ELSEVIER

Journal of Molecular Structure 322 (1994) 83–91

Journal of
MOLECULAR
STRUCTURE

The role of short hydrogen bonds in mechanisms of enzymatic action[1]

N.S. Golubev[a], G.S. Denisov[a,*], V.A. Gindin[b], S.S. Ligay[b], H.-H. Limbach[c], S.N. Smirnov[b]

[a]*Institute of Physics, St. Petersburg State University, Ulianovskaya 1, Peterhof, St. Petersburg 198904, Russian Federation*
[b]*Research Institute of Antibiotics and Medical Enzymes, Rizhsky 41, St. Petersburg 198020, Russian Federation*
[c]*Institut für Organische Chemie, Freie Universität Berlin, Takustrasse 3, Berlin D14195, Germany*

(Received 20 October 1993)

Abstract

^{1}H and ^{13}C NMR spectra of trypsin and ribonuclease, stabilized by chemical modification with a hydrophilic polymer, have been obtained over a wide pH range (1–11). The spectral features, referred to some nuclei of the catalytic sites (the "catalytic triad" for trypsin and the His-12–His-119 pair for ribonuclease), have been identified using different NMR techniques as well as chemical modification with selective reagents. It is found that monoprotonation of these systems leads to symmetrical (or quasi-symmetrical) H-bonds formed between the basic groups. This allows us to explain the discrepancies between experimental data obtained by different authors on the protonation sites in these catalytic systems. The simulation of the catalytic triad by a ^{15}N labeled low molecular weight model has led us to the conclusion that external agents do not cause any discrete proton transfers but do cause a smooth shift of the bridging protons from one basic atom to another, with the quasi-symmetrical H-bonds being formed in intermediate cases. On the basis of these experimental data, a new concept has been proposed for the mechanism of acid–base catalysis performed by the pairs of weak basic groups like His–Im and Asp(Glu)–COO^- (pK_a 3–7) which are not capable of proton abstraction from alcoholic or water OH groups ($pK_a > 13$). This catalysis may consist on the one hand of changing the charge densities on reacting groups due to strong H-bonding and, on the other hand, of facilitating the free movement of a proton in the field of several basic atoms when going along the reaction coordinate. The energy of the very strong H-bonds thus formed diminishes the activation energy of the reaction.

1. Introduction

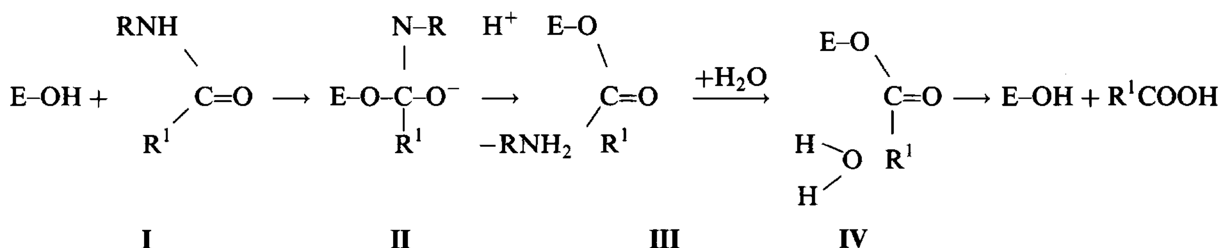
This research is concerned with the problem of acid–base catalysis in enzymatic reactions [1–4]. Extremely high rates of enzymatic reactions require fast chemical steps to be accompanied by at least as fast proton transfers, although unfavorable in terms of free energy (ΔpK_a). The acid–base catalysis in these reactions can be performed only by functional groups of very low basicity (acidity), pK_a 3–8, but its effectiveness is unexpectedly high. Actually, no delay caused by unfavorable proton transfer has ever been found experimentally. A typical example of such a reaction is enzymatic hydrolysis of peptides, proteins and nucleic acids. In the case of

[1] Presented at the Xth Workshop "Horizons in Hydrogen Bond Research", Autrans, France, 12–17 September 1993.
* Corresponding author.

