The mechanism of enzyme action

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As it is impossible to cover the whole field of enzyme mechanisms in a comparatively brief presentation, it is proposed to illustrate the present state of knowledge by reference in detail to one particular enzyme, namely, bovine pancreatic ribonuclease. There is now an enormous amount of information available about this enzyme, including the complete three-dimensional structure of the protein (Kartha, Bello, and Harker, 1967) and a modified derivative (Richards and Wyckoff, 1968). There is also a great deal of chemical and kinetic information which sheds light on the mechanism of its catalytic action and it is now possible to suggest tentatively the nature of the reaction pathway. The physical basis of the rate enhancement factors, which are of the order of magnitude of 10^{10} , is still problematical and will not be discussed.

The enzyme consists of a single chain of 124 amino acid residues; in general, the molecule is kidney-shaped containing a depression, and there is good reason to believe that the active site is in the depression. Several of the amino acid residues in the region of the active site have been implicated in the catalytic process. Whilst histidines 12 and 119 are the most important, both lysine 41 (Murdock, Grist, and Hirs, 1966) and aspartate 121 (Anfinsen, 1956) are also essential. Lysine 41 is implicated because the effect of fluorodinitrobenzene, which reacts rapidly with the lysine residue and inactivates the enzyme, is prevented by competitive inhibitors; aspartate 121 is implicated because, whereas removal of the end three amino acids from the C-terminus has no effect on catalytic activity, removal of the next one, ie, aspartate 121, results in complete loss of catalysis. The exact function of these two residues is unknown.

By far the most important residues have been shown by experiments with haloacetic acids to be two histidine residues, namely 12 and 119 (see Rabin and Mathias, 1963 for review). Negatively charged alkylating reagents, such as iodoacetic acid and bromoacetic acid, inhibit ribonuclease, but this does not occur with neutral alkylating agents such as iodoacetamide, despite the fact that the latter are generally much more reactive than the former. The reaction of the enzyme with the halo-

acetic acids is extraordinary, as either one of the two histidines will react with the reagent but never both in the same molecule. Moreover the rate of this reaction is several orders of magnitude greater than that of haloacetic acid with a simple imidazole in aqueous solution.

If the rate of alkylation of ribonuclease by iodoacetic acid is measured as a function of pH, a typical bell-shaped curve, resembling an idealized pH profile for enzyme activity, is obtained. The reaction of a simple imidazole with iodoacetic acid does not vary with pH in the same way, but follows a simple titration curve inflecting about the pK of the reacting group. There is obviously an ancillary acid group required for the reaction of the enzyme with iodoacetic acid.

As a result of experiments of this sort the concept emerged that in the enzyme these two histidines must be located close together three-dimensionally, in such a way that one of them in the acid form can promote the reactivity of the other towards alkylating reagents. One of the histidines, in the positively charged form, could attract and bind the negative end of the alkylating reagent and juxtapose the reactive carbon atom of the latter to the nitrogen of the other histidine thus promoting its alkylation. Clearly, one imidazole acts as a base and the other as an acid; their pKs are in the region of 6 so that in this pH range there will be an equilibrium mixture of acid and base forms. Which histidine is alkylated would depend amongst other things on the distribution of the charges. This general picture would explain why either of these two histidines, but never both in the same molecule, is alkylated by iodoacetic acid. Competitive inhibitors, which presumably sit on the active site, protect these histidine residues against the action of the alkylating reagents.

There is also other evidence which implicates these two histidines in the catalytic activity of the enzyme. That they are indeed not many nanometers apart has been confirmed more recently by x-ray crystallographic studies (Richards and Wyckoff, 1968).

