc does not promote the breaking of the C–N bond, at least not directly via polarization of the charge density in the substrate. The bottom row provides a hint at an explanation for this. These numbers are the sum of the net atomic charges for everything on the nitrogen side of the peptide bond. Thus, by comparing the first and second columns, we see there is a net transfer of electronic charge across the peptide bond toward CH_3CO when the zinc ion is present. If one considers the two most important resonance structures of NMAA, one can see that



the positive charge on zinc will increase the contribution of the resonance structure on the right giving rise to the changes in Mulliken overlap population noted.

We have also calculated the contribution each residue makes to the total CPA–substrate binding energy. Since we are dealing with the interaction between a molecule and point charges our calculated interaction energy can only hope to represent the electrostatic and polarization contribution and not charge transfer and exchange.[16] The total energies in Tables I and II are a sum of electronic (the energy of the electrons moving in the field of the nuclei and the point charges), nuclear–nuclear, and nuclear–point charge contributions. The interaction energy is just the difference in the total energies in the absence and presence of the charges. Both STO-3G and 4-31G calculations predict strongly attractive interactions between the substrate and the zinc ion.

We can carry out an analysis like the above for each of the residues thought to be important in catalysis. Thus, the electrostatic fields of Arg 145 and Tyr 248 cause a slight decrease in the overlap populations at the peptide bond while the polarizing field of Glu 270 leads to a strengthening of this bond. Also, both Arg 145 and Tyr 248 interact with NMAA in a very weakly repulsive way while the interaction with Glu 270 is slightly attractive. Including the ligands on zinc (His 69 and 196 and Glu 72) in our calculations leads to some modulation of the results with zinc alone but the results are qualitatively the same. We included these ligands to see if they altered the electrostatic field in the vicinity of the zinc ion sufficiently to cause important changes in the way this ion affects the substrate. The seventh column in Tables I and II shows the combined effects of all the previously studied residues. Comparison of this column with the first two shows that the combined effect of all the aforementioned residues on NMAA lies between NMAA alone and NMAA + Zn^{2+}. In other words, the net effect of the other residues is to decrease the perturbation due to the metal ion. Finally, the last column gives the results from including all the previously mentioned charged groups plus the remaining 49 CPA residues which carry either a net +1 or −1 charge. Here again, the electronic structure changes are similar to those found for Zn^{2+} alone. In addition, the similarity between these results and those obtained without the 49 additional charges is quite striking and indicates that the major influence in the electrostatic perturbation of the substrate comes from a few residues in the active site rather than the more numerous but more distant charged residues.

Since the zinc ion is quite close to the substrate carbonyl oxygen, we would expect substantial charge transfer between these species. This should have an important effect on the electronic structure of the substrate. Unfortunately, our computer program is not able to do calculations in which d orbitals are included in the basis set and, thus, we could not study this effect directly. It should be possible, however, to obtain a reasonable approximation to the charge-transfer effect by replacing zinc with Mg^{2+}.[17] Such a calculation is possible at the STO-3G level with the Gaussian 70 program and the results are shown in the last column of Table I. The charge on magnesium is reduced by 0.271 electronic units due to transfer of charge from the substrate. The negative charge on oxygen is increased relative to isolated NMAA but not by as much as when charge transfer is not allowed. The effect of CT on the other substrate atoms is to make them more positive than when only polarization is operative. The Mulliken overlap populations show a further weakening of the C–O bond and strengthening of the peptide linkage relative to the point charge calculation. Regardless of these differences, the trends are the same as for the point charge calculations.

Since the catalytic reaction involves the hydrolysis of the C-terminal peptide bond of the substrate, we were interested in the effect point charges would have on the structural changes likely to occur during this reaction. The changes we decided to study were (1) stretching of the peptide bond and (2) bending of the substituents on nitrogen and carbon away from planarity to give an atom which is more sp^3-like in character. If the carboxylate of Glu 270 attacks the carbonyl carbon of Gly-Tyr in a nucleophilic addition, as suggested by Lipscomb and co-workers,[11-13] then we would expect rehybridization to occur at this center. Likewise, if Tyr 248 transfers its phenolic OH proton to the substrate nitrogen, we would also expect rehybridization from sp^2 to sp^3 to occur. More explicitly, we rehybridized at N by letting the methyl move out of the plane defined by O=C—N in the direction away from the incoming proton. At the carbonyl carbon, rehybridization also involved moving the adjacent methyl out of the plane defined by O=C—N but this time in a direction away from COO^- of Glu 270. The geometry of the OCNH moiety was not allowed to change during rehybridization so as to avoid prejudicing our results by disrupting the Zn^{2+}···O and NH···X interactions. The results of these calculations on all three geometries at R (C–N) = 1.49 Å and in the presence and absence of external charges are shown on the right side of Table III. Since we also wanted to study the effect of stretching the peptide bond and since 1.49 Å is a reasonable representation of the "stretched" state of the peptide bond, we did the above set of calculations over again and at 1.32 Å which is the experimental peptide bond distance.[18] These results are shown on the left side of Table III. In addition, Table IV shows the degree of stabilization experienced by each NMAA geometry due to the presence of the charges. The major conclusion to be drawn from this table is that the point charges stabilize the rehybridized structures more than the planar structure—the greatest stabilization coming with an approximately sp^3 nitrogen. These results can be viewed in a different way. Thus, Table III shows that at R (C–N) = 1.49 Å it costs roughly 15 kcal to rehybridize at the carbon atom in either the absence or presence of the point charges. On the other hand, rehybridization of nitrogen is exothermic to the extent of 9 kcal in the absence of charges and 20 kcal in their presence.