0022-2860/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0022-2860(93)08119-O

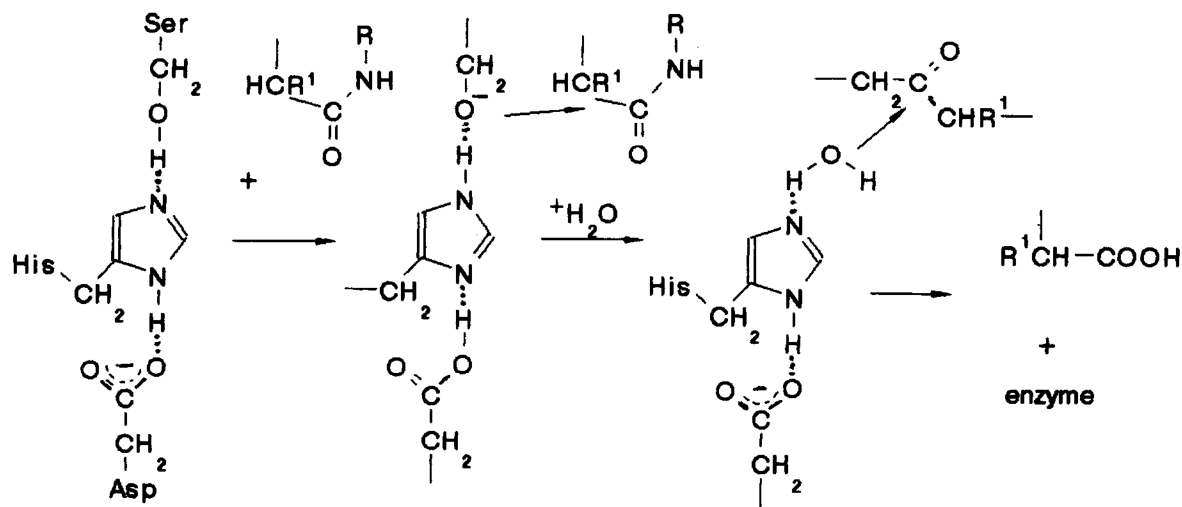
"fast" enzymes like serine proteases (trypsin, chimotrypsin) the enzymatic reaction rate is close to the diffusion limit, while the non-catalyzed reaction under the same conditions (pH 7–8, $T = 300–310\,K$) requires many years for it to go to completion.

Let us consider a general scheme for enzymatic hydrolysis of an amide group [4] (Scheme 1).



Scheme 1.

This reaction goes via the intermediate formation of an acylenzyme, **III**, followed by its fast hydrolysis. The assumed unstable tetrahedral intermediate, **II**, has never been detected experimentally, although there is some indirect evidence for its existence [5,6]. The stage of the acylenzyme formation is rate-limiting, and its fulfilment requires the OH proton to be transferred onto the R^1–NH– group to be eliminated. In the case of the serine proteases the acylenzyme formation state is performed by the OH group of a serine amino acid residue attacking the amide C=O group. When in the neutral state, the reactivity of this alcoholic OH group is insufficient for the nucleophilic addition to proceed with a rate close to the diffusion limit. Deprotonation of the group by a weak basic catalyst (imidazole group of a histidine residue) at pH 7–8 seems to be unlikely. As one of the possible arguments for its high reactivity, the concept of strong dependence of pK_a on medium polarity has been used. In the "charge relay system" postulated by Blow



Scheme 2.

