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Sigmoid Kinetics of the Monomeric Ribonuclease I Due to Ligand-Induced Shifts of Conformation Equilibria

Helga Rübsamen, Rahim Khandker und Herbert Witzel

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Dedicated to Prof. Dr. Th. Wieland on the occasion of his 60th birthday

Summary: Bovine pancreatic ribonuclease I, a monomeric enzyme, shows sigmoid kinetics in the reaction with some of its substrates at pH values above 6.6. These kinetic anomalies are explained on the basis of the proposed reaction mechanism by postulating a conformational equilibrium between two enzyme species, E_a and E_i, which form the kinetically relevant complex ES at different rates via two pre-complexes, E_aS and E_iS. From ES, the enzyme is released predominantly or exclusively in the more active species E_a.

The transition from E_a to E_i is slow compared with the rate-determining steps for the formation and breakdown of ES, so that the equilibrium between E_a and E_i cannot be achieved with increasing substrate concentrations. Finally, only the faster reacting species E_a is involved in the formation of ES. The molecular structures in the catalytic center of the species E_a, E_i, E_aS, E_iS and ES are defined taking into account the results of previous experiments on binding and catalysis.

The rate equation derived from the corresponding kinetic scheme contains s^2 terms. By a mathematical analysis of this equation it is shown that it depends on the relations among three constants (composed of the individual rate constants) whether substrate activation, substrate inhibition or normal saturation kinetics can be expected. With respect to the proposed reaction mechanism of ribonuclease I it is possible to predict experimental conditions under which the degree of sigmoidicity should increase or decrease.

Address: Prof. Dr. H. Witzel, Institut für Biochemie der Universität, D-44 Münster, Orléansring 23a.

Abbreviations and Symbols

Up-Nph = uridylyl-3′→1-*O*-naphthalene
Up-NphOH = uridylyl-3′→1-*O*-naphthalene-5-ol
Up-PhOMe = uridylyl-3′→1-*O*-(4-methoxy)phenol.

s = substrate concentration
e_0 = total enzyme concentration
v = initial reaction velocity
V = maximal reaction velocity
E = enzyme
ES = enzyme-substrate complex
EI = enzyme-inhibitor complex
K_m = Michaelis constant

In this paper, overall velocity constants which cannot a priori be related to individual reaction steps are marked with an asterisk, for example k_{+2}*.

Enzymes:

Guanyloribonuclease *(Aspergillus oryzae)*, ribonucleate 3′-guanylo-oligonucleotidohydrolase also known as ribonuclease T_1 (EC 3.1.4.8; formerly EC 2.7.7.26)

Ribonuclease I (bovine pancreas), ribonucleate 3′-pyrimidino-oligonucleotidohydrolase, also known as ribonuclease A (EC 3.1.4.22; formerly EC 2.7.7.16)

Ribonuclease II *(Aspergillus oryzae)*, ribonucleate 3′-oligonucleotidohydrolase, also known as ribonuclease T_2 (EC 3.1.4.23; formerly EC 2.7.7.17).

In agreement with theoretical considerations, modified substrates, which have enhanced electrophilic character at the phosphorus, show an increase of the sigmoid pattern in the kinetics due to an alteration in the ratios of the rate-determining constants. Furthermore, as is also expected theoretically, an increase of the pH value leads to a shift in the equilibrium between E_a and E_i in favor of E_i and thus increases sigmoidicity, while sulfate ions shift the equilibrium in favor of E_a and lead to a complete normalization of the kinetics at high concentrations.

The kinetic counterpart of substrate activation has been found in the substrate inhibition kinetics of the ribonucleases from *Aspergillus oryzae*, guanyloribonuclease (T_1) and ribonuclease II (T_2), for which the proposed reaction mechanisms require the preferential release of the enzyme in the less active species.

The concept of "ligand-induced shifts of conformational equilibria" (LISCE) in "multipathway" enzymes is based on direct interactions of the ligands with the active site. Indirect interactions with effectors, which can also affect the crucial rate constants, are not excluded. The model can be applied to monomeric as well as oligomeric enzymes, if for the active site of each subunit a slow equilibrium between at least two distinct conformations and the predominant release of the enzyme in only one of them are assumed. This non-equilibrium model, therefore, can be considered to be an alternative to the concepts of subunit interactions derived for non-hyperbolic kinetics of oligomeric enzymes.

Sigmoide Kinetiken bei einer monomeren Ribonuclease I durch Liganden-induzierte Verschiebung eines Konformationsgleichgewichts

Zusammenfassung: Rinderpankreas-Ribonuclease I ist ein monomeres Enzym und zeigt sigmoide *v/s*-Kurven bei der Reaktion mit einigen Substraten im pH-Bereich oberhalb 6.6.

Die Erklärung dieser kinetischen Anomalie ergibt sich aus dem Reaktionsmechanismus, der ein sich langsam einstellendes Gleichgewicht postuliert zwischen zwei Spezies E_a und E_i, die mit unterschiedlicher Geschwindigkeit über die Vorkomplexe E_aS und E_iS den kinetisch relevanten ES-Komplex bilden. Aus dem ES-Komplex wird das Enzym vorwiegend oder ausschließlich in der E_a-Form entlassen.

Da die Umlagerung von E_a nach E_i langsam ist, kann sich bei höheren Substratkonzentrationen das Gleichgewicht nicht mehr einstellen, die Bildung von ES läuft schließlich nur noch über den schnelleren E_a-Cyclus.

Die molekularen Strukturen der Spezies E_a, E_i, E_aS, E_iS und ES lassen sich auf der Basis vorangegangener Untersuchungen zur Bindung und Katalyse definieren.

Hieraus läßt sich ein kinetisches Schema aufstellen, von dem eine Geschwindigkeitsgleichung mit einem s^2-Term abgeleitet werden kann. Durch eine mathematische Analyse der Gleichung können die Bedingungen aufgezeigt werden, wann anomale Kinetiken in Form von „Substrataktivierung" oder „Substrathemmung" zu erwarten sind. Es können aus dieser Analyse weiterhin Bedingungen vorausgesagt werden, unter denen das Ausmaß der Sigmoidität zu- oder abnehmen soll.

Experimente mit entsprechend modifizierten Substraten, bei denen das Verhältnis bestimmter Geschwindigkeitskonstanten zueinander verschoben ist, sowie Experimente, bei denen das Verhältnis der Species E_a zu E_i durch Effektoren verschoben wird, zeigen die erwarteten Resultate. So kann durch Sulfat-Ionen in direkter Wechselwirkung mit dem aktiven Zentrum die anomale Kinetik voll normalisiert werden.

Das Gegenstück zur „Substrataktivierung" bei der Rinderpankreas-Ribonuclease I wurde bei den Ribonucleasen aus *Aspergillus oryzae*, Guanyloribonuclease (T_1) und Ribonuclease II (T_2), gefunden, bei denen vom Mechanismus her eine bevorzugte Entlassung des Enzyms in Form der E_i-Species postuliert werden muß. Beide Enzyme zeigen auf der Basis derselben Geschwindigkeitsgleichung den entsprechenden Kinetik-Typ einer „Substrathemmung".

Das Konzept der Liganden-induzierten Verschiebung eines Konformationsgleichgewichts beruht auf direkten Wechselwirkungen der Liganden mit dem aktiven Zentrum. Das Konzept kann auch auf oligomere Enzyme übertragen werden, wenn für das aktive Zentrum einer jeden Untereinheit ein Gleichgewicht zwischen wenigstens zwei Konformationsisomeren angenommen wird und beim Zerfall des ES-Komplexes bevorzugt die eine Spezies zurückgebildet wird.

Damit kann dieses „non-equilibrium"-Modell als eine Alternative zu den Modellen betrachtet werden, die zur Erklärung der nichthyperbolischen Kinetiken bei oligomeren Enzymen von einer indirekten Wechselwirkung zwischen den Untereinheiten ausgehen.

Several attempts have been made to explain sigmoid kinetics. In terms of the models developed by Monod *et al.*[1] as well as by Koshland *et al.*[2,3] this type of kinetics is interpreted by assuming cooperative binding of the ligands to allosterically interacting subunits of the enzyme. According to their theories, sigmoid kinetics are restricted to oligomeric enzymes. In contradiction to this viewpoint, Anderson and Weber[4] put forward the idea that molecular relaxation effects might account for sigmoid behavior and that the binding of ligands to proteins occurs in a stationary state rather than under true equilibrium.

On the other hand, models have been worked out for two-substrate enzymes which do not require subunit interactions[5–7]. Ferdinand[8] has shown for phosphofructokinase that sigmoid kinetics can result from two-substrate reactions, if the substrates bind to the enzyme under non-equilibrium conditions, and that this kinetic behavior may lead to the phenomena of substrate activation as well as substrate inhibition, a concept mentioned before by Dalziel[9].

By mathematical analysis of the steady-state rate equations that were derived from possible kinetic schemes for two-substrate enzymes, Sweeny and Fisher[10] demonstrated that any model that conforms to a rate equation of the form $1/v = a + b \times s^x$ (v = initial velocity, s = substrate concentration), where a and b are constants and x is equal to or less than -2, will give rise to sigmoid relationships between velocity and substrate concentration and that any model requiring a single, independent active site will do so, if there is more than one pathway leading to the binding of one substrate molecule to the single active site. This idea was subsequently reinforced by a series of communications on two-substrate enzymes[11–14].

For one-substrate enzymes, Keleti[15] showed that any cyclic model containing two different enzyme species will give a rate equation with s^2 terms, and that consequently a sigmoid relationship between reaction velocity and the substrate concentration may be obtained without any further assumptions of cooperative interactions between subunits.

The suggestion that substrate-induced conformation isomerization might be a model for cyclic processes leading to sigmoid kinetics was put forward by Rabin[16].

Frieden[17] has shown that when two conformational states of a single-substrate enzyme exist, both of which bind the substrate with different affinities, bursts or lags of enzyme activity in the progress curve can result, and he introduced the term "hysteretic enzyme" for cases in which the enzyme responds slowly to rapid changes in ligand concentration. In addition, Ainslie *et al.*[18] recently showed by analyzing the mathematical expressions for progress curves and steady-state velocities that whenever a transient is found in a progress curve, cooperativity of the kinetics should also be seen and that it must be considered as a necessary condition for cooperativity that both the binding and the isomerization steps of the mechanism should be in a steady state but not at equilibrium. So far, no *molecular* model has been presented for the interpretation of non-hyperbolic kinetics of monomeric enzymes.

In this paper, it is shown that Ribonuclease I, a monomeric enzyme with one active site and acting on one substrate, gives non-linear Lineweaver-Burk plots and sigmoid v/s curves with certain substrates at pH values above 6.6, which can be normalized by sulfate ions acting as positive effectors[19–21]. On the basis of the proposed mechanism for this enzyme we present a molecular model, which explains sigmoidal v/s curves as a consequence of a slow equilibrium between two enzyme species which form the same ES complex but at different rates. From the ES complex the enzyme is exclusively or predominantly released as the more active species. The application of this non-equilibrium model to substrate inhibition kinetics and to the kinetics of oligomeric enzymes is indicated.

Materials and Methods

Synthesis of uridylyl-3′ → 1-O-(4-methoxy)phenol (Up-PhOMe), uridylyl-3′ → 1-O-naphthalene (Up-Nph) and uridylyl-3′ → 1-O-naphthalene-5-ol (Up-NphOH)

The esters of 3′-uridylic acid were synthesized according to the route given in Fig. 1, which corresponds to a modified procedure of Zan-Kowalczewska *et al.*[22].