We next decided to study the basis set dependence of these results. Thus, Table V summarizes the results of STO-3G

Table III. Energies of Several Geometries of NMAA Calculated at the 4-31G Level

	Energy, kcal/mol	
	R (C-N) = 1.32 Å[a]	R (C-N) = 1.49 Å[b]
Rehybridized carbon	7.1	14.6
Approximate planar	−7.3	0.0[c]
Rehybridized nitrogen	−14.5	−8.9
Rehybridized carbon plus point charges	−56.6	−44.6
Approximate planar plus point charges	−70.2	−58.4
Rehybridized nitrogen plus point charges	−87.1	−78.2

[a] R (C-N) = 1.32 Å is the standardized OC-N bond length suggested by Pople and Gordon.[17] [b] R (C-N) = 1.49 Å is the peptide bond length reported by Lipscomb et al.[13] [c] The energy of this structure in the absence of point charges is our reference energy.

Table IV. Stabilization of Various NMAA Structures Due to the Point Charges[a]

Geometry	$-\Delta E$, kcal/mol, 4-31G
Planar, R (C-N) = 1.49 Å	58.4
Rehybridized at nitrogen, R (C-N) = 1.49 Å	69.3
Rehybridized at Carbon, R (C-N) = 1.49 Å	59.2
Planar, R (C-N) = 1.32 Å	62.9
Rehybridized at Nitrogen, R (C-N) = 1.32 Å	72.6
Rehybridized at Carbon, R (C-N) = 1.32 Å	63.7

[a] Net interaction energy of different *N*-methylacetamide structures in the presence of the point charges of the enzyme. For example, the difference between the planar [R (C-N) = 1.49 Å] and rehybridized at nitrogen [R (C-N) = 1.49 Å] stabilization (69.3 − 58.4 = 10.9 kcal/mol) tells one that the electrostatic field of the enzyme stabilizes the rehybridized structure relative to the planar one by 10.9 kcal/mol.

Table V. Energies of Several Geometries of NMAA Calculated at the STO-3G Level

	Energy, kcal/mol	
	R (C-N) = 1.32 Å[a]	R (C-N) = 1.49 Å[b]
Rehybridized carbon	16.7	15.0
Approximate planar	1.7	0.0[c]
Rehybridized nitrogen	−8.9	−13.0
Rehybridized carbon plus point charges	−29.3	−25.5
Approximate planar plus point charges	−43.9	−39.9
Rehybridized nitrogen plus point charges	−60.0	−59.5

[a] R (C-N) = 1.32 Å is the standardized OC-N bond length suggested by Pople and Gordon.[18] [b] R (C-N) = 1.49 Å is the peptide bond length reported by Lipscomb et al.[13] [c] The energy of this structure in the absence of point charges is our reference energy.

Table VI. Energies of Several Idealized Geometries of NMAA Calculated at the STO-3G Level

	Energy, kcal/mol	
Geometry	R (C-N) = 1.32 Å	R (C-N) = 1.49 Å
Planar	1.0	0[a]
Rehybridized at N	12.2 (11.2)[b]	0.5 (0.5)[b]
Rehybridized at C	63.1 (62.1)	59.7 (59.7)
Rehybridized at N and C	58.4 (57.4)	49.1 (49.1)
	Include Point Charges	
Planar	−46.3	−41.9
Rehybridized at N	−40.3 (6.0)[b]	−48.5 (−6.6)[b]
Rehybridized at C	18.5 (64.8)	19.3 (61.2)
Rehybridized at N and C	4.6 (50.9)	−1.6 (40.3)

[a] All energies, except those in parentheses, are with respect to the planar geometry with R (C-N) = 1.49 Å in the absence of point charges. [b] The numbers in parentheses are calculated barriers to out-of-plane bending.

calculations on the same structures discussed above. The ordering of the various geometries with respect to energy is the same at the STO-3G level of calculation as at the 4-31G level. Thus, rehybridization at carbon costs energy while at nitrogen it is exothermic. The behavior of NMAA with respect to C-N stretching in the absence of charges varies somewhat between the two sets of calculations.