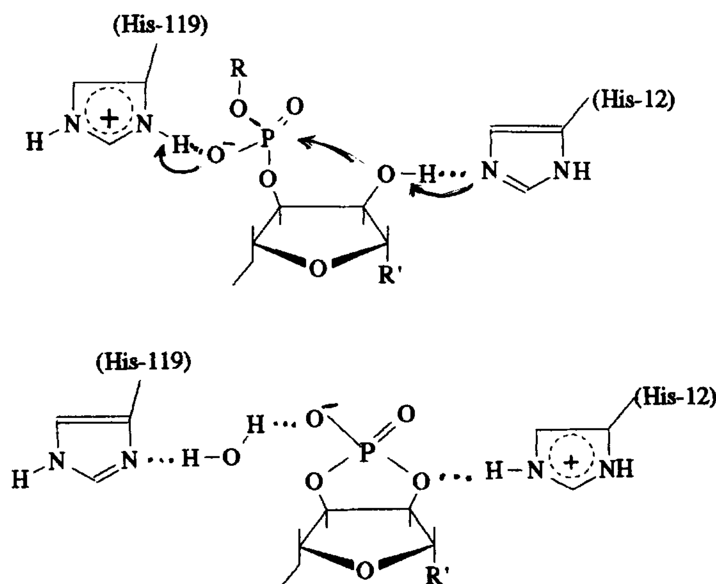
and co-workers, [7,8] and including the H-bonded Asp-His-Ser "catalytic triad" (see Scheme 2) the Asp–COO^- group is "shielded" in the non-polar area of the enzyme globule and is supposed to be characterized by a very high pK_a value. Thus, in an enzyme–substrate complex a double proton transfer might occur through two H-bonds which would enable the nucleophilic addition to proceed. In the following stage of the acylenzyme hydrolysis the same mechanism might activate a water molecule via the double proton transfer from it.

Numerous attempts to verify Blow's hypothesis have not led to an unambiguous conclusion [9–14]. The scheme requires the carboxylate ion but not imidazole (Im) to be protonated preferentially. Spectroscopic data gives contradictory results. At present, most authors believe that the Im ring is more basic than the COO^- group. However, in this case the role of the catalytic triad, as well as of the similar H-bonded systems of basic groups in other enzymes, seems to be unclear.

The mechanism of acid–base catalysis performed by the "charge relay" and similar systems has been discussed above. Let us now consider the conventional mechanism of acid–base catalysis of nucleic acids with ribonuclease [15,16]. The limiting step is the formation of a reactive cyclic phosphate. Here the nucleophilic attack on the phosphoryl group is also performed by a non-ionized OH group. It has been assumed that one His residue induces deprotonation of this group. Another His, while being in the protonated form, performs the stabilization of the group to be eliminated by means of proton transfer onto it (Scheme 3). Here we are faced with the same problem as in the previous case: the catalyzing Im (ImH^+) groups are too weak for these proton transfers to be performed without any delay caused by unfavorable ΔpK values. If the concerted double proton transfer takes place, the pH-optimum of the reaction rate would be close to $pK_a(Im) = 6 \pm 0.5$, instead of the experimental value $pH_{opt} = 8.0$. The latter value obviously requires both the Im groups to be in the neutral form.

In this work we intend to clarify this problem, as well as related questions concerning the mechanism of acid–base catalysis in enzymatic reactions, by means of spectroscopic study of the enzymes and model low molecular weight fragments simulating their catalytic sites.

The pH dependences of ^{13}C and ^{15}N NMR spectra combined with other spectroscopic data could provide the most significant information concerning proton localization inside H-bonded systems in aqueous solutions [17]. This requires a long accumulation of the spectra (for not less than

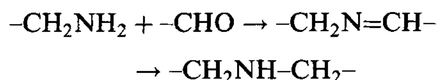


Scheme 3.

several days). As a rule, the native enzymes are not stable enough for their activity and conformational structure to be unchanged when stored under physiological conditions. A considerable stabilization of enzymes may be achieved by their chemical modification with some hydrophilic polymers [18]. In this way, we have succeeded in obtaining NMR spectra of the modified enzymes when accumulating for a week, with no loss of activity.