The reaction catalysed by ribonuclease is shown in Figure 1. RNA is hydrolysed in two stages

Fig. 1 The reaction catalysed by ribonuclease. R refers to the next nucleoside which is linked through the oxygen attached to its 5' carbon atom to the phosphorus atom shown.

involving the formation of a cyclic phosphate intermediate. The phosphorus atom links the 5' oxygen of one nucleoside residue R with the 3' oxygen of another nucleoside as shown, and the specificity of the reaction requires the 3' oxygen of the phosphorus diester link cleaved to be provided by a pyrimidine nucleoside. In the first stage of the reaction nucleophilic¹ attack on the phosphorus by the 2'-hydroxyl results in the formation of the cyclic phosphate and release of the group -OR. The intermediate is then hydrolysed by further nucleophilic exchange, with water attacking the phosphorus atom, opening the cyclic structure, and reconstitution of the 2' hydroxyl. The reaction is basically a very simple one, the complexity being derived entirely from the specificity, namely, the absolute requirement for the pyrimidine base which indicates the existence of a specificity site referred to as the primary specificity site. The nature of the group -R does not influence the specificity of the reaction, but it does have a very considerable effect on the reaction rate which is very much higher when R is a purine than

¹When two unlike atoms such as oxygen and phosphorus form a covalent bond, the shared electrons are never shared equally. The electrons tend to be attracted a little more towards one of the atoms, which is said to be the more 'electronegative' and has a fractional negative charge; the other has a relative deficiency of electrons and therefore a fractional positive charge. Similar changes can also be caused by other mechanisms, and in some circumstances the resulting change in charge density may be distributed over a larger part of the molecule. Though small, these changes influence the type of reagent which will react with a particular site in the molecule. Thus, a site with a low electron density (positive charge) will be susceptible to attack by a negatively charged reagent, and reactions of this type are called nucleophilic reactions, the reagent being a nucleophile. In the reaction under consideration, as oxygen is more electronegative than phosphorus, the latter will acquire a positive charge and thus be susceptible to attack by nucleophilic reagents; the attraction of the imidazole for the hydrogen atom in the 2'-hydroxyl results in a higher electron density around the hydroxyl oxygen atom, thus facilitating its reaction with the phosphorus atom.

when it is a pyrimidine nucleoside. It is convenient to refer to the site which confers this kinetic selectivity as the secondary specificity site.

Kinetic studies of the hydrolysis of the cyclic phosphate showed clearly that two groups are required for catalysis, one acting as an acid and one as a base (Herries, Mathias, and Rabin, 1962). The *pK* values of these two groups in the free enzyme are 5.22 and 6.78, which suggests that they are derived from imidazole groups of histidine residues and this has been confirmed by experiments in organic solvents (Findlay, Mathias, and Rabin, 1962a). Both of these *pK*s increase on substrate binding. In the case of the group acting as an acid this is probably due to hydrogen-bonding with the attacking nucleophile, water; the existence of a water-binding site on the protein has been demonstrated (Findlay, Mathias, and Rabin 1962b).

On the basis of these facts it was possible nearly a decade ago to suggest a mechanism for the hydrolysis of cyclic phosphates. The essence of this suggestion is that a primary specificity site binds the pyrimidine, thus suitably positioning the phosphorus with respect to the two catalytic groups histidine 12 and 119 (Fig. 2). One of these, since shown by x-ray crystallography to be number 12, then acts as an acid by protonating the 2'-oxygen as shown in Figure 2. This weakens the corresponding P-O bond making the phosphorus more susceptible to nucleophilic attack. The other imidazole 119 acts as a base, hydrogen-bonding the attacking nucleophile and thus enhancing its nucleophilic character. We have, therefore, combined acid-base catalysis made possible by molecular configuration which selected

Fig. 2 Enzyme-substrate complex for pyrimidine nucleoside cyclic phosphates suggested in 1961 (Findlay et al, 1961, 1962C). Note that the group on the 5' carbon of ribose is shown as OH. The oxygen could equally well be linked to another nucleoside instead of to an hydrogen atom.

Fig. 3 Enzyme-substrate complex for esters of pyrimidine nucleoside-3'-phosphates suggested in 1961 (Findlay et al, 1961; 1962C). Note that for convenience no substituent is shown on the 4' carbon of the ribose ring.

aligns the phosphorus as to favour a simple nucleophilic displacement reaction.

