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Cryptic Functions of Enzymes in Chemical Catalysis**

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Using a number of examples, this article demonstrates how the functional groups responsible for the catalytic activity of an enzyme must be studied within the context of the enzyme-substrate complex. Very often a substrate will actively cooperate with the enzyme to bring about its own transformation. The so-called cryptic functions of enzymes are considered in the case of seryl proteases which, according to the type of substrate or structural modification introduced in the enzyme, may exhibit esterase, amidase, protease, racemase or dehydratase activity. The cryptic functions may possess a physiological significance which reflects the evolutionary history of the protein. Alternatively they may offer a simple way of exploiting the enzymes as catalysts capable of taking part in the chemical reactions of biotechnological interest but little physiological importance.

(Keywords: Biotechnology; Racemase; Synthetase)

Die kryptischen Funktionen der Enzyme in der chemischen Katalyse

Es wird an einer Reihe von Beispielen gezeigt, daß die funktionellen Gruppen, die für die katalytische Aktivität eines Enzymes verantwortlich sind, im Zusammenhang mit dem gesamten Enzym—Substrat-Komplex betrachtet werden müssen, da sehr oft das Substrat einen aktiven Einfluß auf die eigene Transformation nimmt. Die sogenannten kryptischen Funktionen der Enzyme werden für den Fall von Proteasen untersucht, die entsprechend dem Substrat (und den substratinduzierten Modifikationen im Enzym) als Esterasen, Amidasen, Proteasen, Racemasen oder Dehydratasen wirken können. In den kryptischen Funktionen und der physiologischen Signifikanz kann sich die geschichtliche Evolution des Proteins äußern. Andererseits können die Enzyme auch als Katalysatoren bei chemischen Reaktionen von biotechnischem Interesse aber geringer physiologischer Bedeutung eingesetzt werden.

** This work is dedicated to Prof. *E. Wünsch* (München) at the occasion of his 60th birthday.

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Introduction

One of the fundamental concepts of chemistry is that of chemical potential which can be regarded as a way of defining the relationship between a chemical species and its environment. The more extended but analogous concept of „biochemical potential" may be used to express the relationship between a biomolecule and its physiological or artificial environment. We wish to discuss here the cryptic functions of certain enzymes, described as "masked" functions in an earlier publication[1]. Such functions can in fact be unmasked by particular types of environment, acting through intermolecular and/or intramolecular changes, which might also be considered as an example of what we generally define as "biochemical potential".

Functional properties of enzymes, resulting from evolutionary changes, can be both *general* and *specific*[2]. *General* ones depend on the aminoacid side chains, responsible for properties such as solubility, charge density or distribution, isoelectric point, polarity and structural stability. These are additive properties of the various aminoacid residues regulating intramolecular interactions. Specific functions of enzymes are those responsible for their catalytic power and, unlike the preceding ones, depend on more than additive, i.e. cooperative, properties of the chemical residues constituting the active center of the molecule, the site of amplification effects[3].

We would like to point out that a dialectical way of considering the active site, i.e. a site becoming "active" only after interaction with a substrate, is more suitable than a deterministic one for understanding its true nature.

Results and Discussion

Chemical Modification of Enzymes

Comparison of enzymes with similar catalytic functions, found in different species, has shown that several aminoacids can be changed without appreciable modification of their catalytic power[4]. Chemical modification of enzymes has also shown that the enzyme molecule can accept modifications, sometimes massive, without loss of function[5].

The catalytic activity of an enzyme may indeed be modulated by changes in the environment of the active site: these include structural modifications of the enzyme molecule, changes induced by the substrate on the active configuration and changes induced by the surrounding medium. The physiological counterpart of such artificial manipulations is the modification of enzyme activity by factors such as protein—protein and lipid—protein interactions and the allosteric phenomena.

The seryl proteases illustrate different ways by which the biochemical potential of biomolecules separated from their physiological environment can be unravelled. Trypsin, which we have been studying for some time[6], specifically catalyses the hydrolysis of carboxyl derivatives of *L*-Arg and *L*-Lys, the same active site being responsible for the cleavage of both amides and esters.