2. Experimental

Modification of trypsin from bovine pancreas (sequencing grade, Sigma) with an acrolein–vinylpyrrolidone co-polymer (MM 9 KDa, 7% of CHO groups) was performed in accordance with Ref. 18. The scheme of the reaction was as follows:

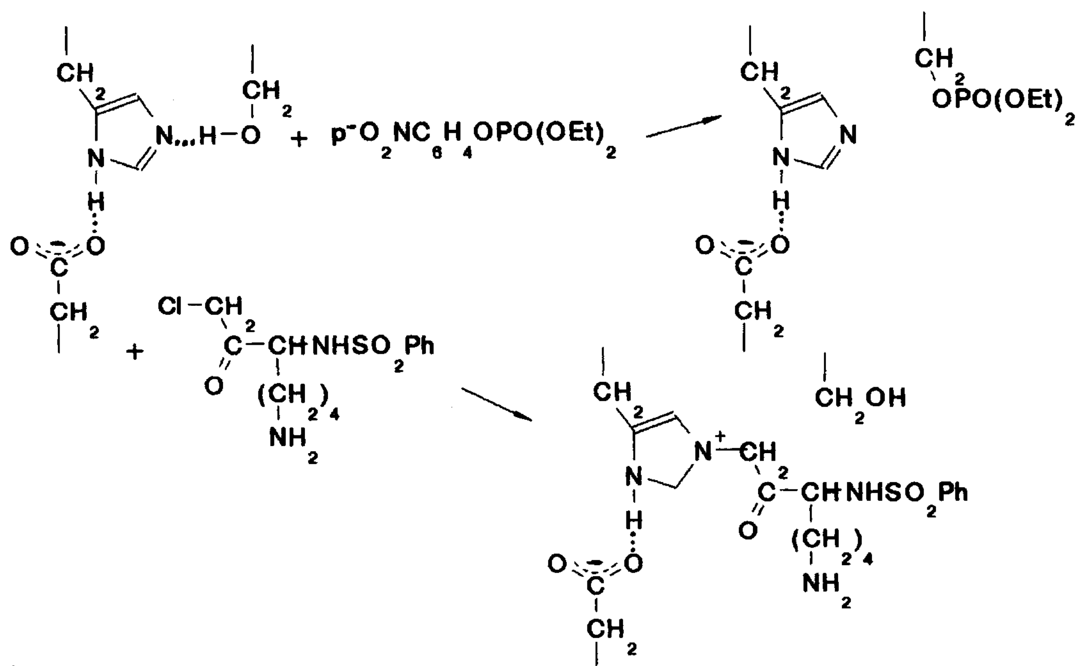


$$-CH_2NH_2 + -CHO \rightarrow -CH_2N{=}CH- \rightarrow -CH_2NH{-}CH_2-$$

A fraction of the conjugate (MM 7 ± 1 KDa) was separated using gel chromatography on a G-150 Sephadex column with 0.01 M HCl as eluent. The concentration of the active sites was determined by means of ^{31}P NMR titration of the serine OH group with *p*-nitrophenyldiethyl phosphate. Any changes in the protein secondary structure upon modification were detected using CD spectra in the 180–320 nm spectral region on a JASCO J-500 spectropolarimeter. The NMR spectra were recorded on a Bruker AC-200 instrument (200.1 MHz for ^1H; 50.3 MHz for ^{13}C; 20.3 MHz for ^{15}N; 81.0 MHz for ^{31}P nuclei) in different regimes (CPD, DEPT, GATEDEC, SELDEC and others) at a concentration of the active sites of 10^{-4}–10^{-5} M. From 2×10^4 to 10^5 scans were required to obtain a good ^{13}C spectrum under these conditions.

3. Results and discussion

The ^{13}C spectrum of the modified trypsin is extremely complicated, consisting of several hundred signals broadened to different extents. Its interpretation was carried out in several steps: (a) separation of signals belonging to the co-polymer as well as the enzyme signals altered as a result of the modification using differential spectra; (b)



Scheme 4.

separation of the signals belonging to the Ser, Asp and His amino acid residues using the numerous data available [6–9,11–16]; (c) identification of several signals which can be referred to these residues forming the catalytic sites. The last step was made on the basis of selective chemical modification of these groups with some special NMR techniques (selective decoupling, nuclear Overhauser effect, heteronuclear 2D correlation spectra, etc.). For example, the signals of the Ser and His functional groups in the catalytic triad of trypsin were attributed to the selective reactions shown in Scheme 4.

