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SYNERGISM BETWEEN STEREOELECTRONIC AND STEREOPROTONIC EFFECTS IN THE ENZYMIC PEPTIDE BOND FORMATION

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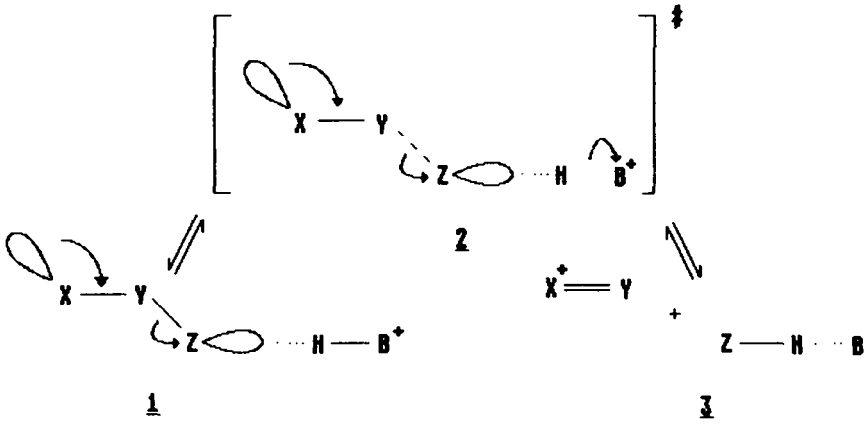
Abstract - *Synergism between stereoprotonic (an inherent preference for protonation/deprotonation in the lone pair direction) and stereoelectronic effects on the formation and breakdown of the tetrahedral intermediate has been demonstrated during the kinetically controlled enzymic aminolysis of specific esters by N-nucleophiles. While both N-methylated and N-unmethylated nucleophiles promote chymotrypsin anilide hydrolysis, the N-methylated nucleophiles do not aminolyse detectably acylchymotrypsin.*

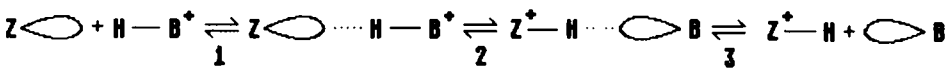
The favourable interaction between a lone pair orbital *n* of the atom X (Scheme I) and the antibonding orbital σ^* of an antiperiplanar polar bond Y–Z (n-$\sigma^*_{Y\text{-}Z}$ interaction) proves to be essential for the stabilization of the ground state (thermodynamic effect) or transition state (kinetic effect) of the mo-



Scheme I

lecular conformation 1[1]. The thermodynamic effect is expressed by the conformational preferences of sugars (anomeric effect[1]) and nucleic acids (gauche effect[2]), the kinetic effect is observed in the stereoelectronic acceleration of the polar Y–Z bond cleavage/formation[3]. On the other hand, a protonation/

deprotonation (acid-base catalysis) of Z in the ground states 1 and 3 in Scheme I or a partial protonation/deprotonation (general acid-base catalysis) of Z in the transition state 2 in Scheme I leads to a weakening/strengthening of the same Y–Z bond[4]. Due to the lone pair directionality[5] this effect is stereoprotonic. Actually, the protonation/deprotonation (step 2, Scheme II) is preceded by hydrogen bonding[6] (proton transfer in ice is ca. 60 times faster than in water[6]). Recent geometrical analysis of