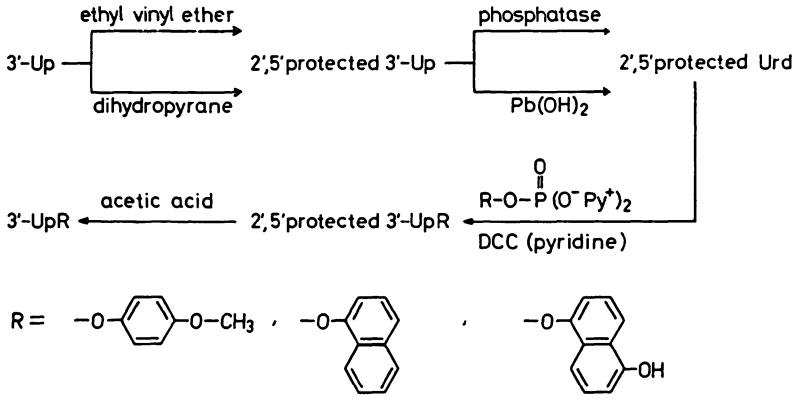


Fig. 1. Procedure for the synthesis of activated esters of 3'-Up.

a) Synthesis of 2',5'-bis-O-(1-ethoxyethyl)uridine

1360 mg (4 mmol) uridine 3'-phosphate (3'Up × H_2O) (Boehringer Mannheim GmbH) were evaporated 3 times with 5 ml of dry dioxane. To the dry powder were added: 24.6 ml dry dioxane, 13.2 ml ethyl vinyl ether and 0.53 ml dimethylformamide saturated with HCl. The solution was stirred for 2.5 h, then 10 ml 4N NH_4OH was added and the resulting mixture was evaporated. The remaining 2',5'-bis-*O*-(1-ethoxyethyl)uridine 3'-phosphate[23] was dissolved in a minimum of dry ethanol and was added dropwise to 200 ml of dry ether with vigorous stirring. The precipitate was collected by centrifugation. The supernatant was evaporated and the residue was dissolved in ethanol and reprecipitated. The combined precipitates were dried under vacuum and dissolved in 20 ml 0.05M NH_4OAc − 0.2M $MgCl_2$ (pH 9.0). After readjusting the pH to 9.0 with 4N NH_3, 10 mg of crude alkaline phosphatase was added. The mixture was kept at 37°C for about 24 h until completion of the reaction. The progress of the dephosphorylation was monitored by thin-layer chromatography on silica gel using chloroform/methanol 9:1 as a solvent; 2',5'-bis-*O*-(1-ethoxyethyl)uridine 3'-phosphate and 2',5'-bis-*O*-(1-ethoxyethyl)uridine have R_F values of 0 and 0.92, respectively.

After completion of the reaction the solution was evaporated to dryness, dissolved in a minimum of methanol and applied to a silica gel column (4 cm × 25 cm). The substance was eluted with chloroform/methanol 9:1. The first peak contained the protected nucleoside in 60 − 80% yield.

For large scale preparations (15 mmol of uridine 3'-phosphate) the dephosphorylation was performed with $Pb(OH)_2$ according to the procedure of Dimroth *et al.*[24]. For this procedure the tetrahydropyranyl derivative was used since the ethoxyethyl groups are unstable under these conditions. The 2',5'-bis-*O*-(tetrahydropyranyl)uridine 3'-phosphate[25] was prepared following the method for the ethoxyethyl derviative, except that the reaction time was increased to 14 h. After addition of 200 ml 1N NH_4OH, a solution of 15 g lead acetate in 40 ml water was added. The precipitate containing the lead salt of the nucleotide in an excess of $Pb(OH)_2$ was collected by centrifugation and washed 3 times with water to remove NH_3. The suspension in 1.5 l of water and 300 ml n-butanol was refluxed for 12 h and then filtered. The phosphate and the unreacted nucleotide remained in the residue, while the filtrate containing the 2',5'-protected nucleoside was evaporated to dryness. The purification on a silica gel column was performed as described before. The yield of the 2',5'-bis-*O*-(tetrahydropyranyl)uridine was 60%.

b) Synthesis of the phosphoric acid esters

1-Naphthyl phosphate and 1-(4-methoxy)phenyl phosphate were prepared according to the methods of Kunz[26] and of Katyschkina and Kraft[27] respectively. For the preparation of 5-hydroxy-1-naphthyl phosphate 32 g (0.2 mol) of 1,5-naphthalenediol and 91.2 g (0.6 mol) of $POCl_3$ were refluxed for 48 h. The remaining $POCl_3$ was evaporated and the residue dissolved in 100 ml of water and filtered. The pH of the filtrate was adjusted to 4.0 with conc. NH_4OH. The solution was evaporated to dryness. The residue containing the monophosphorylated and the diphosphorylated products was dissolved in 100 ml methanol and the diphosphate was precipitated by the addition of 200 ml acetone. For the complete removal of the diphosphorylated product, this procedure was repeated several times. The yield of the monophosphorylated product was 4.8 g = 15%.

c) Condensation of 2',5'-bis-O-(1-ethoxyethyl)uridine with the phosphoric acid esters

For the synthesis of the esters of uridine 3'-phosphate the general procedure of Gilham and Khorana[28] was used.

1 mmol of the protected nucleoside and 1.4 mmol of the pyridinium salt of the phosphoric acid ester were evaporated three times with dry pyridine. The residue was dissolved in 8 ml of dry pyridine and 6 mmol of dicyclohexylcarbodiimide were added. The mixture was kept in the dark at room temperature for about two days. After completion of the reaction 10 ml of 0.1N NH_4OH was added. The precipitated dicyclohexylurea was removed by filtration and the filtrate was evaporated to dryness. The crude product was dissolved in 50 ml methanol, to which 50 ml of 0.005M $(C_2H_5)_3NH \times HCO_3$ was added, and applied to a DEAE cellulose column (2 cm × 20 cm). The compounds were eluted using a linear gradient of 500 ml 0.005M $(C_2H_5)_3NH \times HCO_3$/methanol 1:1 in the mixing vessel and 500 ml 0.2M $(C_2H_5)_3NH \times HCO_3$/methanol 1:1 in the reservoir. The first peak following the breakthrough contained the ester of the protected uridine 3′-phosphate. It was pooled and freed from $(C_2H_5)_3NH \times HCO_3$ by repeated entrainment with 50% methanol. The esters were converted into the sodium salts by passage over a Dowex 50 [Na^+] column (1 cm × 12 cm). After evaporation to dryness, they were stored at −20°C without removal of the protective groups. The yields varied from 45 − 70%, depending on the nature of the alcohol.

Before the kinetic measurements, the ethoxyethyl groups were split off by treatment with 5% acetic acid for 2 h at 20°C, and the substance was purified by paper chromatography, using 2-propanol/0.05% acetic acid 7:3 as solvent. The bands, which contained the unprotected esters, were eluted with methanol.

The purity of the compounds was checked by paper electrophoresis. With ribonuclease I and alkaline phosphatase (analytical grade) the esters were completely degraded to uridine and the corresponding alcohols, indicating that they were not contaminated with the 2′-isomers. The molar extinction coefficients were calculated from nucleoside analysis according to the method of Gassen and Leifer[29].

Analytical data

The spectra were registered with a Cary 15 spectrophotometer at pH 7.0 in buffer solution I.

Paper chromatography was performed in 2-propanol/0.05% acetic acid 7:3 as solvent, using uridine as standard ($R_F \approx 0.69$; $R_U = 1.0$) and paper electrophoresis was performed at 40 V/cm in 0.05M HCO_2H/HCO_2NH_4 pH 2.5, using uridylic acid as standard, ($R_E = 1.0$).

5-Hydroxy-1-naphthyl phosphate: λ_{max} = 326, 311, 396 nm; λ_{min} = 321, 308, 249; ε_{296} = 8630, ε_{260} = 1820, A_{280}/A_{260} = 1.8, R_U = 0.36, R_E = 1.0.

Up-PhOMe: λ_{max} = 262.5 nm; λ_{min} = 235 nm; ε_{260} = 10600, A_{280}/A_{260} = 0.49; R_U = 1.2, R_E = 0.66.

Up-Nph: λ_{max} = 317, 303 sh, 265 nm; λ_{min} = 314, 239 nm; ε_{260} = 12100, A_{280}/A_{260} = 0.75; R_U = 1.1, R_E = 0.66.

Up-NphOH: λ_{max} = 325, 311, 297, 265 nm, λ_{min} = 322, 308, 290, 242 nm, ε_{260} = 12300, A_{280}/A_{260} = 0.75; R_U = 1.0, R_E = 0.64.

Kinetic measurements

The kinetic measurements were performed as described earlier[30] using a Cary 15 spectrophotometer.

Ribonuclease I (No. 15410, Boehringer Mannheim GmbH) was used without further purification. Three different buffer solutions were used.

I) 0.05M β,β-dimethylglutaric acid, 0.1M NaOH and 0.1M NaCl, I = 0.2.

II) 0.05M β,β-dimethylglutaric acid, 0.1M tris(hydroxymethyl)aminomethane and 0.1M NaCl, I = 0.2.

III) Buffer II, with different concentrations of $(NH_4)_2SO_4$ but omitting the NaCl.

The pH values were adjusted by addition of HCl or NaOH, and were rechecked after the kinetic measurements.

The measurements were performed at suitable wavelengths: for Up-PhOMe at 277 and 300 nm, for Up-Nph at 315 nm, and for Up-NphOH at 277 nm and 325 − 335 nm.

The initial velocities v were calculated from the progress curves with the aid of a desk computer (Diehl algotronic) assuming pseudo-first order kinetics. The validity of this assumption was checked for each set of measurements.

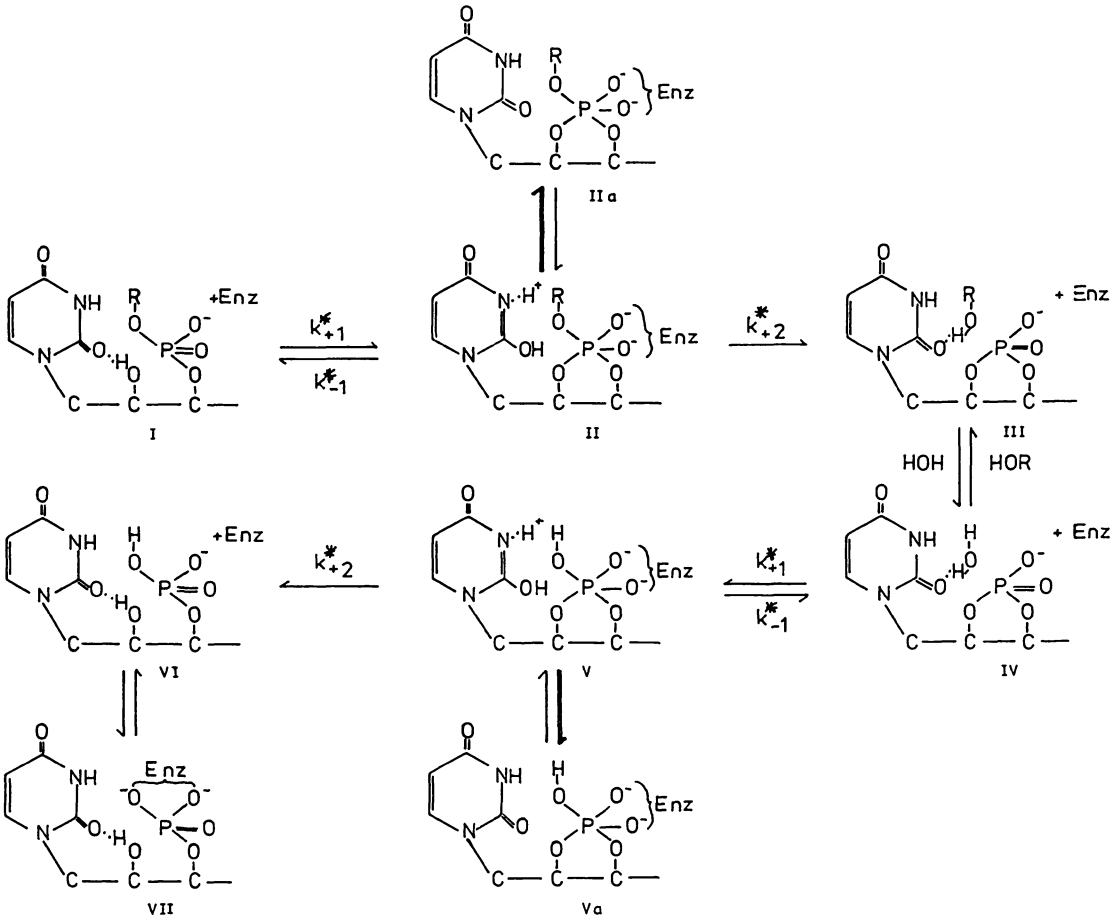
k^*_{+2} ($= V/e_0$) and K_m values were obtained from e_0/v versus $1/s$ plots[31] or s/v versus s plots[32].