Up to now, no selective reagents for the Asp–COO^- group in the catalytic triad are known. However, this carboxylate group is bound to the His Im ring by a strong H-bond. Therefore, chemical modification of the neighboring Im ring proved to cause a marked low-field shift of one of the Asp-$^{13}COO^-$ signals in the spectrum, which enabled it to be assigned. Identification of the signals belonging to the catalytic triad as well as those of the His-12–His-119 system in ribonuclease was confirmed also by means of the nuclear Overhauser effect, based on the identified proton signals [11] for these residues. As an example, Fig. 1 shows the enhancement of the Asp–$^{13}COO^-$ and C2(Im) signals upon selective radio frequency (RF) saturation of the bridging proton signal.

The pH dependences of the chemical shifts for the identified nuclei belonging to the "catalytic triad" of trypsin and the catalytic system of ribonuclease are shown in Figs. 2 and 3, respectively, and are compared with the corresponding shifts for the "normal" amino acid residues lying on the surface of protein globules. The titration curves for these "normal" Im and Asp–COO^- residues reveal the bending points at pH 5.9 and 3.4, respectively, which correspond to the pK_a values for their functional groups. In the case of the groups involved in the catalytic sites, each of the curves contain two bending points. For trypsin, these points indicate pK_as of 7.1 and 1.3, with the $\Delta\delta$ values of the same order of magnitude. This implies that the Asp–$COO^- \cdots$ Im pair acts as a united system, and the question of which of the two bases is protonated preferentially is senseless. The pH maximum for the δ_H function at pH 4–5, equal to 18.1 ppm, corresponds to a short quasi-symmetrical H-bond with the proton delocalized between two basic atoms: $-COO^{\delta-} \cdots H \cdots ImH^{\delta+}$.

As far as ribonuclease is concerned, the corresponding pH dependences show similar behavior. They indicate the formation of a symmetrical H-bond between His-12 and His-119 on monoprotonation of one of these groups: $HIm^{1/2+} \cdots H \cdots ImH^{1/2+}$. (It should be emphasized that no H-bond has been found between these groups

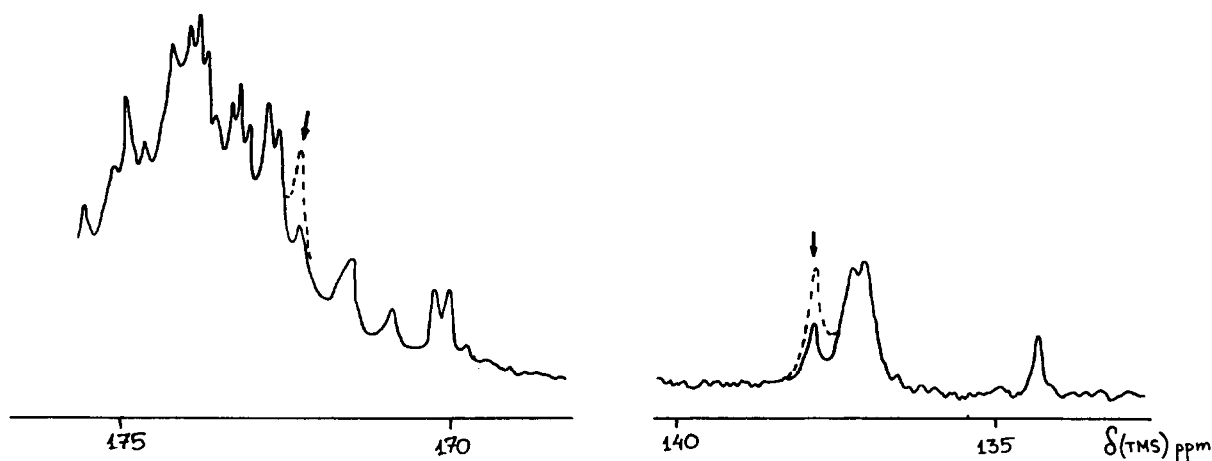
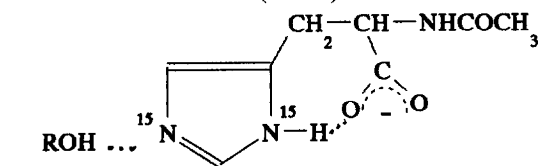


Fig. 1. Part of the ^{13}C NMR spectra (CPD, SELNOE) of modified trypsin (10^{-4} M, aqueous solution, pH 5.0). The broken line shows the enhancement of some ^{13}C signals upon RF saturation of the signal belonging to the bridging proton, δ 18.1 ppm.

