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THEORETICAL STUDY OF THE C-N BOND BREAKAGE CATALYSED BY THE SERINE PEPTIDASES

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ABSTRACT

The conditions of C-N bond breakage by the serine peptidases have been analysed. A two-way table has been generated where either formamide or protonated formamide serves as minimal model of the scissile C-N bond and where either methanol or the couple CH_3O^- H^+ serves as minimal model of the attacking nucleophile. An addition-elimination reaction is proposed which links the enzyme acylation and deacylation steps.

INTRODUCTION

The peptidases act on peptide (peptidase activity) or ester (esterase activity) bonds, catalysing the transfer of the electrophilic group R-C=O of aminoacyl amide or ester substrates $R\text{-}\overset{O}{\overset{\|}{C}}\text{-}NH\text{-}R$ or $R\text{-}\overset{O}{\overset{\|}{C}}\text{-}O\text{-}R'$ to an exogenous nucleophile HY. With the serine proteases, the catalysed reactions proceed through formation of an acyl enzyme where the carbonyl moiety of the substrate is ester linked to a serine side chain of the enzyme active site. Concomitantly, expulsion of the leaving group NH_2-R' or OH-R' (i.e. product P_1) occurs. In turn, attack of the acyl enzyme by HY causes the release of the carbonyl moiety in the form of $R\text{-}\overset{O}{\overset{\|}{C}}\text{-}Y$ (i.e. product P_2) and regeneration of the enzyme active site. Partitioning of the acyl enzyme confers on the serine peptidases their ability to catalyse both carboxypeptidation (HY = H_2O) and transpeptidation (HY = NH_2-R") reactions (ref. 1).

The serine peptidases of known tridimensional structure (chymotrypsin, trypsin, α-lytic protease, elastase, subtilisin and proteinase B) have a common feature which is that the "active" serine is invariantly part of a same triad Ser-His-Asp (known as the charge relay system) (ref. 2). Using the numbering of chymotrypsin, it is thought that acyl enzyme formation involves i) abstraction of the $O\gamma$ proton of Ser^{195} by the $N_{\varepsilon 2}$ of His^{57} (thus activating a nucleophile for attack of the substrate carbonyl); ii) formation of a tetrahedral adduct (whose

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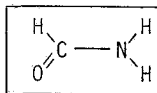
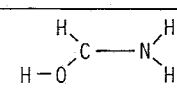
oxyanion hole is stabilized by hydrogen bonding with NH groups of the protein backbone); iii) back delivery of the proton from His^{57} (thus permitting collapse of the adduct, acyl enzyme formation and release of P_1). Enzyme deacylation is also thought to occur through similar steps but this time with H_2O or NH_2-R" acting as nucleophile.

In the above mechanism, the role of Asp is still obscure (ref. 3). Recent NMR (ref. 4) and neutron diffraction (ref. 5) experiments do not support a double proton transfer Ser-His-Asp as initially proposed but rather suggest a single proton shuttle Ser-His with the carboxyl of Asp remaining all the time unprotonated. Moreover, theoretical studies have shown that the triad $Ser^{-}His^{+}Asp^{-}$ is less stable than the triad $Ser^{n}His^{n}Asp^{-}$ (ref. 6). It thus follows that the precise nature of the protein nucleophilic reactant and the exact mode of proton transfer during the reaction remain to be elucidated.

In order to contribute to a better understanding of the catalytic mechanism of the serine peptidases, a theoretical investigation of the reaction paths has been undertaken where formamide (and subsequently formic acid methyl ester) is (will be) used as aminoacyl amide (ester) substrate model and methanol is used as a substitute of the serine residue. Methanol by itself is not nucleophilic. A first attempt to study, at an *ab initio* level, the C-N breakage in formamide and β-lactam used OH^- ion as the nucleophile (ref. 7). In these specific anionic reactions, the internal transfer of the proton cannot be easily interpreted in terms of the structural information of the active sites. With methoxide anion, no proton is available for the internal transfer. Consequently, the product of the first step in the acylation phase is a tetrahedral intermediate with the C-N bond unbroken (ref. 8). Moreover, as Wipff (ref. 9) recently pointed out, other structural features have to be taken into account to describe the acylation reaction. In particular, hydrogen bonds provided by the NH groups of the backbone stabilize the oxyanion hole of the tetrahedral intermediate. Consequently and as a first approximation, protonated formamide has been considered as a crude model of the C=O polarized substrate.