Results

A) The basic mechanism for binding and catalysis

Scheme 1 (I − VII) shows the pathway for the reaction of ribonuclease I with its substrates, as previously postulated[30,33–38]. The enzyme activates the phosphate group by two protonating, positively charged groups, while at the nucleophilic site the 2′-OH group in the first step, or the water molecule in the second step are activated by the 2-oxo group of the pyrimidine base or its equivalents[30]. Details of the processes lowering the activation energy are discussed in ref.[38].

Scheme 2 (VIII-XII), shows the postulated binding process for dianionic monoesters (e.g. the reaction products) which are competitive inhibitors[33,36]. The binding species of the enzyme is that with the triprotonated diimidazole system (IX) in which His 119 is kept at a fixed distance to Lys 41 by a hydrogen bond to His 12. The phosphate group of the inhibitor binds electrostatically to the imidazolium cation of His 119 and to the ammonium

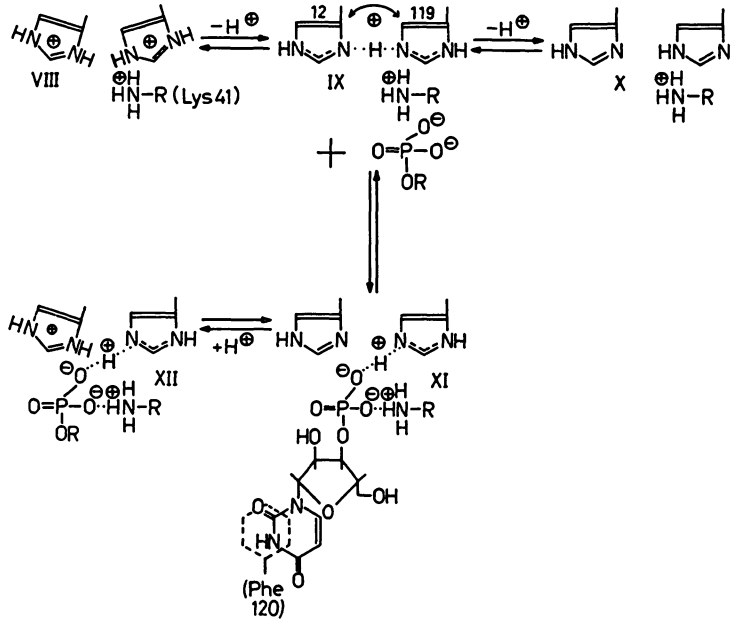


Scheme 1.

group of Lys 41, whereas the base moiety interacts with Phe 120[33,37–39]. The additional proton uptake after formation of this complex, (XI) which is observed in the pH range between 6 and 7, has been explained by an increase of the pK value of His 12, when released from its binding to His 119 in the diimidazole system (XI → XII)[33].

The studies on monoanionic substrates allow one to assume the formation of a similar, but weaker enzyme-substrate complex (E_aS) by an interaction of the phosphate anion with Lys 41 and of the pyrimidine base with Phe 120[39]. The intermediate state (II, V), with a pentacovalent phosphorus, is formed from the E_aS complex only if the pyrimidine base takes up the proton from the 2′-OH group and His 119 protonates the second phosphate oxygen in a simultaneous step. Criteria have been discussed[33], which may be used to decide whether this enzyme-stabilized intermediate is the kinetically relevant enzyme substrate complex ES.

When the complex is formed, His 12 is released. Due to the neutralization of the charges of the imidazolium group of His 119 and of the ammonium group of Lys 41 by the two anionic phosphate oxygens, the pK value of His 12 should increase analogously to that in the binding process with inhibitors. More precisely, the center formed by the three bases His 12, His 119 and the phosphate anion, having only one proton, takes up a further proton which however, as indicated by the NMR data[36], is located more at the His 12 site. Only in this form is the ES complex stabilized.



Scheme 2.

The breakdown of the ES complex is initiated by the deprotonated His 12 which competes for the proton between His 119 and the phosphate oxygen, and a simultaneous proton catalysis by the pyrimidine base in the form of its conjugate acid at the 2'-oxygen (reverse reaction II → I) or at the oxygen of the leaving group (product formation II → III). The breakdown of the stronger enzyme-inhibitor complex with a tetracovalent dianionic structure at the phosphorus and higher basicity should occur analogously, however, at higher pH values[36].

B) The pH dependence of k^*_{+2} and K_m

The pH dependence of $1/K_m$ and k^*_{+2}, in agreement with previous studies[40,41] for the transesterification step (I-III) as well as for the hydrolysis step (IV-VI), follows a bell-shaped curve with maxima at pH 5.6 and pH 7.2, respectively (Fig. 2; K_m is shown instead of $1/K_m$). In the breakdown steps of the ES complex (II → I and II → III), k^*_{-1} and k^*_{+2} are coupled rate constants[35] for alternative paths, which have the same pH dependence. Therefore the pH dependence of k^*_{+1} (overall formation of ES) should be that of k^*_{+2}/K_m and should depend on the concentration of the free enzyme in the active form (IX). The calculated k^*_{+2}/K_m versus pH curve (Fig. 2b) has a maximum at pH 6 and the roughly derived pK values of 5.5 and 6.5 agree well with the pK values obtained with higher acuracy from NMR data for His 119 and His 12 in the free enzyme[36].

Since the breakdown of ES is assumed to be initiated by the deprotonated His 12, the maximum value for k^*_{+2} should be reached after its complete deprotonation. k^*_{+2} therefore should not decrease at pH values above 7.2, provided that with increasing substrate concentration the concentration of ES finally equals the total enzyme concentration e_0. Since there is no reason to assume a higher stability of the ES complex with higher $OH^\ominus$ concentrations, the drop of k^*_{+2} should indicate that, at least at higher pH values, the formation of ES is rate-determining so that with $s \rightarrow \infty$ the concentration of ES is not equal to e_0. The pH dependence curve of k^*_{+2} should be determined in the alkaline range primarily by the concentration of IX. The altered pK of His 12 in the ES complex, however, should shift the profile to higher pH values. Consequently, the maximum of the pH dependence of $1/K_m$ (= pH dependence of the formation of the ES complex over the pH dependence of its break-

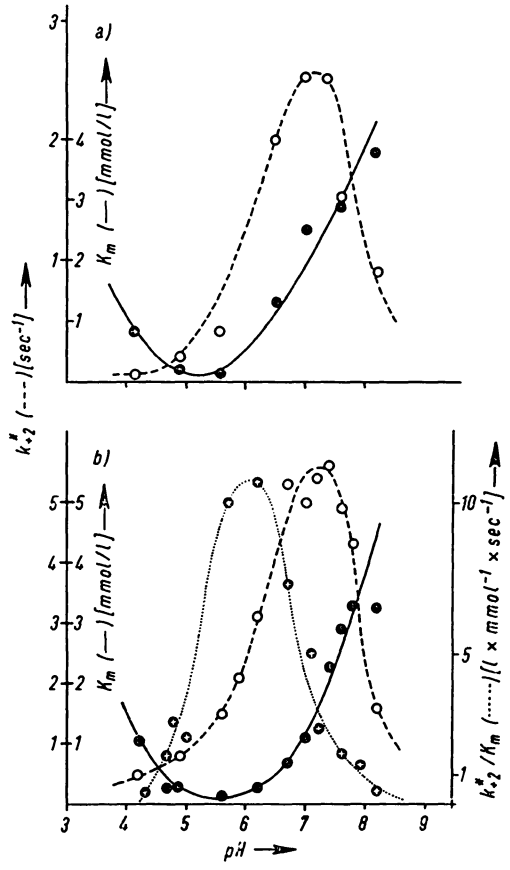


Fig. 2. The pH dependence of the k^*_{+2} values, the K_m values and k_{+2}/K_m for the hydrolysis of 3′,5′-UpA (a) and 2′,3′-cyclic Up (b) by bovine pancreatic ribonuclease I.
The measurements were carried out at 20°C in buffer II.

down) is shifted to a pH value which is lower than that at which the concentration of IX is maximal. An argument in favor of these assumptions may be the observation that dinucleoside phosphates have increased k^*_{+2} values, although the mechanism postulates only an increase of the rate constant for the k^*_{+1} step[34]. Since, however, $K_m \left(\simeq \frac{k^*_{-1} + k^*_{+2}}{k^*_{+1}}\right)$ remains constant for these substrates it follows that when k^*_{-1} and k^*_{+2} are raised by a factor of up to 100, k^*_{+1} must be increased by the same factor or, vice versa, k^*_{+2} and k^*_{-1} must increase with the increase of k^*_{+1}, which means that k^*_{+1} is also the rate-determining step for the breakdown.

C) Non-hyperbolic kinetics and their interpretation on the basis of the proposed mechanism

Fig. 3a shows the v/s curve and the Lineweaver-Burk plot for the hydrolysis of 2′,3′-cyclic cytidylic acid at pH 7.6, extended to high substrate concentrations. Obviously an upward deviation from linearity is found in the e_0/v versus $1/s$ plot. Fig. 3b demonstrates for the v/s curve that two different rate equations are obtained, depending on whether the Michaelis-Menten function (1) is fitted to the

$$v = \frac{e_0 k_{+2}}{\frac{K_m}{s} + 1} \tag{1}$$

$$v = e_0 x_1 \times \frac{s^2 + x_2 s}{s^2 + x_3 s + x_4} \tag{2}$$

experimental values in the range of low or of high substrate concentrations, respectively. For equation 2, which will be derived in this paper and which contains s^2 terms, a good fit results over the whole range of substrate concentrations. Similar results are obtained for 2′,3′-cyclic cytidylic acid at all pH values above 7.2.