Four Trp residues of the molecule can be modified, e.g. reversibly formylated[7], yet the modified enzyme is totally active in ester hydrolysis while amidase and protease activities are completely lost. None of the four Trp residues is known to be involved in the active center[8] so the above modification has probably alter the geometry of the active site following an intramolecular change. The effect of formylation can in fact be reproduced when the solvent is changed and in 50% dioxane, 25% formamide and 37% chloroethanol only the proteolytic activity is completely eliminated[9]. A decrease in hydrophobic interactions may be a common effect of the formylation reaction and of the solvent change.

Active Role of Substrates in Enzyme Reactions

The generally accepted mechanism for the catalytic action of the seryl proteases consists of two steps: the first is the formation of an acyl-enzyme with the liberation of an alcohol or an amine as first product and the second is the hydrolysis of the acyl-enzyme giving the second product[10].

The difference between the mechanisms of hydrolysis for an ester and an amide should depend on a change in the first step, i.e. the acyl-enzyme formation. The substrate can in fact be regarded as an acylating reagent for the active serine and this part of the reaction does not require the participation of water molecules. We have already shown that a peptide bond can be non-enzymatically split in anhydrous conditions by an intramolecular reaction with the vicinal peptide bond and by acid catalysis[11] (Fig. 1).

Rupture of the amide bond is energetically compensated by formation of the O—C bond and a thermodynamically favoured 5-membered ring. The cyclic product can be considered as an internal anhydride or an internal active ester which can react, in a neutral medium, with nucleophilic agents such as amines, alcohol or water.

The mechanism is typical of polypeptide substrates and explains why amides, normally devoid of acylating power, can easily acylate other amides, alcohols and water. We have demonstrated the existence of such cyclic intermediate during chymotryptic activity by freezing

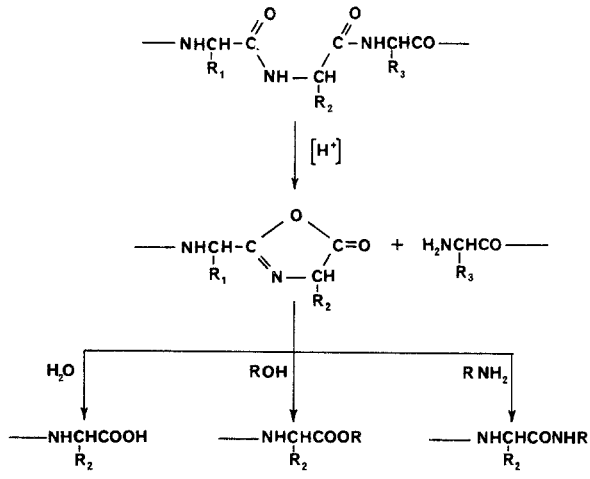

Fig. 1. Participation of amide group in proteolysis

the enzyme-substrate system in weakly acidic media at short time intervals[12] (Fig. 2).

The enzyme induces an intramolecular reaction of the substrate after the formation of the enzyme-substrate complex and the leaving group is an alcohol or an amine, according to the nature of the substrate. The cyclic products reacts with the active serine to yield the acyl-enzyme which is subsequently hydrolyzed. The scheme of Fig. 2 can represent both tryptic and chymotryptic hydrolysis of natural substrates, but not of all substrates and in all conditions. If the substrate is an ester there is no need for cyclic product formation to generate the acyl-enzyme, which is another ester; it is necessary, however, when the substrate is an amide with low acylating activity. The separation of esterase and protease activity of trypsin after formylation or treatment with solvents, above reported, could be attributed to the inability of the modified enzyme to induce the intramolecular reaction of the amide substrate. On the other hand, the abolition of such a step should not and does not affect the transformation of esters.

If a cyclisation of the substrate can be somehow induced, the formation of an acyl-enzyme will not be strictly necessary to insure hydrolysis. While normally the "active" serine oxydryl of the enzyme is in the best position for reacting with cyclic intermediates, in particular conditions attained after chemical modification or addition of powerful nucleophiles, the acyl-enzyme might not be formed at all[13].