The formation of the cyclic phosphate must also be explained (Fig. 3). The group which acts as a base (histidine 119) in the hydrolysis of the cyclic phosphate is now required as an acid, and the group functioning as an acid in hydrolysis of the cyclic phosphate (histidine 12) is now required as a base. The latter hydrogen-bonds the 2'-hydroxyl, weakening the C₂OH bond and turning the hydroxyl group into something akin to an alkoxide and thus favouring its attack on the phosphorus. The departure of the -OR group, with the formation of the cyclic phosphate, is favoured by the acid interaction with histidine 119 shown. Clearly, only histidines could function in this way because they are the only groups which have a *pK* in and around the region of neutrality. Furthermore, the macroscopic *pK*s for the ionizing system of these two histidines are sufficiently close together to enable this inversion of function to occur without gross loss of efficiency.

We have not yet explained why the secondary specificity site confers kinetic selectivity. It is suggested that the enzyme-substrate complex may exist in at least two forms, one as an active complex and the other as an abortive complex (Fig. 4), the latter being produced by rotation of the phosphoric acid moiety which brings the negatively charged oxygen into juxtaposition with the positively charged imidazole. This would be a very favourable interaction chemically and energetically, but in our view

it would be a totally abortive interaction because it would not place the substrate into the right situation for the linking oxygen to be protonated as in Figure 3. For this to occur another interaction between the enzyme and the R group indicated by the arrow in Figure 4 is required. Hence, if the nature of the R group is suitable, ie, if it is a purine nucleoside, then the active complex will be formed with a resulting enhancement of the reaction rate. It can be shown by simple calculation that this will primarily affect *V*_{max} but not the *K*_m.

One of the easiest and most superficial ways of gaining information on enzyme-substrate binding is by studying changes in the dissociation constants which result from modifications of the ligand. This can be done with competitive inhibitors, building up the ligand piece by piece and observing the effect of each modification. Table I shows a series of such data obtained with ribonuclease and competitive inhibitors. As first ribose and then a pyrimidine are added to inorganic phosphate, the dissociation constant falls progressively indicating tighter binding of the ligand. Opening out the cyclic phosphate causes the dissociation constant to fall still further. Consideration of ligands 1 to 5 suggests that the binding of the substrate cytidine 2',3'-phosphate is dependent on at least three weak contributions involving respectively phosphorus, the sugar ring, and the base. The influence of the group R, which is split off, is indicated by the effect of adding a benzyl group to 2'-deoxythymidine-3'-phosphate, the resulting fall

Fig. 4 Suggested forms of the active and abortive complexes between ribonuclease and its substrates.

Number	Ligand	Dissociation Constant (mM)
1	Inorganic phosphate	3,500
2	Methyl D-ribose cyclic phosphate	950
3	Cytidine 2' 3' phosphate (Km)	160
4	Cytidine 3' phosphate	18
5	Cytidine 2' phosphate	1.9
6	2' deoxythymidine 3' phosphate	3.4
7	2' deoxythymidine 3' phosphate benzyl ester	0.8

Table Dissociation constants of enzyme-ligand complexes at pH 5.2, $I = 0.02$, 25°C ¹

¹Values obtained by regression Lineweaver-Burk plots using the ligand as a competitive inhibitor.

in K suggesting a weak interaction with this group also.

The data show that the formation of an enzyme-substrate complex involves a large number of weak interactions, and this is true for all enzymes that have been studied in detail, eg, carboxypeptidase, chymotrypsin, and lysozyme.