Fig. 2. pH dependences of the chemical shifts for some nuclei belonging to the "catalytic triad" of trypsin, compared with those for the shifts (–o–) of the "usual" groups not involved in a H-bonded system.

in the initial, non-protonated state of the enzyme.) We believe that this formation of (quasi)-symmetrical H-bonds upon monoprotonation of the catalytic systems for the two most important hydrolytic enzymes cannot be occasional and must take part in the mechanism of the enzymatic action.

For detailed elaboration of the mechanism of proton transfer processes in the "charge relay system", we have studied H-bonded complexes formed by the model ^{15}N-labeled molecule with a number of OH acids (ROH) shown in Scheme 5.



Scheme 5.

These ROH molecules are intended to simulate the serine OH group when connected to the nitrogen atom by a H-bond. The increase in the acidity of this group by varying the R residue can simulate the enzymatic reaction going along the reaction coordinate. The ^{1}H, ^{13}C and ^{15}N NMR spectra of the complexes of this molecule were produced with a range of OH acids at a temperature low enough for the intermolecular proton exchange to be slowed down and two proton signals, OH and NH, to be observed separately.

Figure 4 shows the dependences of chemical shifts of two ^{15}N nuclei in this molecule as well at two bridging protons on the pK_a value of an ROH acid. It is seen that, with the acidity rising, the ^{15}N signals shift smoothly to the opposite directions. This may be interpreted

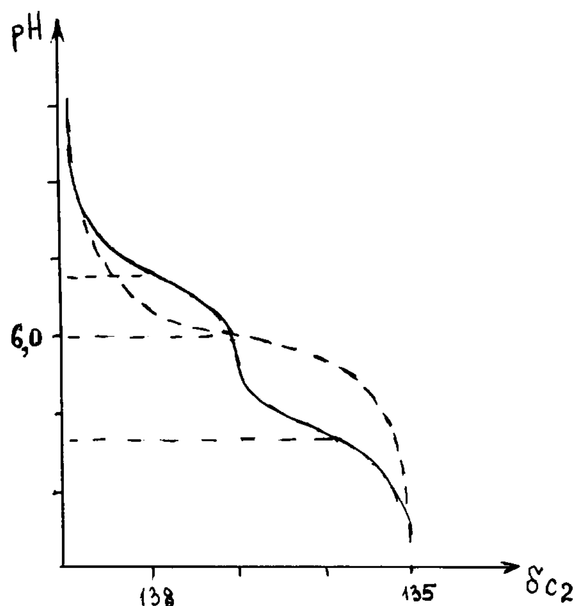
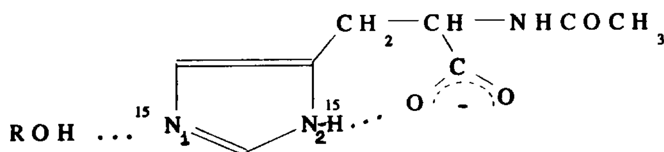


Fig. 3. pH dependence of the chemical shift of the C2(Im) signal belonging to the His residues of the catalytic site of ribonuclease, compared with that for the shift of the "usual" Im groups.

as follows: the O–H···N(1) covalent bond becomes longer and the N(2)H···O^- H-bond becomes shorter. In the intermediate case, when pK_a values for both the OH groups are equal, the system of two quasi-symmetrical H-bonds is formed, $-COO^{1/2-}\cdots H\cdots Im\cdots H\cdots {}^{1/2-}OCO-$. On the subsequent decrease of the pK_a value, the H-bond transforms into a covalent bond. There is no indication of any discrete double proton transfer; this transformation obviously proceeds via a smooth concerted shift of both the bridging protons.