In the present study, either formamide or protonated formamide serves as minimal model of the scissile C-N bond and either methanol or the couple CH_3O^- H^+ serves as minimal model of the charge relay system activated nucleophile. Depending on the nature of the partners, the formamide + methanol reaction can be analysed as shown in the following two-way table.

		
CH_3OH	I	III
CH_3O^- H^+	II	IV

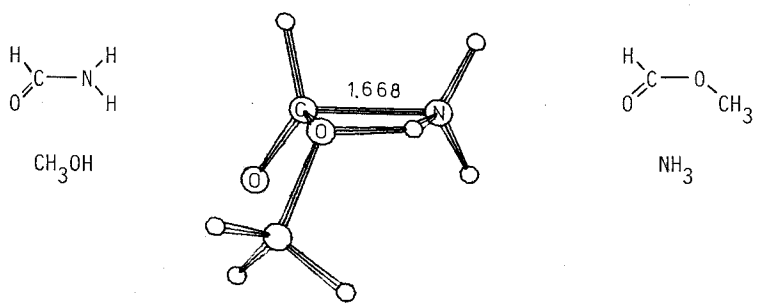
Ab initio STO-3G calculations were carried out using the GAUSSIAN 80 programs (ref. 10). All the degrees of freedom were optimized by analytical gradient methods. The transition state structures (TS) were identified by finding one negative eigenvalue of the force constant matrix computed by finite differences. The reaction coordinate is described by the associated eigenvector. All the energies are expressed relative to the products.

RESULTS

Path I Formamide + CH_3OH

This reaction path which is at variance with all biochemical and structural information accumulated to-day, is the most simple one. In this concerted mechanism, C-N bond breakage and both N-H and C-O bonds formation occur simultaneously. On the basis of previous data (ref. 11), the energetic and geometric features of the transition state structure in the reverse reaction ammonia + formic acid are very similar.

Reactants TS Products



ΔE = 10.89 kcal/mole ΔE = 56.34 kcal/mole ΔE = 0.00 kcal/mole

Path II Formamide + CH_3O^- H^+

With a methoxide anion as nucleophile, the reaction leads to a tetrahedral intermediate (Fig. 1b) stabilized by some 70.49 kcal/mole and the C-N bond (1.576 Å) remains unbroken. This two-step path refers to the charge relay system and therefore the $N_{\varepsilon 2}$ proton is available. If the proton is very close to the N of the amine fragment, the zwitterion (Fig. 1c) is unstable and the C-N bond breaks without barrier.

From X-ray data of native proteins and enzyme-inhibitor complexes, there is not enough evidence for a well-oriented transfer of the His proton to the amino leaving group of the substrate. If, in contrast, the proton is attracted by the negative charge located on the oxygen atom of formamide, the formation of a new stable intermediate (Fig. 1e) takes place. This compound is 30.85 kcal/mole more stable than the reactants formamide + methanol and can also be obtained following

path IV in which the proton refers to the polarized C=O model. It may be emphasized that this proton also constitutes a rough model for the neighbouring hydrogens interacting with the oxyanion hole of the tetrahedral adduct (Fig. 1b) and thus is another illustration of the connection between the two paths.