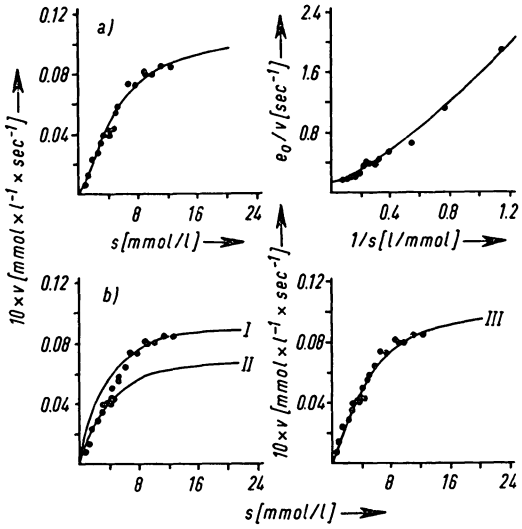
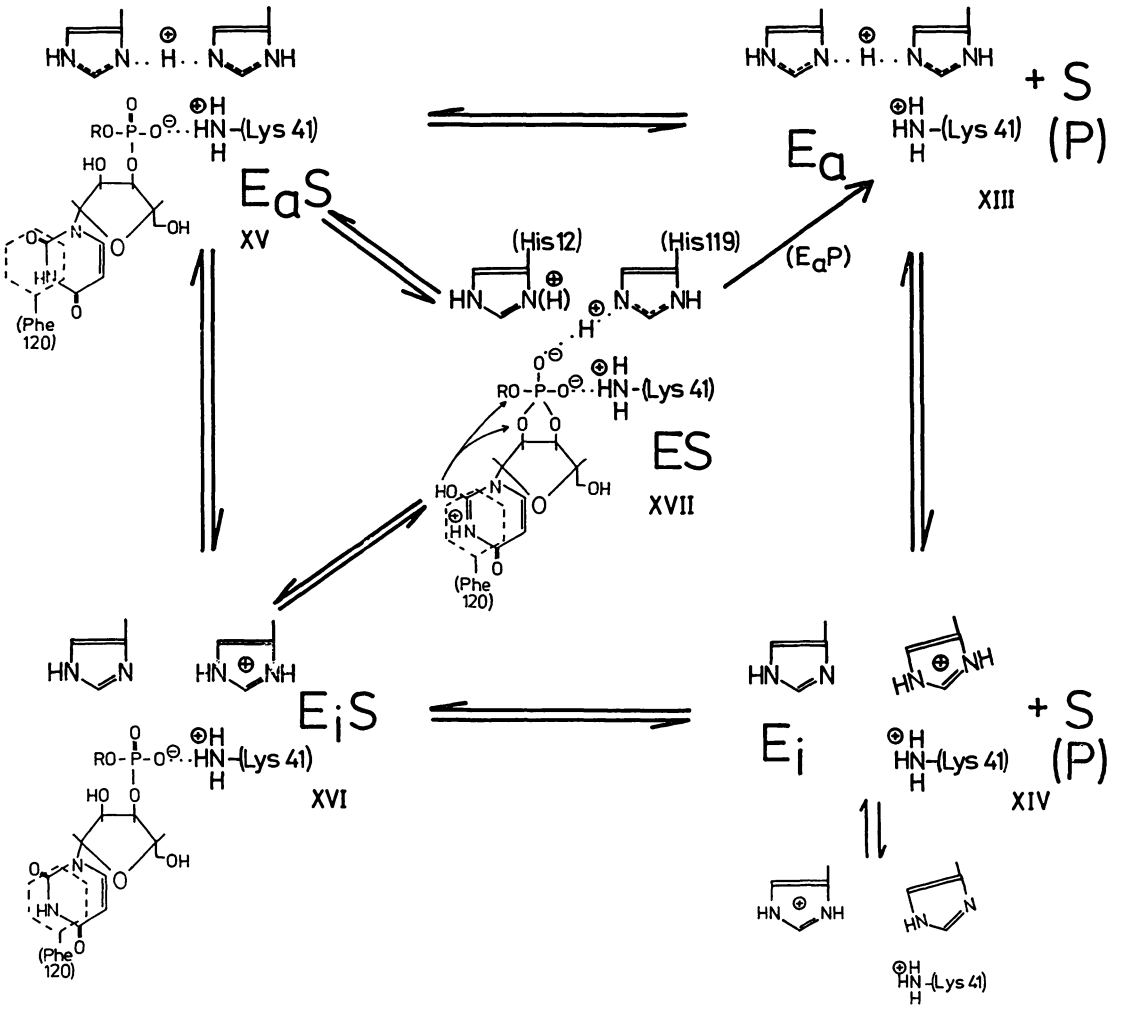


Fig. 3. a) Kinetics of the hydrolysis of 2′,3′-cyclic Cp by ribonuclease I at pH 7.6, buffer II, 20°C, $e_0 = 1.6 \times 10^{-3}$mM.
b) The fitting of equations to the experimental points:
I equation (1): $K_m = 2.0$mM, $k_{+2} = 5.5$ sec^{-1}
II equation (1): $K_m = 3.0$mM, $k_{+2} = 4.1$ sec^{-1}
III equation (2): $x_1 = 6.5$ sec^{-1}, $x_2 = 3.2$, $x_3 = 3.2$, $x_4 = 38.0$.



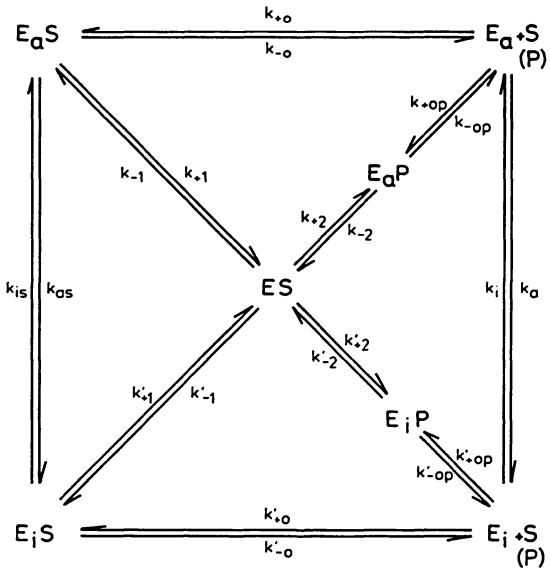
Scheme 3.

From this kinetic behavior we conclude that at least two enzyme species with different catalytic activities exist, which form the ES complex via two different pathways ($E_a + S \rightarrow E_aS \rightarrow ES$ and $E_i + S \rightarrow E_iS \rightarrow ES$), and that in the steady state higher substrate concentrations shift the ratio of these enzyme species in favor of the more active species E_a and E_aS.

On the basis of the described binding and catalytic processes it should be possible to relate the two species which are postulated from the kinetic behavior to defined conformations of the enzyme (Scheme 3). As shown by NMR studies[36] one species with a triprotonated diimidazole system should exist, which contains His 119 hydrogen bonded to His 12 (E_a; XIII). For thermodynamic reasons this species must be in equilibrium with another species (E_i; XIV) in which no hydrogen bond exists; both histidines are therefore mobile.

We assume that both species form the complexes E_aS (XV) and E_iS (XVI), respectively, in which the substrate is bound by an electrostatic interaction with Lys 41 and a hydrophobic interaction of the base with Phe 120.

The formation of E_aS and E_iS is assumed to proceed at nearly the same rates, since the difference in the conformation of E_a and E_i does not affect the two binding sites.



Scheme 4.

As described before, the formation of the ES complex (XVII) requires the simultaneous action of the pyrimidine base at the 2′-OH group (base catalysis step) and of the imidazolium cation of His 119 at the phosphate oxygen (proton catalysis step). In E_aS (XV) the "fixation" of the proton between the two imidazoles facilitates the proton catalysis and consequently should cause a higher rate in the proton catalysis step for the formation of the intermediate ES (XVII), while the more mobile imidazolium in E_iS (XVI) should lead to a lower rate constant for the formation of ES due to a higher entropy term in the activation energy (or by a higher reaction order). In addition, for E_i we expect a lower degree of protonation at His 119, since according to the NMR data[36] His 12 is a stronger base than His 119.

Since the breakdown of the ES complex is predominantly initiated by His 12, which in the deprotonated form competes for the proton between His 119 and the phosphate oxygen, thus destabilizing the intermediate state with pentacovalent phosphorus, the enzyme should be released from the ES complex predominantly or exclusively in the more active species, regardless of whether the reverse reaction ($ES \rightarrow E_aS \rightarrow E_a + S$) or product formation ($ES \rightarrow E_aP \rightarrow E_a + P$) occurred.

Thus two reaction pathways lead to ES, a slower one via E_iS and a faster one via E_aS. At low substrate concentration both pathways contribute to the concentration of ES, of which v is a linear function. Since the enzyme is released from ES predominantly in the E_a species, the transition to the E_i species requires a substrate-independent isomerization. Consequently, in the steady state, the contribution to ES via E_iS is limited by the transition $E_a \rightarrow E_i$, which at high substrate concentration (due to decreasing concentrations of E_a) tends to zero. Thus, at low substrate concentrations, a V value results which is an average of the contributions from both cycles. With increasing substrate concentration the equilibrium in the active center of the enzyme is shifted in favor of E_a, so that finally only the E_a cycle is running and the higher V value of the E_a cycle alone is reached.

From Scheme 3 the complete kinetic Scheme 4 can be derived, and the following estimations on the magnitude and pH dependence of the apparent individual rate constants seem to be justified.

1) k_{+0} and k'_{+0} are the rate constants for the formation of E_aS and E_iS, i. e. the interaction of the phosphate anion with Lys 41 and of the base with Phe 120. k_{-0} and k'_{-0} are those for the corresponding dissociation steps. k'_{+0} and k'_{-0} should have values similar to those of k_{+0} and k_{-0}, since the diimidazole system is not involved in the formation of E_aS and E_iS.

A rough estimate of the values of these constants may be obtained from the observation that 3′,5′-deoxy-CpC, a substrate analog which is bound but does not have

the following k_{+1} step after the formation of E_aS, is a very poor competitive inhibitor with a K_i value between 10^{-1} and 10^{-2}M. If we assume that k_{+0} is in the range of diffusion control (10^8 sec^{-1}), k_{-0} should be at least 10^6 sec^{-1}.

For the constants k_{+op} and k'_{+op}, as well as k_{-op} and k'_{-op}, similar values to those for k_{+0} and k_{-0}, respectively, can be assumed, since the product of the transesterification step, a 2',3'-cyclic phosphate, which is released via k_{+2} and k_{-op}, is the substrate for the hydrolysis step, in which it is released from ES via k_{-1} and k_{-0}. In this case, E_aS and E_aP are identical complexes. The product of the second step, the 3'-monoester, is primarily released in the E_aP (E_iP) complex in which it is monoprotonated and does not interact with His 119. Consequently, the step $E_aP \rightleftharpoons E_i + P$ is reversible. At pH values above 6, however, the deprotonation of the 3'-monoester occurs, so that the dianionic product, now the inhibitor I, binds at different rate constants in the step $E_a + I \rightleftharpoons E_aI$, leading finally to EI (XI). Except for this case, all rate constants described in this section should be pH independent in the range of pH 5 to pH 9, provided that the nucleotide base does not have a pK value in this range. Differences between these rate constants of various substrates should arise mainly from differences in the interaction between the base and Phe 120.

2) k_{+1} and k'_{+1} are complex rate constants for the formation of ES from E_aS and E_iS, respectively. They include the simultaneous action of the pyrimidine base at the 2'-OH group (base catalysis step) and of His 119 at the phosphate oxygen (proton catalysis step) leading to the intermediate with pentacovalent phosphorus.