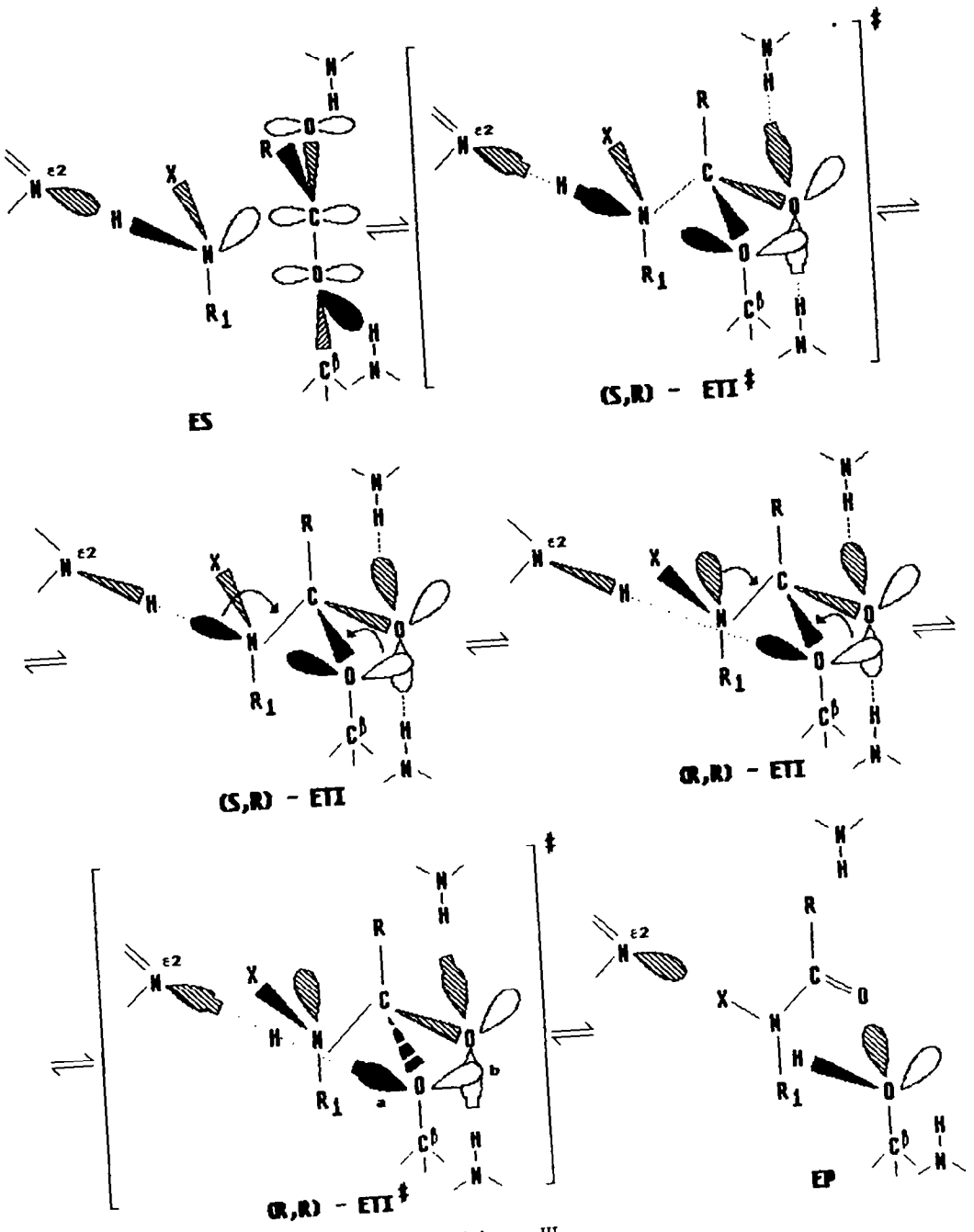
Scheme II

hydrogen bonds in crystal structure[7] reveals a preference of hydrogen bonding in the directions of the conventionally viewed sp^3 and sp^2 lone pairs. Here we analyze the relationship between these stereoelectronic and stereoprotonic effects on the aminolysis of an acylchymotrypsin by N-methylated and N-unmethylated nucleophiles.

The structural and energetic complementarity of the enzyme and substrate may lock the substrate in a reactive conformation 1 (Scheme I). Actually, as it has been deduced from X-ray diffraction studies of the enzyme-inhibitor complexes[7-9], the serine proteinase active site is complementary to a (S,R)-configuration of an enzymic tetrahedral intermediate, ETI (Scheme III, the configuration of the N atom is (S), and that of the former carbonyl carbon - (R)). This ground state configuration is stabilized stereoelectronically since its geometry is favourable for a n-donation by both the Ser-195 γ-oxygen and substrate nitrogen. It is also stabilized stereoprotonically by the hydrogen bonds from the properly oriented imidazole $N^{\varepsilon 2}H$ of His-57 and the backbone NH of Gly-193 and Ser-195 (the "oxyion hole"[10]).