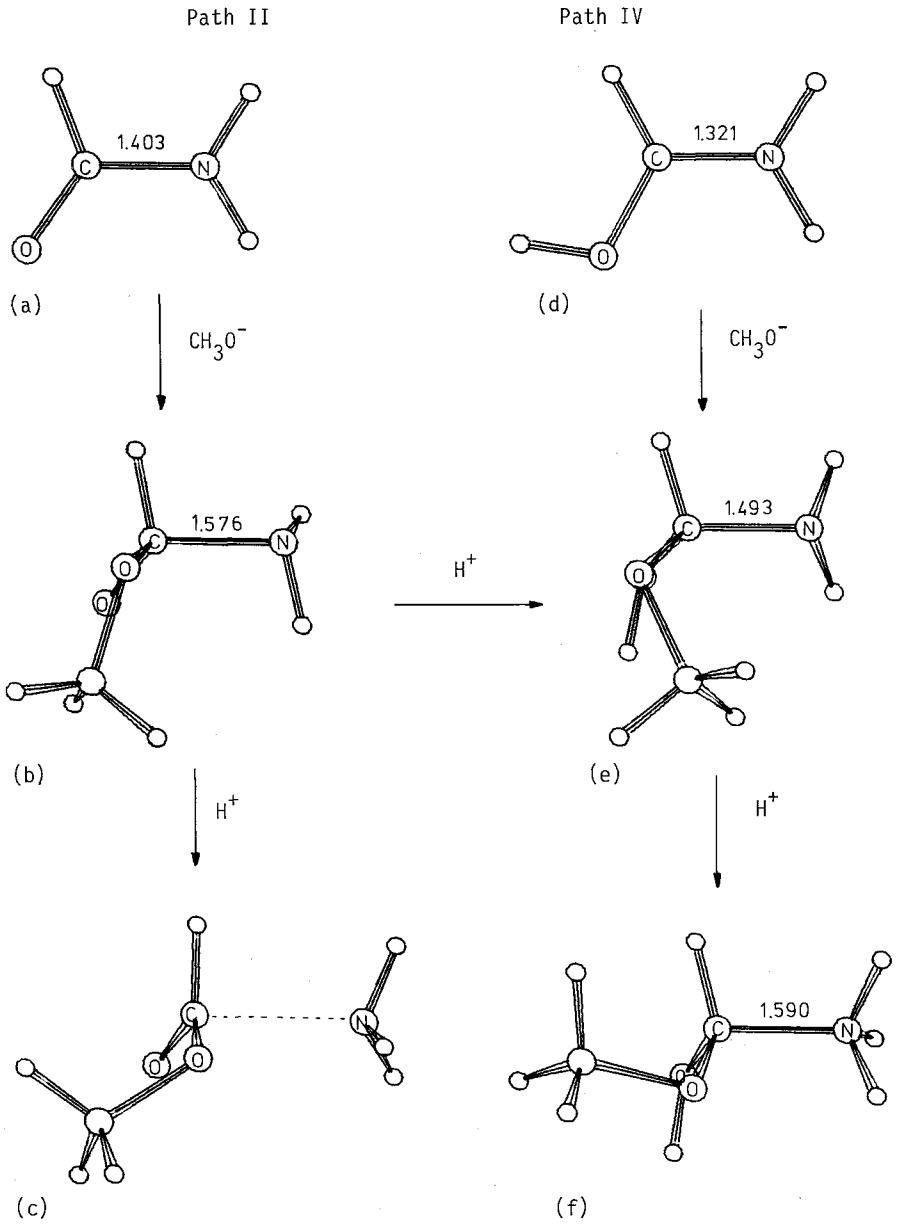
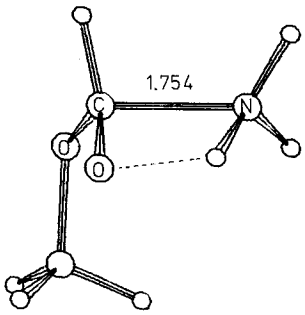


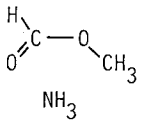
Fig. 1. Evolution of reactions II and IV.

Whatever the origin of this proton, its internal transfer to the N amino group induces the breakage of the C-N bond and the release of ammonia. The activation barrier required in this step is 71.69 kcal/mole.

Intermediate TS Products

See Fig. 1e



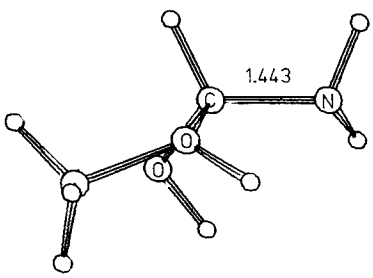


ΔE = - 19.96 kcal/mole ΔE = 51.73 kcal/mole ΔE = 0.00 kcal/mole

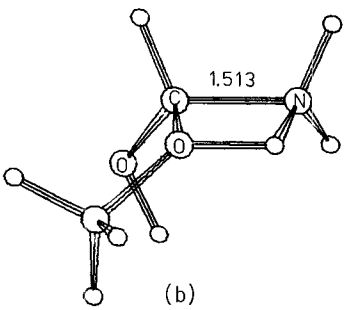
The geometric features of the transition state structure are close to those obtained in the ammonia formic acid reaction (ref. 11).