Further information on substrate binding may be obtained by spectrophotometric studies, because the interaction of ribonuclease with certain ligands produces changes in the ultraviolet absorption

spectrum. Examples of such difference spectra (Deavin, Fisher, Kemp, Mathias, and Rabin, 1969), ie, the difference produced in the absorption spectra of various substances by combination with ribonuclease, are shown in Fig. 5 for a series of guanine nucleotides. They are virtually identical, the different magnitudes of the peaks being entirely due to different binding constants and the particular concentrations used. In addition, the difference spectra produced by changing the pH of guanosine monophosphate in the absence of ribonuclease are also shown. The spectrum resulting from a change in pH from 6.2 to 1.82 is almost identical with that produced by combination of this ligand with the enzyme, a large peak occurring in the same position, and a small secondary peak corresponding with the shoulder present in the other curves. This suggests that the effect of the enzyme is produced by interaction of a guanine ring N with a proton-donating group on the surface of the enzyme. The ring N is suggested as taking part because this is believed to be the normal site of protonation of guanine (Ts'O, Rapaport, and Bollum, 1966). That this is not the mode of interaction of the enzyme with pyrimidine nucleotides is shown by the difference spectra in Fig. 6 in which it can be seen that the difference spectrum of cytidine monophosphate in acid is quite different from that obtained with the ligand and ribonuclease. Cytidine must therefore interact on some other location than the site of interaction of the guanine nucleotide, so there must be a minimum of two binding areas for purines and pyrimidines, which may reasonably be supposed to be the secondary and primary specificity sites respectively.

Recent crystallographic studies (Richards and Wyckoff, 1968) have provided further factual details of the interaction of a substrate with various groups on the enzyme. The important facts are that two histidines are located very close together exactly as we suggested, namely, histidine 12, which probably hydrogen-bonds to the attacking hydroxyl, and histidine 119, which probably hydrogen-bonds to the leaving oxygen. Details of the pyrimidine-enzyme interaction as determined by Richards and Wyckoff (1968) are shown in Fig. 7 for a cytidine guanosine dinucleotide. It involves a number of rather weak hydrogen-bond interactions between the pyrimidine ring and threonine 45 and serine 123 on the enzyme. The function of lysine 41 is not at all clear. The side-chain points towards the two oxygens of the phosphate, but whether it has anything to do with the catalysis is unknown. The role of lysine 7, which is shown protonating the purine ring, is based solely on spectral evidence and though its location is suitable, such a role remains speculative. There may

Fig. 5 Difference spectra of interaction of ribonuclease and guanosine 5' phosphate (GMP), guanosine 5' diphosphate (GDP), and guanosine 5' triphosphate (GTP). Difference spectra resulting from change of the pH of solutions of guanosine 5' monophosphate from pH 6.2 to 10.5 (. . .) and to 1.82 (—), final concentrations 44 μM, temp 25°, ionic strength 0.02, light path 1 cm.

Fig. 6 Difference spectra of interaction of ribonuclease and cytidine 5' phosphate (—), cytidine 5' diphosphate (---), and cytidine 5' triphosphate (···). Difference spectrum resulting from change of pH of a solution of cytidine 5' phosphate from pH 6.4 to 8.2 (— · — · —). Final concentration 50 μ M, temp 25°, ionic strength 0.02, light path 1 cm.

Fig. 7 Suggested enzyme-substrate complex of ribonuclease and a CpG sequence of RNA.

Fig. 8 Suggested trigonal bipyramid intermediate formed between enzyme and substrate during catalysis by ribonuclease.

also be an interaction between aspartate 121 and the other end of histidine 119 although this is far from certain.