We were not entirely happy with the published x-ray coordinates for the substrate because of the unusually long C-N and N-CH_3 bond lengths and also because of the prediction that the structure with a rehybridized nitrogen is of lower energy than the planar structure. Therefore, we decided to generate coordinates for an idealized NMAA using standardized bond lengths and angles. The coordinates for the OCN fragment were not changed from the published ones except that the C-N bond length was allowed to vary as discussed above. The O-C-N-H, O-C-N-CH_3, and H_3C-N-C-CH_3 dihedral angles were set at 180, 0, and 180°, respectively. All bonds to nitrogen formed angles of 120° to each other, and the N-C-CH_3 angle was taken from the x-ray coordinates (108.1°). The hybridization at both methyl groups was tetrahedral with C-H bond lengths of 1.09 Å. The N-H, N-CH_3, and OC-CH_3 bond lengths are 1.02, 1.47, and 1.496 Å, respectively, with the latter value once again taken from the x-ray coordinates. Finally, the methyl groups were placed so that the dihedral angle of the hydrogens with respect to C-N bond were 90, 210, and 330. To generate the coordinates of the N-rehybridized geometry the *N*-methyl group was moved 60° [ϕ ($C_{Me}C_\alpha C{=}O$ = 60°] out of the plane defined by the OCNH moiety and away from the phenolic OH proton of Tyr 248. Similarly, rehybridization occurred at the carbon by a 60° [ϕ ($C_{Me}CC_\alpha N$) = 60°] bending motion out of the plane and away from Glu 270. Figure 1 contains the results for all of these STO-3G calculations. In contrast to the previous calculations, Figure 1 shows that planar NMAA is of lower energy than any of the rehybridized structures. Thus, out-of-plane bending at either C or N costs energy. Table VI also shows the energies of the idealized geometries and from them one can calculate the energy required for various types of out-of-plane bending motions at both R (C-N) = 1.32 and 1.49 Å and in the presence and absence of the enzyme. Thus, it takes 11.2 and 62.1 kcal to rehybridize nitrogen and carbon, respectively, at R (C-N) = 1.32 Å. Rehybridizing nitrogen and carbon simultaneously costs 57.4 kcal indicating a substantial (11.2 + 62.1 − 57.4 = 15.9) cooperative effect between these motions. If we now include the polarizing influence of the enzyme in our calculations, Table VI shows a marked change in the barrier to bending at nitrogen. Thus, this energy is lowered by 5.2 kcal to 6.0 kcal/mol. There is a similar effect on the rehybridization energy at C and N but surprisingly the opposite effect is observed for bending just at C, the energy required for this pro-