For normal substrates the base catalysis seems to be rate-determining, because modifications at the pyrimidine base always affect k_{+1}[30]. In 3'-diesters containing electron-withdrawing alcoholic groups, the protonation by His 119 might become the slower step. This should be expected, since in this case the electrophilic character at the phosphorus is increased, so that the rate for the base-catalyzed attack of the nucleophile is increased and, due to the lower basicity at the phosphate oxygen, the rate for the protonation is decreased. Hence, the ratio k'_{+1}/k_{+1}, and thus the degree of sigmoidicity, should depend on the degree of the conformational alteration at the active site of the enzyme as well as on the electrophilicity of the phosphate group of the substrate.

k_{+1} should be smaller than k_{op}' and k_{-0}. This conclusion comes from the observation that 2',3'-cyclic Up is hydrolyzed at the same rate, independently of whether the reaction is started with the cyclic phosphate or with the dinucleoside phosphate 3',5'-UpA (which is split 500 times faster and yields the cyclic phosphate as the product). Therefore, after formation of E_aP by the decomposition of 3',5'-UpA, as discussed in subsection 1, E_aP formed in the 3',5'-UpA reaction is identical with the complex E_aS resulting from direct reaction of 2',3'-cyclic Up with the enzyme. An immediate release of the substrate must follow in the step k_{-op} (which is identical to k_{-0} of the direct reaction), before formation of the new ES complex in the step k_{+1} can occur.

3) k_{-1} and k_{+2} as well as k'_{-1} and k'_{+2} are the rate constants for the breakdown of ES. They are also complex and include two catalytic steps which are the reverse of those of k_{+1} and k'_{+1}. Since the proton catalysis by the pyrimidine base occurs alternatively at the 2'-oxygen (k_{-1}) or 5'-oxygen (k_{+2}), both steps are analogous and E_aS and E_aP are analogous complexes.

The ratio of the concentrations of E_aS and E_aP formed from ES and hence of k_{+2}/k_{-1} as well as k'_{+2}/k'_{-1} should be different in the transesterification (I → III) and in the hydrolysis (IV → VI) steps. In the first step it should also depend on the leaving tendency of the ester group which is to be split off.

Since E_aP and E_iP as well as E_aS and E_iS are formed from the same complex ES, the ratio k_{+2}/k_{-1} should equal that of k'_{+2}/k'_{-1}. Furthermore, since k_{+2} and k_{-1} as well as k'_{+2} and k'_{-1} are constants for alternative paths and therefore coupled, the ratio of k_{+2} or k'_{-2} to any other constant is determined by the ratio of k_{-1} or k'_{-1} to that constant. Consequently, if the enzyme is released predominantly in the more active species E_a, the ratio k_{-1}/k_{+1} must be greater than k'_{-1}/k'_{+1}. If k_{-1}/k_{+1} were equal to k'_{-1}/k'_{+1}, the concentration of E_a would not be enhanced relative to that of E_i with increasing substrate concentration, so that a hyperbolic saturation function would be obtained, while in the case of $k_{-1}/k_{+1} < k'_{-1}/k'_{+1}$ substrate inhibition would result due to a relative increase of the concentration of E_i.

The pH dependence of k_{+2} (k'_{+2}) and k_{-1} (k'_{-1}) should be determined by the deprotonation of His 12. Thus the four apparent constants should reach a maximum value at pH values higher than 7. The decrease of the overall constant k^*_{+2} described in section B may indicate that $k_{-1} + k_{+2}$ is smaller than k_{+1} below pH 7, and that above pH 7 the step k_{+1} becomes rate determining.

4) k_a and k_i are the rate constants for the isomerization process between E_a and E_i at the free enzyme, while k_{as} and k_{is} are those for the analogous change of conformation between E_aS and E_iS. If the values for k_{as} and k_{is} were considerably higher than those for k_a and k_i, no sigmoid transitions should be found, since the ratio of E_aS/E_iS would be kept constant by the flow from E_aS to E_iS. Therefore k_{as} and k_{is} should be smaller than k_a and $k_i \times k_a$ should be smaller than k_{+1}, otherwise the substrate-independent transition from E_a to E_i after the release of E_a from E_aP or E_aS would not limit the contribution of the E_i cycle to the concentration of ES. The difference between k_{+1} and k_a determines at what substrate concentration the transitions occur.

D) Mathematical treatment of the kinetic Scheme 4

Under steady state conditions and neglecting the formation of E_iP, equation 3 can be derived from Scheme 4*.

$$r = \frac{k_{-op} \times e_0}{1 + \frac{K_3 + K_4 \times s}{K_1 + K_2 \times s} + \frac{K_5}{(K_1 + K_2 \times s) \times s}} \quad (3)$$

where the equilibrium constants $K_1 - K_5$ are defined as shown below.

$$K_1 = k_{+2}\ [(k_a k'_{+0} + k_i k_{+0})\ (k'_{+1} k_{as} + k_{is} k_{+1} + k_{+1} k'_{+1}) + k_{-0} k'_{+1} k_a k'_{+0} + k'_{-0} k_{+1} k_i k_{+0}]$$

$$K_2 = k_{+2} k_{+0} k'_{+0}\ (k_{is} k_{+1} + k'_{+1} k_{as} + k'_{+1} k_{-1})$$

$$K_3 = k'_{+0} \left[(k_{-op} + k_{-2}) \left[\begin{array}{l} k_a[(k_{-0} + k_{as} + k_{is})\ (k_{-1} + k'_{-1}) + k_{+1}(k'_{-1} + k_{is}) + k'_{+1}\ (k_{-1} + k_{as} + k_{+1})] \\ + k_{-0}[k_{is}(k_{-1} + k'_{-1}) + k'_{+1}\ (k_{-1} + k_a)] \end{array} \right] \right.$$
$$\left. + k_{-op} k_{+2}\ [k_a(k_{-0} + k_{as} + k_{is} + k_{+1}) + k_{-0}\ (k_{is} + k'_{+1}) + k_{is} k_{+1} + k'_{+1}(k_{as} + k_{+1})] \right]$$

$$+ k_{+0} \left[(k_{-op} + k_{-2}) \left[\begin{array}{l} k_i\ [(k'_{-0} + k_{as} + k_{is})\ (k_{-1} + k'_{-1}) + k_{+1}(k'_{-1} + k_{is} + k'_{+1}) + k'_{+1}(k_{-1} + k_{as})] \\ + k'_{-0}[k_{as}(k_{-1} + k'_{-1}) + k_{+1}(k'_{-1} + k_i)] \end{array} \right] \right.$$
$$\left. + k_{-op} k_{+2}[k_i(k'_{-0} + k_{as} + k_{is} + k'_{+1}) + k'_{-0} k_{as}] \right]$$

$$K_4 = k_{+0} k'_{+0} \left[\begin{array}{l} (k_{-op} + k_{-2})\ [(k_{as} + k_{is})\ (k_{-1} + k'_{-1}) + k_{+1}(k'_{-1} + k_{is} + k'_{+1}) + k'_{-1}(k_{-1} + k_{as})] \\ + k_{-op} k_{+2}[k_{as} + k_{is} + k'_{+1}] \end{array} \right]$$

$$K_5 = (k_a + k_i) \left[\begin{array}{l} (k_{-op} + k_{-2})\ [(k_{-0} k'_{-0} + k_{-0} k_{is} + k'_{-0} k_{as})\ (k_{-1} + k'_{-1}) + k_{-0} k'_{+1} k_{-1} + k'_{-0} k_{+1} k'_{-1}] \\ + k_{-op} k_{+2}\ [k_{as}(k'_{+1} + k'_{-0}) + k_{is}(k_{+1} k_{-0}) + (k'_{+1} + k'_{-0})\ (k_{+1} + k_{-0})] \end{array} \right]$$

Equation 2 shows that the shape of the v/s curve is determined by x_2, x_3 and x_4 while x_1 determines the value of V. For the case of substrate activation it describes nonhyperbolic v/s curves, which can be, but need not necessarily be, sigmoid. In both cases a deviation from linearity in the Lineweaver-Burk plot occurs, which is often referred to as "positive cooperativity".

The conditions under which positive or negative deviations from linearity occur in the Lineweaver-Burk plot are derived from the first (9) and second (10) derivatives of the reciprocal of equation 2. If the first derivative (9)

$$\frac{d(e_0/v)}{d(1/s)} = \frac{\left(\frac{1}{s}\right)^2 x_2 x_4 + \left(\frac{1}{s}\right) 2x_4 + x_3 - x_2}{x_1 \left[x_2 \left(\frac{1}{s}\right) + 1\right]^2} \quad (9)$$

is a constant, a straight line results, while the second derivative (10)

$$\frac{d^2(e_0/v)}{d(1/s)^2} = 2 \frac{x_4 - x_2(x_3 - x_2)}{x_1 \left(x_2 \left(\frac{1}{s}\right) + 1\right)^3} \quad (10)$$

indicates whether an upward or a downward deviation from linearity occurs. The following relations among x_2, x_3 and x_4 hold for the cases a–d in Fig. 4:

This equation can be rewritten to equation 2 which contains the minimum number of adjustable parameters x**, where

$$x_1 = \frac{k_{-op}\ K_2}{K_2 + K_4} \quad (4)$$

$$x_2 = \frac{K_1}{K_2} \quad (5)$$

$$x_3 = \frac{K_1 + K_3}{K_2 + K_4} \quad (6)$$

$$x_4 = \frac{K_5}{K_2 + K_4} \quad (7)$$

* For simplicity, the pH dependence of the constants is not taken into account, so that the constants for pH-dependent steps (see section C) should be regarded as apparent constants, the magnitude of which are defined by the pH value in question.

** These constants are all positive, since the individual rate constants are positive.

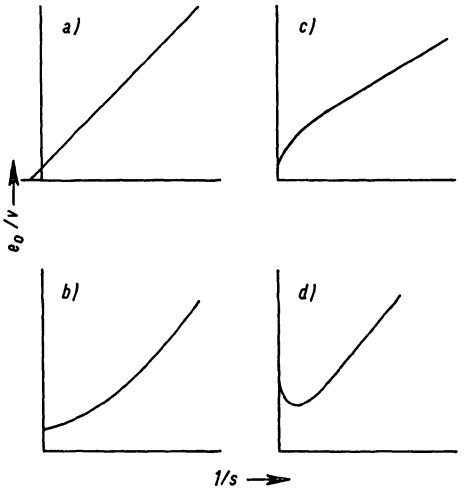


Fig. 4. Possible types of Lineweaver-Burk plots of equation 2.

a) $x_3 > x_2$ and $x_4 = x_2(x_3 - x_2)$ or $x_2 = x_4 = 0$ true Michaelis-Menten function

b) $x_3 \geqslant x_2$ and $x_4 > x_2(x_3 - x_2)$ positive reciprocal nonlinearity ("positive cooperativity")

c) $x_3 \geqslant x_2$ and $x_4 < x_2(x_3 - x_2)$ negative reciprocal nonlinearity ("negative cooperativity")

d) $x_3 < x_2$ substrate inhibition (maximum in the v/s curve and minimum in the Lineweaver-Burk plot).