Path III Protonated formamide + CH_3OH

Intermediate TS Product



(a)



(b)

See Fig. 1f

(c)

ΔE = 14.37 kcal/mole ΔE = 29.38 kcal/mole ΔE = 0.00 kcal/mole

Fig. 2. Evolution of reaction III.

Contrary to what occurs in path I, a stable intermediate appears which possesses a C-O bond between the protonated formamide and methanol (Fig. 2a). The internal transfer of the proton requires only 15.01 kcal/mole. In the product (Fig. 2c), the C-N bond is 1.590 Å long and remains unbroken. The partial positive charge on the carbon atom of the formamide moiety is in electrostatic interaction with the lone pair of ammonia. A stabilization energy of 48.05 kcal/mole with respect to protonated ester and ammonia is found.

Path IV Protonated formamide + CH_3O^- H^+

In this two-step path, the approach of the methoxide anion gives rise to the stable tetrahedral intermediate previously discussed in path II (Fig. 1e). The protonation of the amino leaving group by the proton involved in the charge relay increases the C-N bond length to 1.590 Å. Note that this compound is also the product of path III (Fig. 2c or 1f).

DISCUSSION

In this two-way table description of the formamide + methanol reaction, the three equilibrium structures at the transition state involve the internal transfer of a proton from the oxygen atom of formamide or methanol to the nitrogen atom through a four-membered ring. The activation barrier associated with the C-N bond breakage is very high : 45.45 kcal/mole in path I and 71.69 kcal/mole in path II. In the lower energy consuming path III, the amino leaving group is not released.

With respect to the unstable zwitterion (Fig. 1c), the proton located on the oxygen of the carbonyl strongly stabilizes the molecule (Fig. 1f). As the protonated formamide is a limit case of the polarized C=O , we are presently studying the stability of the C-N bond through optimization of all the degrees of freedom except the O-H bond which is increased step by step. It could then be possible to define the geometric and energetic effects of the hydrogen bonds of the backbone on the stability of the tetrahedral intermediate or the acyl enzyme itself.

The stability of the positive charged product of paths III and IV (Fig. 1f or 2c) can also be related to biochemical information. Indeed, with peptide substrates, enzyme deacylation is more rapid than enzyme acylation which is the rate limiting step. The acyl enzyme does not accumulate detectably at the steady state and cannot be trapped. Therefore, it is not unreasonable to assume a connection between these two reactions via the reactant involved in the deacylation phase. An addition-elimination reaction is especially suitable for this purpose. By a push-pull effect, either ammonia or water partially neutralizes the positive charge on the carbon atom and the amino leaving group is released. The transition state structures (Fig. 3b, 3c) have a sp^2 configuration around the carbonyl. The activation barrier involved in these reactions are very low : 9.13 kcal/mole and 12.35 kcal/mole with NH_3 and H_2O , respectively. Such a mechanism well explains the transpeptidation and carboxypeptidation reactions catalysed by the serine peptidases.