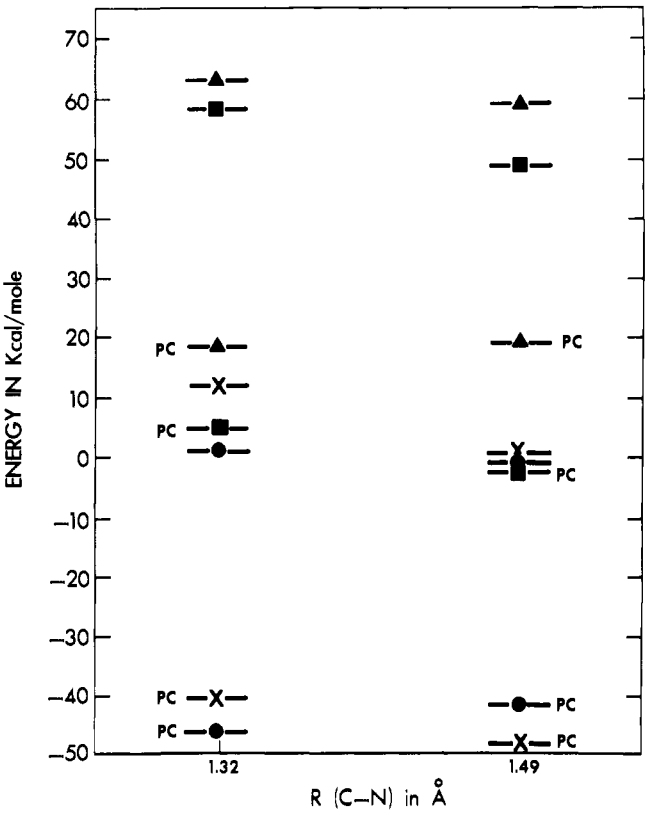


Figure 1. STO-3G energies of several idealized geometries of NMAA. The following symbols are used to identify the geometries: ●, planar structure; X, rehybridized nitrogen; ▲, rehybridized carbon; ■, rehybridized carbon and nitrogen. The letters PC indicate that point charges were included in the calculation. The energy of planar NMAA at R (C–N) = 1.49 Å is the energy reference.

cess rising slightly instead. The cooperative effect is even more pronounced in the presence of point charges. Thus, it takes 5.9 + 64.8 kcal/mol to rehybridize N and C independently but only 50.8 kcal/mol to do so together. We observe the same trends at R (C–N) = 1.49 Å. Stretching the C–N bond lowers the energy of all nonplanar structures relative to planar. Thus, only 0.5 kcal is required to rehybridize nitrogen at the longer bond length and in the presence of point charges this rehybridization is actually predicted to be exothermic. The out-of-plane bending mode at C also requires less energy at the longer C–N bond length but it is still predicted to be much greater than at nitrogen. The cooperative effects noted above also obtain at the longer C–N distance.

We are now in a position to make a few comments concerning the influence of the electrostatic field of the enzyme on the mechanism of the catalytic reaction. In the absence of point charges, Figure 1 shows the most stable NMAA geometry at both R (C–N) = 1.32 and 1.49 Å to be planar. Placing the substrate in the electrostatic field of the enzyme shifts the equilibrium toward the products. Thus, Figure 1 shows the energy of planar NMAA increasing with increasing C–N bond length while the energy of the N-rehybridized geometry decreases and crosses the curve for the planar species. Thus, Figure 1 would predict that the lengthening of the C–N bond is exothermic in the presence of the point charges and goes with concomitant rehybridization at N. It is noteworthy that although rehybridization at N costs 6 kcal and stretching of the C–N bond requires 4.4 kcal, the coupled motions have a ΔE which is negative (−2.2 kcal). We thus have one more example of the importance of cooperativity in this region.

Recent work by Johansen and Vallee[19] and a reevaluation of the x-ray data by Lipscomb[12] have called into question the

Table VII. Effect of Tyr 248 on the Energy of Rehybridization of Substrate Nitrogen

Geometry	Energy, kcal/mol,[a] R (C–N) = 1.32 Å
Rehybridized at N	12.2
Planar	1.0
Rehybridized at N plus all point charges	−40.3
Planar plus all point charges	−46.3
Rehybridized at N plus Tyr 248 charges	11.8
Planar plus Tyr 248 charges	1.5
Rehybridized at N plus all point charges except Tyr 248	−40.4
Planar plus all point charges except Tyr 248	−47.4

[a] For the energy reference, see Table VI, footnote *a*.

role of Tyr 248 in the catalytic reaction. In light of this work, we carried out additional calculations to determine the influence of Tyr 248 on the structural changes studied above. Table VII shows the results of these calculations and a comparison with Table VI for the cases when all the point charges are absent and when they are present. Thus, rehybridization at nitrogen in the absence of all charges costs 12.2–1.0 = 11.2 kcal, while in the presence of all the charges the energy required is −40.3 + 46.3 = 6.0 kcal. With only the charges due to Tyr 248 present, the corresponding energy is 11.8 − 1.5 = 10.3 kcal. If all the point charges except Tyr 248 are included, we require −40.4 + 47.4 = 7.0 kcal. Therefore, our electrostatic calculations find only a small Tyr 248 effect on the activation energy of hydrolysis. Of course, we are only allowing the Tyr 248 to interact with the peptide via an electrostatic H bond and have not simulated possible tyrosine proton transfer within this model.

Conclusions

It is important to reiterate that we have only focused on the electrostatic effect on the substrate of CPA using the x-ray structure of the CPA–Gly-Tyr complex to define the location of the scissle peptide bond. It was our intent to investigate this effect on the electronic structure of the substrate and on the energetics of distortion of the substrate during the earliest stages of reaction (before the transition state is reached). It is our conclusion that electrostatic environmental effects do contribute to lowering the transition state energy of the reaction. This lowering does not appear to be due to weakening of the C–N bond by the polarizing effect of the zinc ion but, as Figure 1 shows, to the enhanced stability of an sp^3 vs. sp^2 nitrogen.