The condition for sigmoidicity, which is contained in the more general condition for positive, reciprocal nonlinearity (4b), is derived from inspection of the first (equ. 11) and second (equ. 12) derivatives of equation 2.

$$\frac{dv}{ds} = e_0 x_1 \times \frac{s^2(x_3 - x_2) + 2x_4 s + x_2 x_4}{(s^2 + x_3 s + x_4)^2} \qquad (11)$$

$$\frac{d^2v}{ds^2} = -2e_0 x_1 \frac{s^3(x_3 - x_2) + 3s^2 x_4 + 3s x_2 x_4 - (x_4 - x_2 x_3)x_4}{(s^2 + x_3 s + x_4)^3} \qquad (12)$$

Since for a sigmoid curve v only increases with s but never decreases, the first derivative must be positive, hence $x_3 \geqslant x_2$. Furthermore, since the sigmoid curve has one point of inflection, the second derivative must pass through zero only once, so that x_4 must be greater than $x_2 x_3$.

Therefore

$$x_3 \geqslant x_2 \text{ and } x_4 > x_2 x_3 \qquad (13)$$

are necessary and sufficient conditions for sigmoidicity. In a similar manner as described by Ainslie, Shill and Neet[18], it can be shown that these conditions hold if the relations among individual rate constants which are given in section C are obeyed. A detailed analysis of the influence of relations among individual rate constants on sigmoidicity for equation 3 will be given in a subsequent paper.

E) Experimental results confirming the proposed model

The following consequences of the considerations in section C have been tested[19,20]:

a) Alterations in the degree of sigmoidicity should be found if, according to subsection C 2, the difference between k_{+1} and k'_{+1} is changed by a chemical modification of the substrate which affects the electrophilic properties at the phosphorus.

b) Alterations should also be found if the ratio of the concentrations of the species E_a and E_i is shifted by effectors. A shift in favor of E_i should increase the degree of sigmoidicity, while a shift in favor of E_a should decrease the degree of sigmoidicity and finally normalize the v/s curves. This behavior corresponds to that of negative and positive effectors in the kinetics of allosteric enzymes.

In order to test consequence a) we synthesized substrates with increasing electrophilic character at the phosphorus using phenolic alcohols with decreasing pK values in the ester group which was to be split off. As discussed in subsection C 2, the difference between k'_{+1} and k_{+1} and, in consequence the degree of sigmoidicity, should increase for these substrates. As Fig. 5 demonstrates for the v/s curves and Lineweaver-Burk plots, the degree of sigmoidicity increases in the expected order.

For testing consequence b) experiments were carried out at different pH values and different sulfate concentrations. If the pH value is increased, the ratio of the concentrations of E_a and E_i should shift in favor of E_i. This follows from the fact that in the equilibrium $IX \rightleftharpoons X$, X should mainly exist in the conformation in which the hydrogen bond is broken. Protonation of X therefore predominantly leads to the species E_i. The higher contribution to ES from the E_i cycle therefore increases the differ-

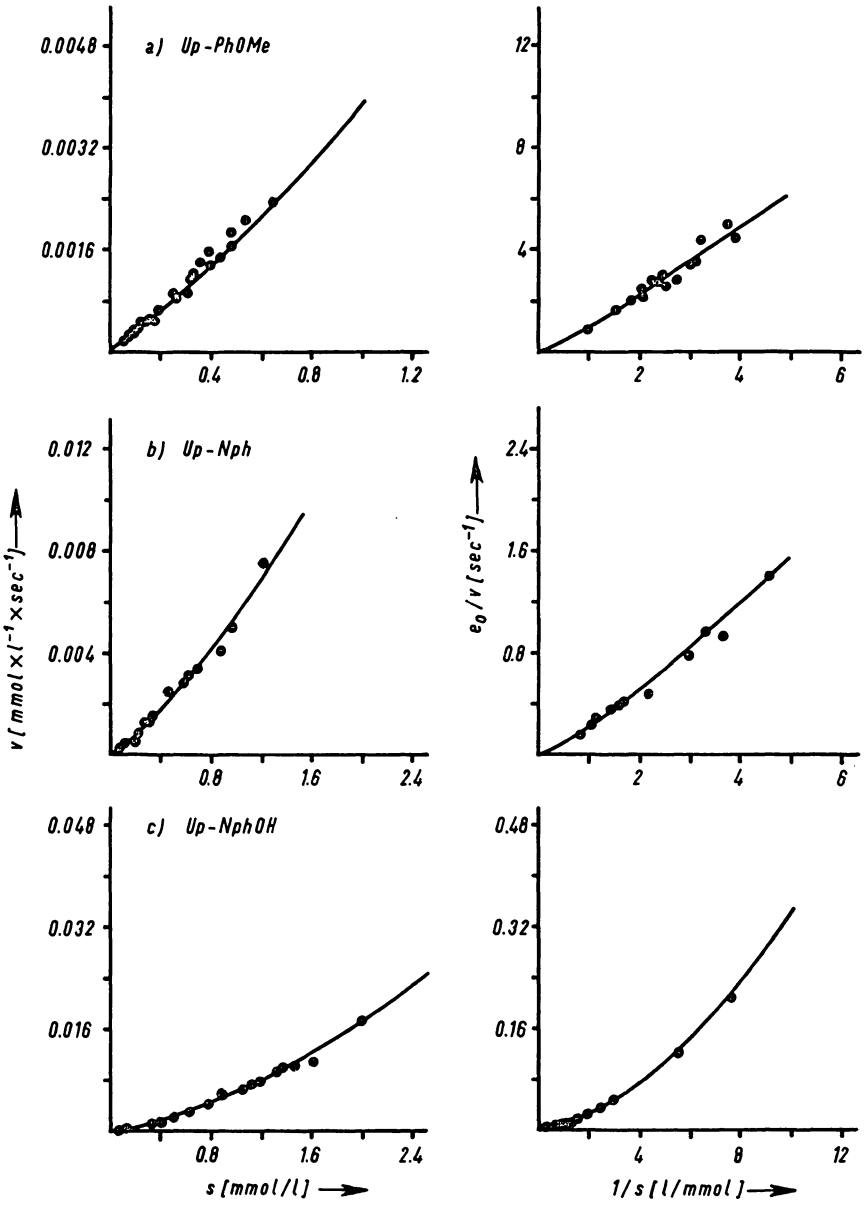


Fig. 5. Kinetics of the hydrolysis of esters of 3′-uridylic acid by pancreatic ribonuclease I at pH 7.8, buffer II, 20°C in the low substrate concentration range.

a) Up-PhOMe, $e_0 = 4.2 \times 10^{-3}$мм (pK = 10.0)

b) Up-Nph, $e_0 = 1.3 \times 10^{-3}$мм (pK = 9.3)

c) Up-NphOH, $e_0 = 0.57 \times 10^{-3}$мм (p$K$ = 8.7).

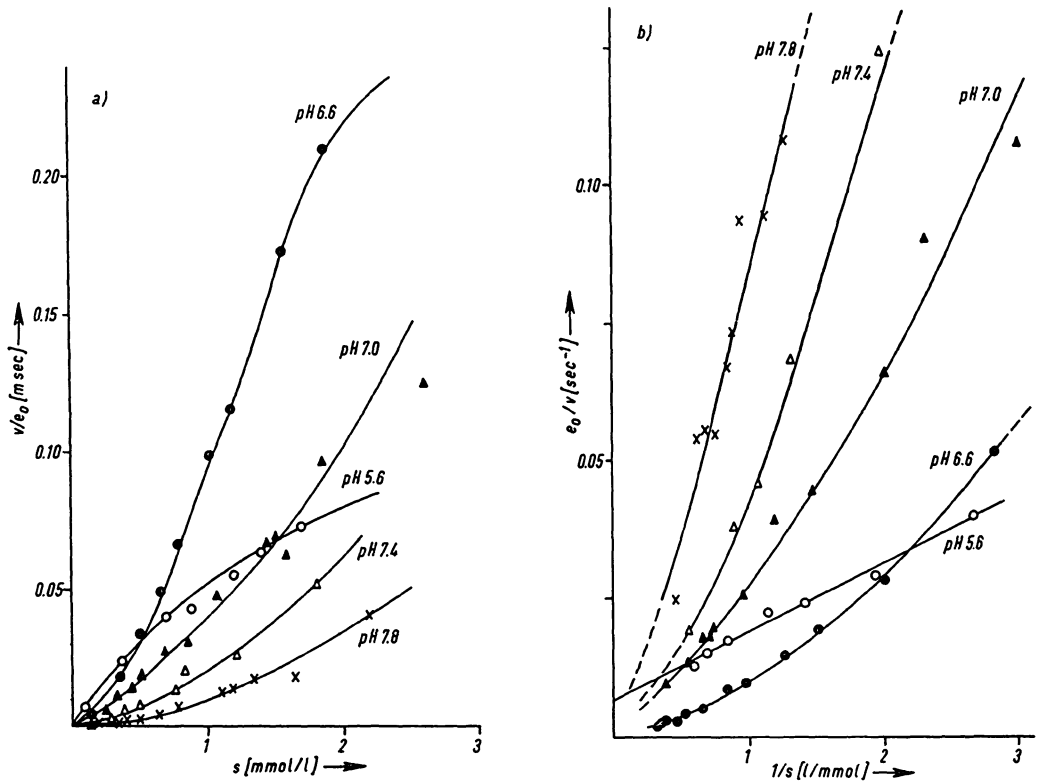


Fig. 6a) and b). pH dependence of the degree of sigmoidicity for the kinetics of the hydrolysis of UpNphOH by ribonuclease I at 20°C, buffer II.

ence between the V value of the E_a cycle, which is finally reached, and the V value extrapolated from low substrate concentrations. Therefore all substrates should show a higher degree of sigmoidicity at higher pH values. As an example Fig. 6a and b show the v/s curves and the Lineweaver-Burk plots for Up-NphOH at different pH values. At pH 5.6 no sigmoidicity can be observed, while at pH 6.6 a marked effect is found. Less electrophilic substrates need higher pH values to give sigmoidicity; e.g. for 2′,3′-cyclic Cp sigmoidicity is found only at pH values above 7.2.

A shift of the ratio E_a/E_i in favor of the species E_a should be found if an effector binds weakly to the enzyme and releases the enzyme predominantly or exclusively in the E_a species. High concentrations of such a weak competitive inhibitor should transfer all enzyme molecules into the E_a species, so that in the reaction with substrates only the E_a cycle is running. We have used sulfate as a weak competitive inhibitor. In analogy to the substrate, it should form a pre-complex (E_aI, E_iI) by an electrostatic interaction with Lys 41 which is then transformed into an EI complex by a further electrostatic interaction with His 119.

Compared with 2′- or 3′-nucleotides, however, this EI complex is weak, since it is not stabilized by an additional interaction of the nucleotide base with Phe 120. The breakdown of the complex should be initiated by His 12, which being the stronger base competes with the sulfate ion for the imidazolium cation. As a consequence, mainly E_a is released from the EI complex.