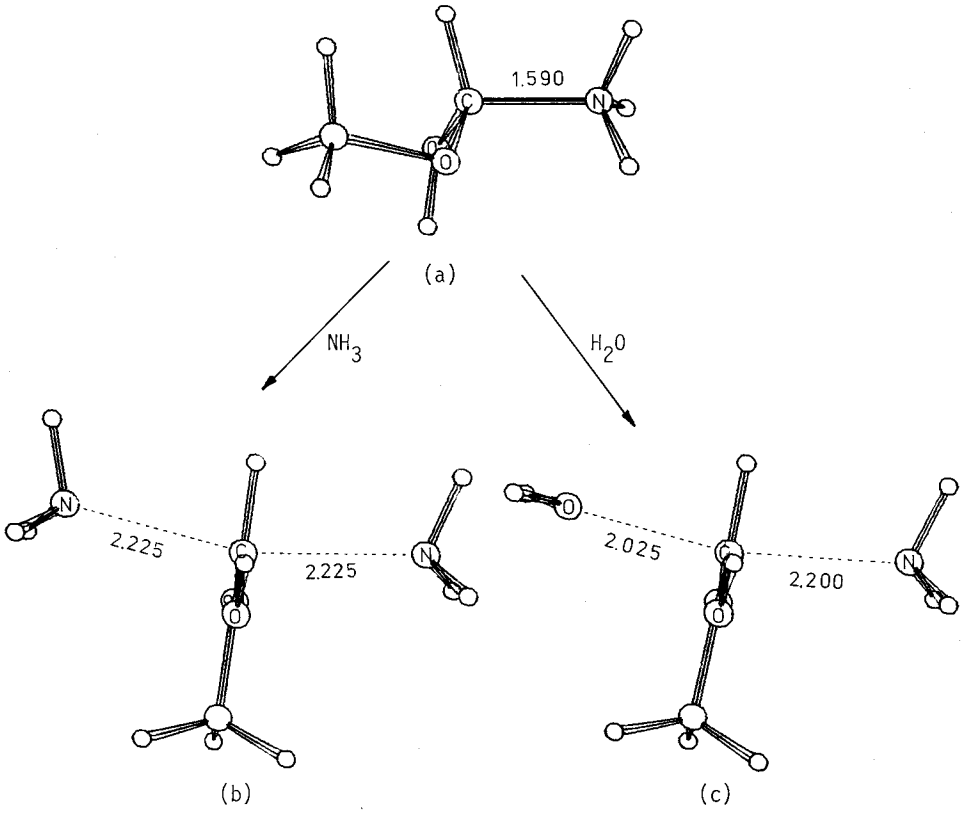
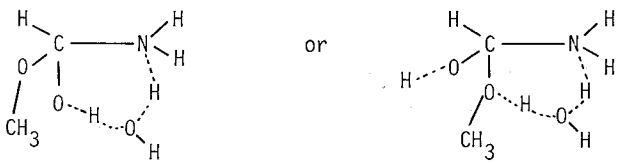


Fig. 3. Addition-elimination reaction.

In addition, water can interact in another way. Indeed, water molecules have been recognized in the active sites of most serine peptidases, and at least one of them is located between the serine and the histidine of the charge relay. Considering the previously described transition state structures, this water molecule can serve both as proton donor and proton acceptor in an extended six-membered ring :



Walking on the flat potential energy hypersurface is revealing (Fig. 4). Depending on the initial geometries (Fig. 4a, b, c), the product looks like a