(S,R)-ETI is still an unstable intermediate and according to the *Hammond* postulate[11], the transition state (S,R)-ETI‡ in its formation during the kinetically controlled enzyme aminolysis[12] (Scheme III), will resemble the structure of this intermediate. Moreover, the factors stabilizing the ground state configuration will stabilize the transition state configuration as well. Actually, the energy of (S,R)-ETI‡ is decreased stereoelectronically by the favourable interactions between the developing oxyion and γ-oxygen lone pair orbitals oriented antiperiplanar to the incipient polar C^{α}-N bond. Furthermore, this late transition state is relieved stereoprotonically by partial deprotonation of the entering nucleophile by the imidazole $N^{\varepsilon 2}$ (general base catalysis) and by partial protonation of the "oxyion hole" NH (general acid catalysis). These energetic changes are conjugated with a selective strengthening of the incipient C^{α}-N bond: the $n\text{-}\sigma^{*}_{C-O^-}$ interaction of the N lone pair orbital developing after partial N-deprotection and the antiperiplanar C-O^- bond should lead to some double bond character of this bond.

In order to break down to a peptide, (S,R)-ETI requires a $N^{\varepsilon 2}$ proton transfer to γ-oxygen of Ser-195. Since the inversion of pyramidal nitrogen atom is a very fast process[13], the lowest energy pathway available for this proton transfer is the nitrogen inversion. The latter gives rise to an invertomer (R,R)-ETI (Scheme III) whose nitrogen is no longer hydrogen bonded and the $N^{\varepsilon 2}$ proton can be easily transfered to the γ-oxygen of Ser-195.



Scheme III

The model building studies of *Dutler* et al.[8,14] have revealed that when X in (R,R)-ETI is bulkier than H, this intermediate experiences severe unfavourable interactions between X and the His-57 imidazole ring. The fact that N-methylated peptides[14] and anilides[7] are not substrates for chymotrypsin has been attributed to this steric hindrance, preventing the formation of (R,R)-ETI during chymotryptic peptide hydrolysis. This interpretation has been recently supported by crystallographic and NMR experiments with elastase and a hexapeptide containing N-methylated peptide bond[15].

The configuration of the leaving group nitrogen has been postulated[7,8] by an axiomatic application of *Deslongchamps'* stereoelectronic theory[3]. This is a principal argument of *Sinnot*[16] questioning the stereoelectronic control in serine proteinase action. It is clear from the above discussion that when enzymic aminolysis is considered, the configuration of the nitrogen leaving group follows from the requirements for stereospecific general acid-base catalysis coinciding with the requirements for an effective stereoelectronic catalysis.

We studied the chymotryptic aminolysis of specific esters by an approach we have used previously[17,18]. The values for the aminolysis/hydrolysis ratio β, equal to the rate constant ratio for aminolysis and hydrolysis of acetyl-Phe(NO_2)-chymotrypsin in the presence of some N-methylated and N-unmethylated nucleophiles are summarized in the Table. Characteristically, H-Gly-Phe-$NHCH_3$ and H-Gly-NH_2 are very effective nucleophiles, while H-Sar-Phe-$NHCH_3$ and H-Sar-NH_2 do not aminolyse the acylenzyme detectably even at a nucleophile concentration higher than 1 M. Furthermore, it is known that N-nucleophiles increase the rate of chymotrypsin hydrolysis of glutaryl-Phe-p-nitroanilide by nucleophile binding preventing the nonproductive binding of the anilide portion[19]. Both the N-methylated and the N-unmethylated nucleophiles do promote the chymotrypsin hydrolysis of this anilide (see Table) suggesting a productive binding in the initial enzyme-nucleophile complex. Since this behaviour should be expected from the principle of microscopic reversibility, these results support the earlier observation in the reverse reaction[7,14].

The close inspection of the ground and transition state structure of the (S,R)-isomer of the enzymic tetrahedral intermediate does not provide arguments in favour of the N-methyl nucleophile non-reactivity. Moreover, if the effect is in the next step, the (S,R)-ETI should be a dead-end product and accumulate, provided it is enough stabilized[20]. Since the space between the imidazole hydrogen and nucleophile nitrogen in (R,R)-ETI is not accessible for a X bulkier than H[8], the next step, the N-inversion, should be highly endergonic for N-methylated nucleophiles accounting for their non-reactivity.

The (R,R)- as well as the (S,R)-invertomer is stabilized in the ground state both stereoelectronically and stereoprotonically. The n-donation of nitrogen, however, is changed from C^{α}-O^{-} to C^{α}-O^{γ} polar bond and instead of nitrogen, the γ-oxygen of the Ser-195 is hydrogen bonded to the imidazol $N^{\varepsilon 2}$ of His-57 (Scheme III).