The experimental data considered above allowed us to suggest the following concerted mechanism of acid–base catalysis in reactions catalyzed by serine proteases (Scheme 6).

When the electrophilic C=O group approaches the Ser oxygen atom, a cooperative strengthening of both the H-bonds occurs, i.e. a synchronous shifting of the two bridging protons to the left. This leads to an increase in electron densities both on the oxygen atom and the nitrogen atom



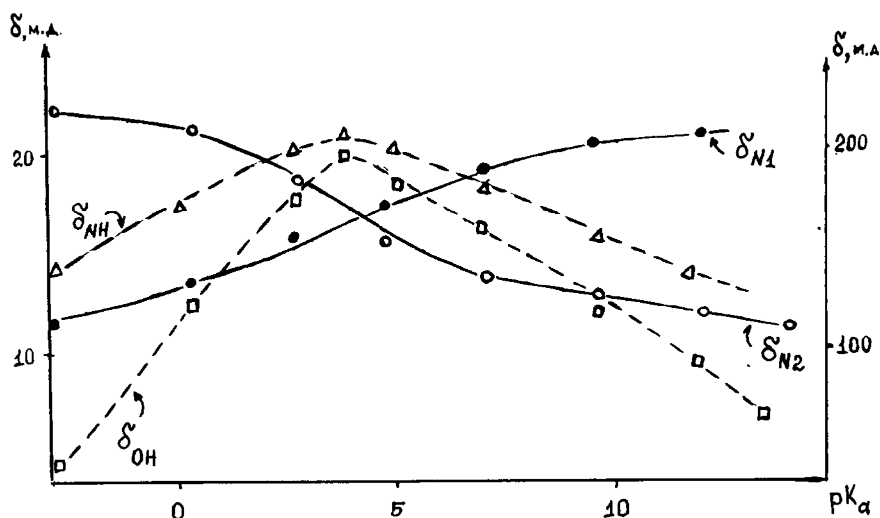
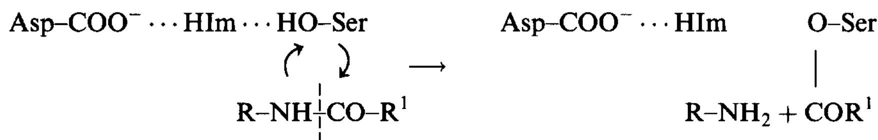


Fig. 4. Dependences of the chemical shifts of the OH, NH, $^{15}N(1)$ and $^{15}N(2)$ nuclei in the complexes of the model molecule with a range of ROH acids on the pH (ROH) values. ROH = CH_3OH, CF_3CH_2OH, $(CF_3)_2CHOH$, $(CF_3)_3COH$, CF_3COOH, CF_3SO_2OH.



Scheme 6.

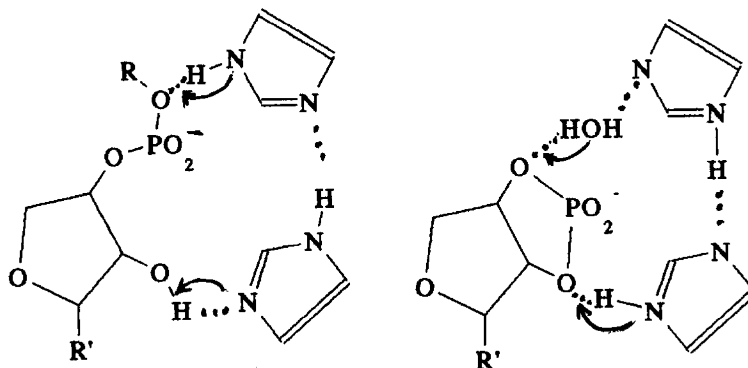
of the catalytic site. Thus, the effective basicity of this nitrogen atom is a function of the reaction coordinate.