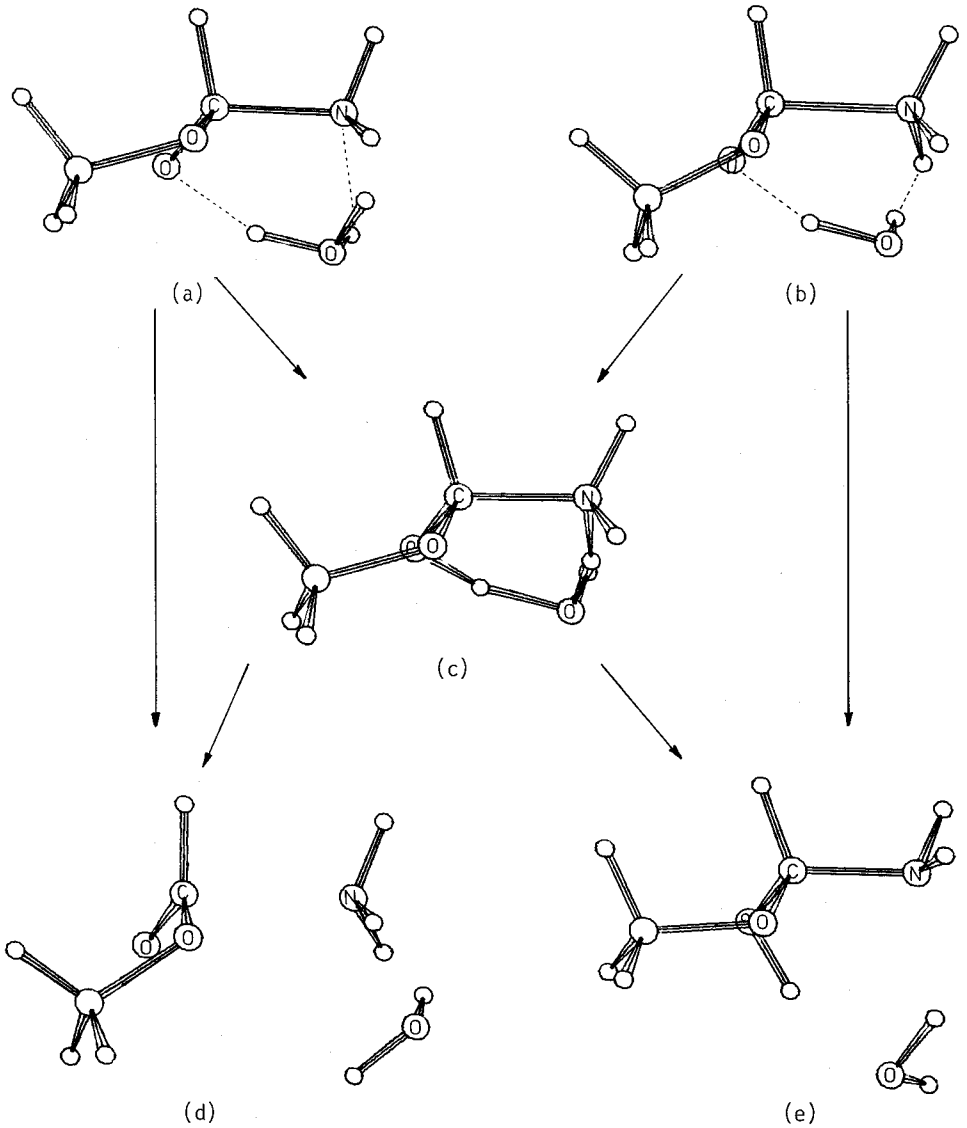


Fig. 4. Attempts to give an idea on the way the system may reorganize.

hydrated form of the stable intermediate (Fig. 4e) or a trimer : ester + NH_3 + H_2O (Fig. 4d). Moreover, the hydrated zwitterion of path II (Fig. 4b) is not necessarily unstable and can also go back to the hydrated intermediate. Although refinements on this hypersurface are still in progress, the results so far accumulated emphasize the important role that a well-oriented water molecule may fulfill in the biochemical system. In other words, water may become the catalytic relay of the proton involved in the charge relay system.

CONCLUSION

Experimental studies have emphasized the active involvement of several co-factors of the serine peptidases active sites in the catalysed C-N breakage of the carbonyl donor substrates. Our preliminary results attempt to classify the relative influence of these co-factors on the sequence of the reactions which take place in the enzymes active sites. Thus the stabilization of the oxyanion hole in the tetrahedral intermediates (paths II, III, IV) does not necessarily induce C-N breakage even if the role of the charge relay is assumed. In addition, the geometric orientation of the water molecule governs the reaction path. Since water acts simultaneously as hydrogen donor and acceptor, its role can be interpreted as proton carrier in the charge relay itself. Finally, H_2O and NH_3 may be important features of the addition-elimination reaction. Consistent with the experimental data, this model links the acylation and deacylation phases in the carboxypeptidation and transpeptidation reactions. Note that all the molecules in this study have been fully optimized. On this basis, it is now possible to analyse the influence of specific constraints involved in the active site such as the orientation of the substrate towards the active serine.

Study of the charge relay system on the one hand and of the tetrahedral intermediate on the other, neglects their respective interactions. These two systems do not have the same electric charge : the triad methanol-imidazole-formate is negative while the described intermediates are positive or neutral. Consequently, the system formamide-water-triad will be investigated as a coherent entity.

ACKNOWLEDGEMENTS

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