The decomposition of (R,R)-ETI to the enzyme-product complex EA should proceed through an early transition state (R,R)-ETI‡ (*Hammond* postulate[11]). This means that this transition state should be relieved by interactions stabilizing the ground state configuration, the stereoelectronic and stereoprotonic effects being more pronounced in the transition state. The partial direct protonation of the γ-oxygen lone

TABLE

RATE-ENHANCEMENT ACTIVITY AND AMINOLYSIS/HYDROLYSIS RATIO OF SOME N-NUCLEOPHILES AND THEIR N-METHYLATED DERIVATIVES IN CHYMOTRYPSIN ANILIDES AND ESTER AMINOLYSIS

NUCLEOPHILE	RATE ENHANCEMENT $(v_n/v_o)^a$	AMINOLYSIS/HYDROLYSIS $(k_n/k_w)^b$
H-Gly-Phe-$NHCH_3$	3.0	4000
H-Sar-Phe-$NHCH_3$	3.0	<1
H-Gly-NH_2	1.1	3960
H-Sar-NH_2	1.2	<1

[a] Initial rate ratio for the hydrolysis of Glutaryl-Phe-NHPh(NO_2) in the presence (v_n) and absence (v_o) of a nucleophile.

[b] Rate constant ratio for the aminolysis (k_n) and hydrolysis (k_w) of the acylenzyme.

pair (**a** in Scheme III) is probably sufficient for the partial C^{α}-O^{γ} bond cleavage since the proteinase active site does not provide a general acid/base for the direct partial protonation/deprotonation of the other lone pair (**b** in Scheme II). The elimination of the stabilizing effect of this lone pair orbital on the C^{α}-O^{γ} bond by a torsion about the Ser-195 bonds proposed by *Gorenstein* and *Taira*[21] is thus not tenable since such conformational changes should eliminate the effective stereoprotonation of the Ser-195 γ-oxygen.

In conclusion, the synergism between stereoelectronic and stereoprotonic effects is an effective catalytic mechanism used by serine proteinase to accelerate the peptide bond cleavage/formation. Furthermore, the formation of a dead-end tetrahedral intermediate (S,R)-ETI suggest new ideas for the design of proteinase inhibitors.

EXPERIMENTAL

Bovine α-chymotrypsin (chymotrypsin A) was obtained from Boehringer Mannheim and used without further purification. The normality of the enzyme stock solution was determined by an active-site titration with N-trans-cinnamoylimidazole[22].

Substrates. Acetyl-Phe(NO_2)-OMe[17] and glutaryl-Phe-p-nitroanilides[23] were prepared as described previously.

Nucleophiles. Glycinamide and sarcosinamide hydrochlorides were obtained from Sigma. H-Gly-Phe-$NHCH_3$ and H-Sar-Phe-$NHCH_3$ hydrochlorides were prepared by aminolysis of Z-Gly-Phe-OMe and Z-Sar-Phe-OMe in saturated methanolic methylamine and subsequent decarbobenzoxylation by catalytic hydrogenation. The dipeptide esters have been obtained with high yields by acylation with Z-Gly-Cl and Z-Sar-Cl of H-Phe-OMe. H-Gly-Phe-$NHCH_3$·HCl: m.p. 147-9° C, $[\alpha]_D^{20} = 20°$ (c=0.1 H_2O), ^1H NMR (250 MHz, Me_2SO-d_6) data: 8.17 (2H, Gly NH), 7.97 (1H, Phe NH), 4.47 (1H, Phe $C^{\alpha}H$),

3.59 (2H, Gly $C^{\alpha}H_2$), 2.58 (3H, amide-CH_3). H-Sar-Phe-$NHCH_3$·HCl: m.p. 80-83° C, $[\alpha]_D^{20}$= 10° (c=0.1 H_2O). ^1H NMR (250 MHz, Me_2SO-d_6) data: 8.00 (1H, Sar NH), 7.80 (1H, Phe NH), 4.50 (1H, Phe $C^{\alpha}H$), 3.75 (2H, Sar $C^{\alpha}H_2$), 2.72 (3H, Sar CH_3), 2.58 (3H, amide-CH_3).

Rate-enhancement activity of the nucleophiles in the chymotrypsin (1 μM) hydrolysis in 0.2 M Tris-HCl buffer pH 7.8 of glutaryl-Phe-p-nitroanilide (1 mM) was evaluated by the ratio v_a/v_o, v_a and v_o is the increase of the absorption at 410 nm within 10 min in the presence and absence of a N-nucleophile (50 mM).

Aminolysis/hydrolysis ratio β for different nucleophiles has been determined spectrophotometrically as described earlier[18].

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