Subsequent advancement of the reaction involves a falling-off of the electrophilic influence of the carbonyl group which results in a reverse movement of both the protons thus weakening the H-bonds. However, since the nitrogen atom of the substrate molecule is now more basic than the oxygen atom, the first bridging proton is shifted towards the group to be eliminated. The transition state in this scheme is stabilized by two quasi-symmetrical H-bonds, which may be considered as the main factor decreasing the activation energy. The concerted mechanism briefly described here does not require any unfavorable proton transfers and can be designated as "acid–base catalysis by H–bonding". It accounts for both the basic and the acidic catalysis performed by the Im group in the neutral form (at pH > pK_a(Im)).

For the hydrolysis of nucleic acids performed by ribonuclease, the similar concerted mechanism shown in Scheme 7 may be supposed instead of that in Scheme 3. In the proposed mechanism, two Im groups react simultaneously, both being in the neutral form, which is in accordance with the pH-optimum of the reaction, pH > pK_a(Im). The transition state is stabilized by a very strong symmetrical H-bond.

4. Conclusions

In the studied systems acid–base catalysis is accomplished via a smooth shift of the protons involved, with no discrete proton transfers according to the conventional Broensted scheme. Such strongly coupled H-bonded systems, partially or fully isolated from the aqueous medium, are widely spread in the catalytic sites of enzymes. We believe that the role of these conjugated systems may be a transformation of the potential energy surface in a way that would involve a single minimum corresponding to the equilibrium position of the bridging protons. This minimum is strongly influenced by external agents and, therefore, can follow the reaction coordinate. The proposed scheme of acid–base interaction could explain the excellent efficacy of rather weak basic groups, such as Im (His), in acid–base catalysis.



Scheme 7.

5. Acknowledgment

This work was performed with financial support from the Russian Foundation for Fundamental Research (project code: 93-03-5664).

6. References

[1] G. Zundel. J. Mol. Struct., 45 (1978) 55–73.
[2] D. Hadzi, Proton Transfers in Biological Mechanisms, J. Mol. Struct., 177 (1988) 1–21.
[3] A. Fersht, Enzyme Structure and Mechanism, Freeman, San Francisco, 1980, pp. 165–189.
[4] V.K. Antonov, Chemistry of Proteolysis, Moscow, Nauka, 1984 (in Russian).
[5] M.F. Hegazi, D.M. Quinn and R.L. Schowen, Transition State in Biochemical Processes, Plenum Press, New York 1978, pp. 355–428.
[6] F. De Los de Tar, J. Am. Chem. Soc., 103 (1981) 107–110.
[7] D.M. Blow, J.J. Birktoft and B.S. Hartley, Nature, 221 (1969) 337–340.
[8] D.M. Blow and T.A. Steitz, Annu. Rev. Biochem., 39 (1970) 63–81.
[9] W.W. Bachovchin, R. Kaiser, J.H. Richards and J.D. Roberts, Proc. Natl. Acad. Sci. U.S.A., 78 (1981) 7323–7326.
[10] R.E. Koeppe and R.M. Stroud, Biochemistry, 15 (1976) 3450–3458.
[11] M.W. Hunkapiller, S.H. Smallcombe, D.R. Whitaker and J.H. Richards, Biochemistry, 12 (1973) 4732–4743.
[12] W.W. Bachovchin and J.D. Roberts. J. Am. Chem. Soc., 100 (1978) 8041–8047.
[13] J.L. Markley and M.A. Porubcan, Mol. Biol., 102 (1976) 487–509.
[14] G. Robilliard and R.G. Schulman, J. Mol. Biol., 71 (1972) 507–511.
[15] F.M. Richards and H.W. Wickoff, The Enzymes, 4 (1971) 647–652.
[16] J.L. Markley, Biochemistry, 14 (1975) 3546–3549..
[17] N.S. Golubev, V.A. Gindin, E.V. Maksimov and S.N. Smirnov, Khim. Fiz., 10 (1991) 1659–1662.
[18] T.M. Taratina, V.E. Potapenko, S.P. Klenin, S.P. Bartoshevic, L.A. Volkova, I.G. Deiisov and B.V. Moskvichev, Biophys. Chem., 38 (1990) 1–10.