It is suggested that the catalysis of the nucleophilic exchange involves an intermediate with a structure shown in Figure 8. It seems from recent work on the chemistry of phosphorus (Dennis and Westheimer, 1966; Frank and Usher, 1967) that many of its reactions involve trigonal bipyramid intermediates, with the attacking and departing nucleophiles in the apical positions, and with the cyclic phosphate ring spanning one apical and one basal position. The suggested intermediate in a ribonuclease-catalysed reaction fulfils these criteria (Fig. 8). The cyclic phosphate does span one apical and one basal position. The two histidine residues bond to the 2' oxygen and the leaving group respectively, so that the most electronegative groups are indeed in the apical positions. Catalysis requires simply the movement of protons along the hydrogen bonds between the imidazole nitrogens and the phosphorus oxygens. Cyclic phosphate formation requires simultaneous proton movement towards histidine 12 and away from histidine 119; one vibration then forms the cyclic phosphate. The hydrolysis of the cyclic phosphate, where R is H in the illustration, requires the reverse movements of one proton towards histidine 119 and away from histidine 12 to the 2' oxygen; one vibration of the phosphorus in the middle of the trigonal bipyramid will give the second stage of reaction hydrolysis.

This has been a very brief survey of the chemistry and action of ribonuclease, which I hope has made it clear that we have considerable understanding of this catalyst. More is probably known about this enzyme, carboxypeptidase, and lysozyme than for any simple organic chemical reaction in solution, and this is, I think, a measure of the extent of the achievements of the crystallographers and the biochemists in combination.

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References

- Anfinsen, C. B., (1956). The limited digestion of ribonuclease with pepsin. *J. biol. Chem.*, **221**, 405-412.
- Deavin, A., Fisher R., Kemp, C. M., Mathias, A. P., and Rabin, B. R. (1969). Bovine pancreatic ribonuclease-spectrophotometric investigations of the interaction of the enzyme with nucleotides. *Europ. J. Biochem.*, **7**, 21-26.
- Dennis, E. A., and Westheimer, F. H. (1966). The rates of hydrolysis of esters of cyclic-phosphinic acids. *J. Amer. chem. Soc.*, **88**, 3431-3432.
- Findlay, D., Mathias, A. P., and Rabin, B. R. (1962a). The active site and mechanism of action of bovine pancreatic ribonuclease. 5. The charge types at the active centre. *Biochem. J.*, **85**, 139-144.
- Findlay, D., Mathias, A. P., and Rabin, B. R. (1962b). The active site and mechanism of action of bovine pancreatic ribonuclease. 4. The activity in inert organic solvents and alcohols. *Biochem. J.*, **85**, 134-139.
- Findlay, D., Herries, D. G., Mathias, A. P., Rabin, B. R., and Ross, C. A. (1962c). The active site and mechanism of action of bovine pancreatic ribonuclease. 7. The catalytic mechanism. *Biochem. J.*, **85**, 152-153.
- Frank, D. S., and Usher, D. A. (1967). A mechanism involving pseudo-rotation for the hydrolysis of dimethylphosphoacetoin. *J. Amer. chem. Soc.*, **89**, 6360-6361.
- Herries, D. G., Mathias, A. P., and Rabin, B. R. (1962). The active site and mechanism of action of bovine pancreatic ribonuclease. 3. The pH-dependence of the kinetic parameters for the hydrolysis of cytidine 2',3'-phosphate. *Biochem. J.*, **85**, 127-134.
- Kartha, G., Bello, J., and Harker, D. (1967). Tertiary structure of ribonuclease. *Nature (Lond.)*, **213**, 862-865.
- Murdock, A. L., Grist, K. L., and Hirs, C. H. W. (1966). On the dinitrophenylation of bovine pancreatic ribonuclease A: kinetics of the reaction in water and 8 M urea. *Arch. Biochem.*, **114**, 375-390.
- Rabin, B. R., and Mathias, A. P. (1963). In *14th Colloquium der Gesellschaft für physiologische Chemie*, p. 7. Springer, Berlin.
- Richards, F., and Wykoff H. W. (1968). Data communicated by Dr N. M. Allowell at the December 1968 meeting of the British Biophysical Society. Some of this information is summarized by Dr L. N. Johnson (1969). In *FEBS Letters*, **2**, 201.
- Ts'O, P'O'P. Rapaport, S. A., and Bollum, F. J. (1966). A comparative study of polydeoxyribonucleotides and polyribonucleotides by optical rotatory dispersion. *Biochemistry*, **5**, 4153-4170.