Fig. 7 shows that at low inhibitor concentration (0.5M) inhibition in the v/s curve is observed. Multiplying the substrate concentration by 0.75, the curve obtained with sulfate added can be superimposed on the curve obtained without the ad-

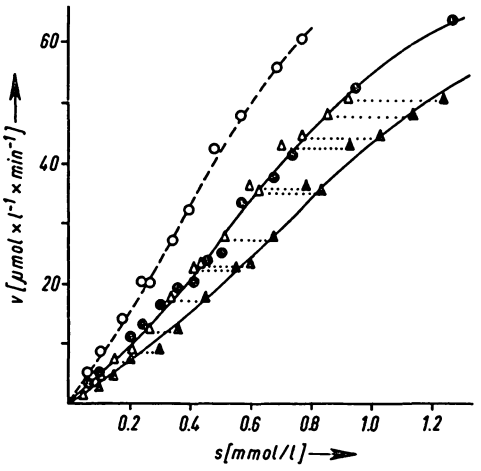


Fig. 7. Kinetics of the hydrolysis of Up-Nph by ribonuclease I at different concentrations of $(NH_4)_2SO_4$, 20°C, pH = 7.6, buffer III, $e_0 = 0.51 \times 10^{-3}$mM. ●, no $SO_4^{2\ominus}$; ○, 1.5M $SO_4^{2\ominus}$; ▲, 0.5M $SO_4^{2\ominus}$; △, 0.5M $SO_4^{2\ominus}$ ($s \times 0.75$).

dition of sulfate, indicating the competitive type of inhibition*. In contrast, at 1.5M sulfate v is increased compared with the curve obtained without the addition of sulfate. The curve is still sigmoid. These results indicate that the increase in the concentration of the species E_a overcompensates for the inhibition. Finally Figures 8a and b show for two other substrates that the sigmoid or nonhyperbolic curves are normalized at 3.6M sulfate**. Thus, in the reaction of ribonuclease I, sulfate acts as a positive effector in the kinetics of allosteric enzymes. In a similar way, the $OH^{\ominus}$ ion can be regarded as the simplest negative effector[44] in our system, shifting the equilibrium in favor of E_i. The function of sulfate as an effector and a quantitative treatment of the binding equilibrium in terms of our model will be given in a subsequent paper***.

Discussion

The sigmoid transitions in the kinetics of the reaction of ribonuclease I from bovine pancreas with some of its substrates cannot be interpreted on the basis of subunit or multisite models. The enzyme is monomeric and has only one active center. Obviously, alternatives for the interpretation of sigmoid kinetics must exist, which hold for the interaction of a single substrate with a single active site of the enzyme.

Theoretical models which fit the kinetic data can be put forward, even if nothing is known about the mechanism. On the other hand, if details of a mechanism are known, one should be able to derive the particular kinetic behavior as a consequence of it. In this paper, we have developed a kinetic model, which is primarily based on the proposed mechanism for ribonuclease I. Instead of examining other theoretical possibilities to explain the sigmoid kinetics found with this enzyme, we therefore first discuss the question of how reliable is the proposed mechanism.

The essentials of the mechanism that were derived from experimental results are the participation of the base in the catalysis step[30] and the existence of a triprotonated diimidazole system in the binding step[33,36]. The intermediate with a pentacovalent phosphorus stabilized by the two protonating groups of the enzyme is a reasonable postulate and is in accordance with the present conceptions of phosphorus chemistry[45]. If this intermediate is accepted, its breakdown should be catalyzed in steps which are exactly the reverse of those in its

* For simple competitive inhibition the following equation for the initial velocity:

$$v = \frac{V}{1 + \frac{K_m}{s}\left(1 + \frac{i}{K_i}\right)}$$ holds[42], where i is the concentration of inhibitor and K_i is the equilibrium constant of its binding to the enzyme. In the case of constant inhibitor concentration, $1 + i/K_i$ can be treated as a constant f, so that v of the inhibition kinetic equals that of the normal curve

$$\frac{V}{1 + \frac{K_m}{a}} = \frac{V}{1 + \frac{K_m}{s} \times f}, \text{ if } a = s \times 1/f.$$

** The higher v values and hence the normalization of the v/s curves in the presence of high sulfate concentration can be attributed to a specific effect of sulfate rather than to effects of ionic strength, since according to results of Winstead and Wold[43], higher concentrations of monoanionic ions do not increase the v values. It can also be shown that only the nonhyperbolic kinetics are normalized by addition of sulfate, while the hyperbolic kinetics below pH 7 are only inhibited by higher sulfate concentrations.

*** Rübsamen, H., Khandker, R. & Witzel, H., in preparation.

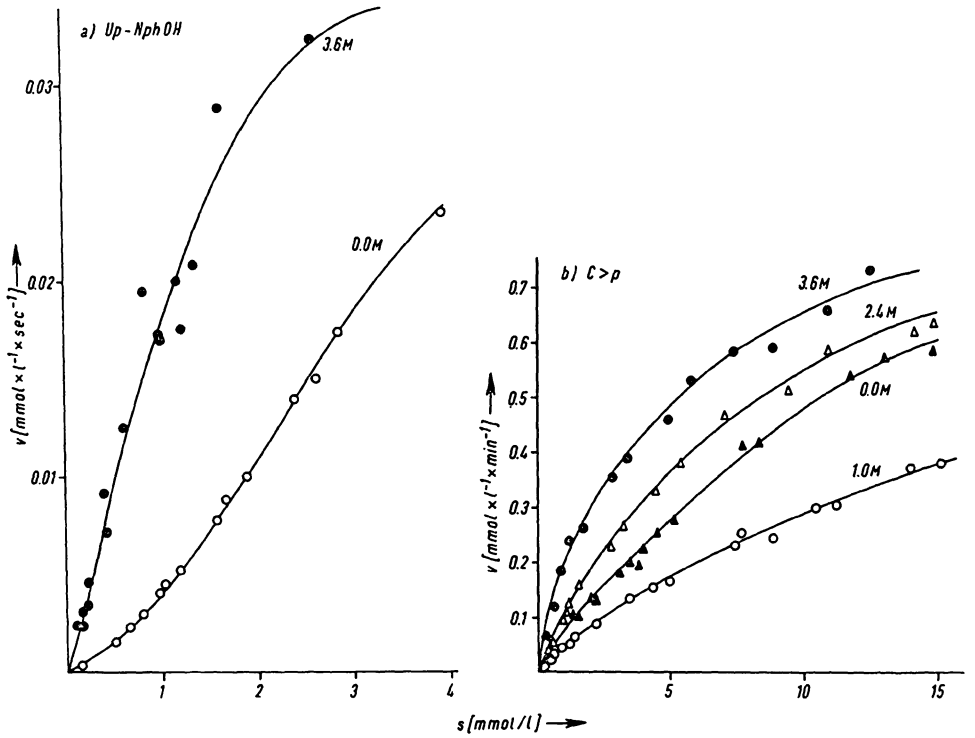


Fig. 8. The complete normalization of non-hyperbolic v/s curves at high concentrations of $(NH_4)_2SO_4$ (given at the curves).

a) Up-NphOH (10°C) pH 6.6, buffer III, $e_0 = 4.5 \times 10^{-5}$mM.

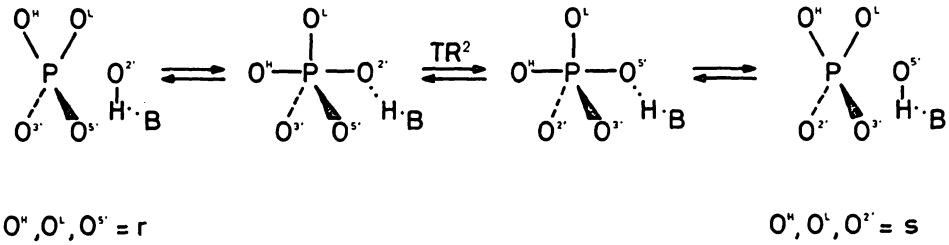
b) 2′,3′-cyclic Cp, 20°C, pH = 7.6, buffer III, $e_0/ = 1.4 \times 10^{-3}$mM. The nonhyperbolic character of the curve without sulfate is shown in Fig. 3.

formation. These are a base catalysis at His 119 by His 12, which restores the triprotonated diimidazole system, and a simultaneous proton catalysis by the pyrimidine base acting in the form of the conjugate acid alternatively at the 2′-oxygen or at the 5′-oxygen.

This alternative action at the 2′- or 5′-oxygen requires an "adjacent" mechanism[46]. Further evidence besides the previous arguments in favor of "adjacent"[30,41,47] can be obtained from results demonstrating that in dinucleoside phosphates the 5′-nucleoside is released from a stacked position and that the synthesis of dinucleoside phosphates from 2′,3′-cyclic nucleotides and nucleosides occurs only if the entering nucleoside can stack with the nucleotide[48]. In a stacked conformation the 5′-oxygen, however, cannot be located opposite to the 2′-oxygen. The dinucleoside phosphonate cocrystallized in the open form with the enzyme[49] does not necessarily invalidate the interpretation of these results.

Objections to the "adjacent" mechanism required for this concept were recently raised, since Usher *et al.*[50] and Eckstein *et al.*[51] had demonstrated that inversion of the absolute configuration at the phosphorus occurs during the transesterification and the hydrolysis reactions. It had been concluded that these results were only compatible with an "in line" mechanism[52].

It has been shown[45] however, that in contrast to the situation in carbon chemistry, the ligands at a pentacovalent phosphorus are able to exchange their positions by rotating in groups against each other (turnstile rotation = TR). In such a case the inversion of the absolute configuration is a consequence of an adjacent mechanism (Scheme 5):

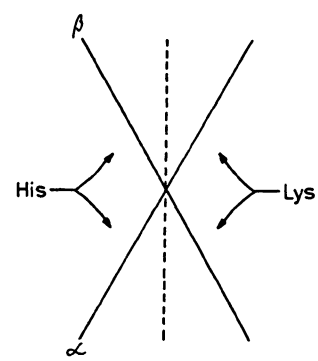


Scheme 5.

It will be demonstrated in connection with studies on dinucleoside phosphates that in the E_aS complex the 2′-OH group obviously attacks the phosphorus opposite to that oxygen which is protonated by His 119. In this case, base and proton catalysis for the formation of ES as well as proton and base catalysis for the breakdown of ES, now polarizing in the opposite direction, operate "in line" (O_H-P-$O^{2'}$) and act on the apical positions. Therefore the k_{-1} step is the exact reverse of the k_{+1} step. In the alternative k_{+2} step the proton of the pyrimidine base is transferred to the adjacent 5′-oxygen instead of the 2′-oxygen. In contrast to the three ester oxygens, which are fixed in their positions, the two oxygens protonated by the enzyme are able to follow the shift in polarity by a double turnstile rotation, so that His 119 is now opposite to the protonated 5′-oxygen, which can leave from an apical position.

The turnstile mechanism instead of the Berry pseudorotation[53] should be preferred due to the relatively fixed position of the trio with the 2′-, 3′- and 5′-oxygens and a certain mobility of the two protonated oxygens as a duo, reined by the two flexible amino acid side chains of Lys 41 and His 119*. We leave open the question of whether the turnstile rotation causes protonation at the 5′-oxygen or whether protonation at the 5′-oxygen causes turnstile rotation. The process is in good agreement with the general rules discussed by Marquarding *et al.*[45]. It is also in agreement with their suggestion that multiple protonations (in our case at three oxygens) at the pentacovalent phosphorus in form of a trigonal bipyramid might lower the electron density around the phosphorus so that the five-membered 2′,3′-ring system need not prefer exclusively an equatorial-apical position. Energy differences between the equatorial-apical and equatorial-equatorial position should exist and should be reflected in the ratio of the k_{-1} and k_{+2} values.