In the work just described, we have used a point-charge representation for all the residues of CPA. Further studies of the catalytic mechanism might profitably be carried out by relaxing this approximation to the extent that certain residues such as Glu 270 and Tyr 248 are included in the full quantum mechanical calculation. If analogues of the side chain were used, such as formate for Glu 270, these calculations would still be feasible at both the ab initio and semiempirical MO level. A more complete study might even involve an investigation of the actual reaction pathway and its energetics. All but the most limited study would involve many degrees of freedom and, thus, one might combine empirical methods to estimate energy changes due to movement of protein residues with a quantum mechanical approach to calculate energy changes for groups near the site of reaction.

However, we would like to stress that the use of the electrostatic potential for the groups at the active site not directly involved in the reaction allows one to represent the protein

environment and its possible influence on enzyme catalysis. The importance of using an adequate representation of environmental effects on enzyme catalysis is clearly pointed out by Rogers and Bruice,[20] who, in their model experimental studies on possible α-chymotrypsin mechanisms, suggest that if there is a kinetically significant role of the COO^- it "must be ascribed to little understood factors not duplicated in the model studies (e.g., the heterogeneous and ordered surroundings of the triad functional groups at the active site)". A comparison and consideration of possible catalytic mechanisms of other peptidases including environmental effects are underway in this laboratory.

Acknowledgments. P.A.K. is grateful to the National Institutes of Health, General Medical Sciences, for research support (GM-20654) and for a career development award (GM-70718).

References and Notes

(1) H. Kistenmacher, H. Popkie, and E. Clementi, *J. Chem. Phys.*, **61,** 799 (1974).
(2) P. A. Kollman and I. D. Kuntz, *J. Am. Chem. Soc.*, **96,** 4766 (1974).
(3) G. N. J. Port and A. Pullman, *Theor. Chim. Acta*, **31,** 231 (1973); A. Pullman, G. Giuliano, and J. Tomasi, *ibid.*, **33,** 87 (1974).
(4) M. D. Newton, *J. Chem. Phys.*, **58,** 5833 (1973).
(5) J. Hylton, R. E. Christofferson, and G. G. Hall, *Chem. Phys. Lett.*, **26,** 501 (1974).
(6) S. Ray, *Chem. Phys. Lett.*, **11,** 573 (1971).
(7) S. Yamabe, S. Kato, H. Fujimoto, and K. Fukui, *Theor. Chim. Acta*, **30,** 327 (1973).
(8) J. Almlöf and U. Wahlgren, *Theor. Chim. Acta*, **28,** 161 (1973).
(9) C. Ghio, E. Scrocco, and J. Tomasi in "Environmental Effects on Molecular Structure and Properties", D. Reidel, Dordrecht, Holland, p 329.
(10) J. O. Noell and K. Morokuma, *Chem. Phys. Lett.*, **36,** 465 (1975).
(11) F. A. Quiocho and W. N. Lipscomb, *Adv. Protein Chem.*, **25,** 1–78 (1971).
(12) W. N. Lipscomb, *Proc. Natl. Acad. Sci. U.S.A.*, **70,** 3797 (1973).
(13) W. N. Lipscomb, J. A. Hartsuck, F. Quiocho, P. H. Bethge, M. L. Ludwig, T. A. Steitz, H. Muirhead, and J. C. Coppola, *Brookhaven Symp. Biol.*, **21,** 24 (1968).
(14) D. M. Hayes and P. A. Kollman, *J. Am. Chem. Soc.*, **98,** 3335 (1976).
(15) The Gaussian 70 program was developed at Carnegie Mellon University in the group of J. Pople (QCPE No. 236) to carry out molecular orbital calculations. We thank W. J. Hehre for the use of the CDC 7600 version of the program.
(16) The studies of Morokuma et al. [see, for example, *J. Chem. Phys.*, **55,** 1236 (1971)] find that at the minimum energy geometry, the electrostatic plus polarization contribution is often a good qualitative estimate of the actual ΔE, with the exchange and charge transfer partially cancelling each other.
(17) This may not be such an unreasonable approximation since the Pauling ionic radii for Zn^{2+} and Mg^{2+} are similar (0.74 and 0.65 Å, respectively: F. A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry", Wiley, New York, N.Y., 1972, p 52).
(18) J. A. Pople and M. Gordon, *J. Am. Chem. Soc.*, **89,** 4253 (1967).
(19) J. T. Johansen and B. L. Vallee, *Biochemistry*, **14,** 649 (1975).
(20) G. A. Rogers and T. C. Bruice, *J. Am. Chem. Soc.*, **96,** 2473 (1974).