The reaction of the 2′-3′-cyclic nucleotide with water or another alcohol occurs analogously. As shown in Scheme 6, in the E_aS complex of the cyclic nucleotide the two free oxygens are in the β-position with respect to the two enzyme groups, rotated by 120° compared with the α-position in the 3′-diesters before the transesterification step. Thus the nucleophile enters exactly into the same position from which the original 5′-ester oxygen had left the phosphorus. The 2′-ester bond is split only after a new turnstile rotation has occurred. The product,

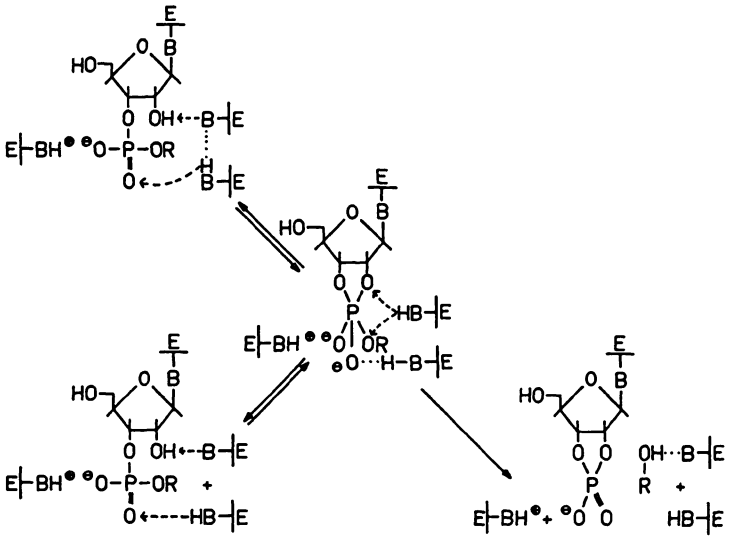


Scheme 6.

now a 3′-monoester, has again the configuration of the original 3′-diester before the inversion. This means that the inversion of the absolute configuration alone cannot decide in favor of "in line" or "adjacent".

In addition to these arguments, further support for the interpretation of the kinetic anomalies of ribonuclease I by our mechanism arises from the kinetics of guanyloribonuclease and ribonuclease II, which show substrate inhibition[54,55].

* It is postulated that in the ES complex only the duo with the two protonated oxygens rotates. In Scheme 5, however, the trio is rotated by 120° in order to get equivalent positions for the ligands on the drawings.



Scheme 7.

According to C, 4 and D, d substrate inhibition should be found, if $k_{-1}/k_{+1} < k'_{-1}k'_{+1}$, i.e. if the enzyme is released preferentially in the E_i species. Our studies on the mechanisms of the two *A. oryzae* enzymes guanyloribonuclease and ribonuclease II[54,55], both catalyzing the same reactions, indicate that first a complex is formed in which the substrate is bound to the enzyme by an electrostatic interaction of the phosphate group with a positively charged amino acid side chain and by a specific (guanyloribonuclease) or an unspecific (ribonuclease II) interaction of the base with the enzyme.

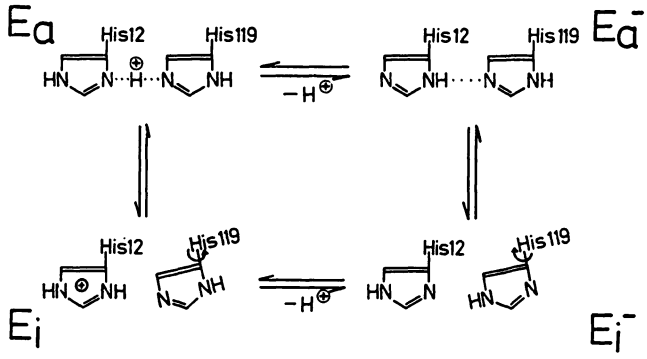
Furthermore it can be shown that, in contrast to the reaction of ribonuclease I, the nucleoside base is not involved in the catalytic process. The function of the pyrimidine base is taken over by the base B of a B × HB system at the enzyme, while HB takes over the function of His 119 (Scheme 7). The formation of the intermediate with pentacovalent phosphorus occurs only when B and HB act simultaneously. Therefore the hydrogen-bonded system B × HB should have faster rates for the formation of ES than the system B + HB.

During the breakdown of the ES complex of guanyloribonuclease and ribonuclease II, HB should leave the substrate, when B is still bound either to the 2′-OH or to the 5′-OH group which must be released before the B × HB system can be formed again. The probability of a concerted reaction restoring immediately the B × HB species is therefore much lower than the probability of the formation of B + HB from which B × HB is formed in a slow isomerization process. This is exactly the case: $k_{-1}/k_{+1} < k'_{-1}/k'_{+1}$. In fact substrate inhibition is found for both ribonucleases from *A. oryzae* (Fig. 9)[54,55]. The reaction of the ribonucleases I, II and guanyloribonuclease can be treated by the same kinetic Scheme 4 and the same rate equation (2)*.

Due to this analogy, we would expect that ribonuclease I should also show substrate inhibition, if (as proposed by the mechanisms of Findlay *et al.*[52] and of Henderson and Wang[56]) one histidine were to act at the nucleophilic site while the other acts at the electrophilic site simultaneously, since in this case the enzyme would be released in the form in which the two histidines are not fixed to each other by a hydrogen bond and since this species would be the less active one.

Besides the mechanism itself, the second point of discussion should be the assumption of the conformational equilibrium $E_a \leftrightharpoons E_i$, which should have rate constants below those of the rate-determining steps for the formation of ES. In a series of communications[57–60] on temperature jump and

* Turnstile rotation and inversion of the absolute configuration in the transesterification and hydrolysis step, as in the case of ribonuclease I, are further consequences.



Scheme 8.

stopped-flow experiments performed on ribonuclease I, Hammes and coworkers described an isomerization process in the active center of the enzyme. Since the existence of XIII seems to be confirmed[36,61] and since, for thermodynamic reasons, this species must be in equilibrium with XIV, it should be justifiable to assume that the observed isomerization process corresponds to the transition XIII ⇋ XIV. A few arguments in favor of this assumption may be mentioned.

1) The isomerization process is observed in the pH range 5.5 to 7.5. From the evidence of its ^2H_2O effect this can be attributed to the making and breaking of a hydrogen bond[58,59].

2) The isomerization involves a group with a pK value of about 6.1[59].

3) Substrates and inhibitors specifically interfere with the isomerization. The finding that the percentage of saturation at which the amplitude of the relaxation process disappears decreases in the series: 2′,3′-cyclic cytidylic acid (a slowly hydrolyzed substrate), 3′,5′-cytidylyl cytidine (a fast hydrolyzed substrate) and 3′-cytidylic acid[60] could mean that in this order the equilibrium is shifted more easily in favor of E_a.

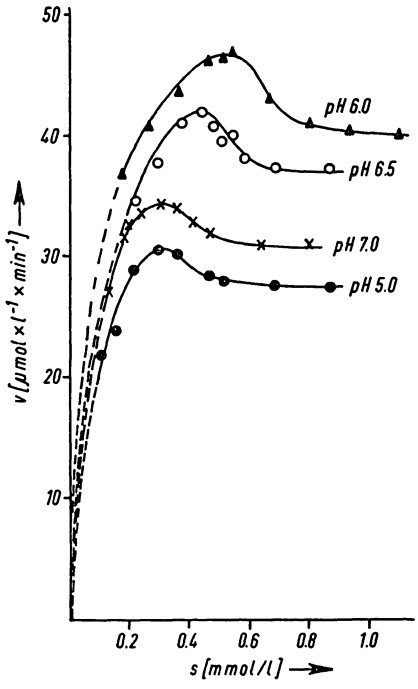


Fig. 9. Substrate inhibition kinetics of guanyloribonuclease, GpA as substrate, 20°C, buffer II, e_0 = 2.14 × 10^{-6} mM.

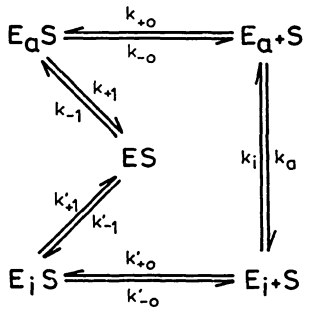
A possible objection to this interpretation of the relaxation process is that the pH dependence of the relaxation time seems to indicate an isomerization between only two species[59], while in the pH range 5 to 7.5 the isomerization $E_a \leftrightharpoons E_i$, described in this paper, should involve at least four species (Scheme 8). The equation for the pH dependence of the relaxation time for a four-species isomerization[62], however, can be reduced in a limited pH range to the type derived by Cathou and Hammes[59]. The rate constants k_{12} and k_{21} calculated from the pH dependence of the relaxation time by these authors[59] are of an order of magnitude which would meet our requirements for a slow process. It should be pointed out, however, that for the four-species model they cannot be attributed to the steps $E_a \leftrightharpoons E_i$, so that the order of magnitude for the apparent constants k_a and k_i in Scheme 4 cannot be directly derived from these data.

The concept of "ligand-induced shifts of conformational equilibria" (LISCE) in "multipathway" enzymes, which has been developed in this paper, is based on the assumption of a steady state. It is not restricted to monomeric enzymes, since a slow conformational equilibrium between two (or even more than two) defined conformations at the active site, which show differences in the rate-determining steps for the formation of the kinetically relevant enzyme-substrate complex (or complexes), and the preferential release of the enzyme in only one of these conformations, can just as well be assumed for each subunit of an oligomeric enzyme.

The number of functionally different conformation isomers of an enzyme determines the number of different pathways in the kinetic scheme which lead to the formation of the product. As the analysis of various kinetic schemes by the King-Altman method[63] shows, the highest power of s in the rate equation for the steady-state initial velocity equals that number of pathways. Since the highest power of s is also equal to the maximal value of the Hill coefficient that can be expected from a given rate equation, the number of enzyme species with different catalytic activities, which are assumed for the interpretation of special kinetics, must never be smaller than the highest Hill coefficient found for the reaction. On the other hand, the degree of sigmoidicity which is predicted from a rate equation derived from a given kinetic scheme is determined by the ratios of the individual rate constants.

In terms of our model, shifts in the equilibrium between the enzyme species by positive or negative effectors are explained by direct interactions with the active site. If $k_{+2} \ll k_{-1}$ holds (and k_{as} and k_{is} are neglected for simplicity), Scheme 4 reduces to Scheme 9, in which no product formation occurs, and which describes an overall binding equilibrium between the enzyme and an inhibitor I. If the enzyme is released from the EI complex preferentially in the E_a species, a nonhyperbolic, saturation function which can be sigmoid should be obtained due to a shift of the equilibrium in favor of the species E_a. This will be shown in a subsequent paper. As demonstrated in section E, sulfate should act according to this principle, since competitive inhibition is found at low concentrations of the effector, while at high concentrations the competitive inhibition in the kinetics is overcompensated by the activation.

Although direct interaction between the effector and the active site of the enzyme is postulated,



Scheme 9.

chemical similarity between the effector and the substrate is not required. It is not necessary that the effector binds to all groups of the enzyme which interact with the substrate (sulfate for example does not interact with Phe 120). A structure which is chemically different from the substrate thus may meet all functional requirements for shifting the equilibrium.

Besides the direct interactions of substrates and effectors with the active site, our model does not exclude the binding of effectors to a site distinct from the active site. Thus, an indirect interaction influencing the conformation at the active site may also cause a change in the rate constants of the equilibrium $E_a \leftrightharpoons E_i$. If association of the subunits of an oligomeric enzyme leads to alterations in these rate constants resulting in sigmoid kinetics, each subunit may be considered to be an "effector" of the other.

The concept of ligand-induced shifts of conformational equilibria by direct (or in the case of effectors even indirect) interactions of the ligands with the active site of a "multipathway" enzyme *can be considered as an alternative to models which assume only subunit or multisite (i. e. indirect) interactions for the interpretation of nonhyperbolic kinetics.* It can be applied to monomeric as well as oligomeric enzymes.

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