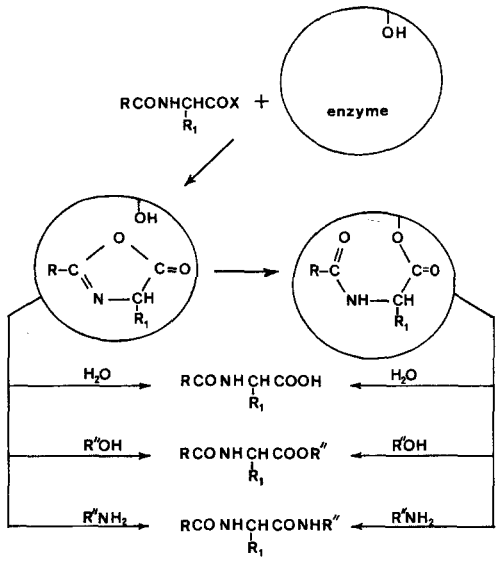

Fig. 2. Possible enzymatic pathway; X = —NHR or —OR

A crucial point in such a context is the active role of the substrate in implementing its own transformation. The intramolecular process consists of a reaction between two functional groups of the same molecule, the enzyme acting as a possible acid catalyst. Within the molecule of a substrate one may thus identify a *geometric* portion, required for specific with the enzyme, and a *functional* portion which is a prerequisite of the catalytic mechanism. While the geometric portion is untouchable, the functional portion can undergo considerable modifications without loss of susceptibility to enzyme action.

Unexpected Enzyme Reactions

If unusual chemical functions are introduced in the enzyme—substrate complex, unexpected reactions can be catalysed by the enzyme. Examples are the phenylthiocarbamyl derivatives of *L*-Phe, *L*-Trp or *L*-Tyr reacted with chymotrypsin[14] (Fig. 3).

The geometric portion of the substrate (R) ensures the specificity of the binding of such substrates to the chymotrypsin molecule. The carboxyl group is activated through an acid catalysis by His-57 which takes part in the active site of the enzyme[15]. The chemical group best placed to exert a nucleophilic attack on the carboxyl carbon is the

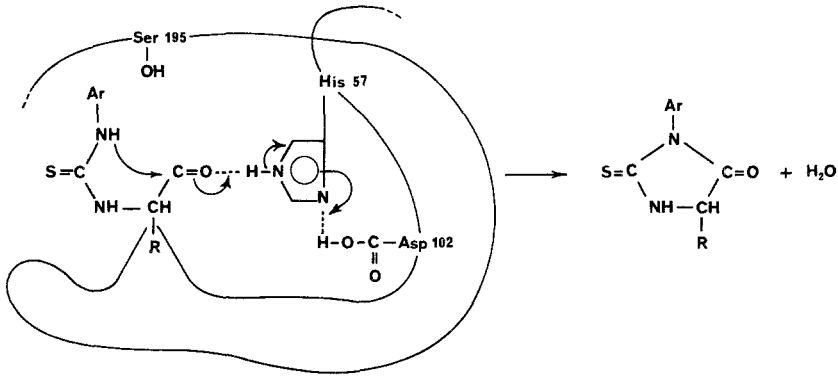

Fig. 3. Intramolecular reaction of substrate induced by the enzyme; R = geometric portion of the substrate

thioamide nitrogen. The resulting cycle, in contrast to experience with natural substrates, is chemically inert and is not further transformed. The global chymotrypsin-catalyzed reaction in the synthesis of this heterocyclic compound is a *dehydratation*. *L*-Tyr, *L*-Trp and *L*-Phe are transformed at a rate comparable to that of the natural substrates and similar results are obtained by reacting the phenylthiocarbamyl derivative of *L*-Arg with trypsin[16]. Study of the *pH*-activity diagrams allowed us to infer yields of the cyclisation reaction and to observe that, while the reaction of synthesis (intramolecular dehydration) occurs at an optimum *pH* of 4.5, that of hydrolysis has an optimum *pH* value of 8. The non-cyclic species acts as a substrate and the cyclic derivative is the reaction product at acid *pH*, while the opposite is true at alkaline *pH*. By shuttling between the two *pH* values it is possible to switch repeatedly from the non-cyclic to the cyclic species by enzymatic catalysis without major decrease in yield[17] (Fig. 4).

The *pH* optimum should not therefore be taken as a characteristic of the enzyme alone, but rather of the enzyme—catalyzed reaction.

The above example shows another unusual aspect of trypsin catalysis. The substrate with an optimum *pH* value of 4.5 is a linear

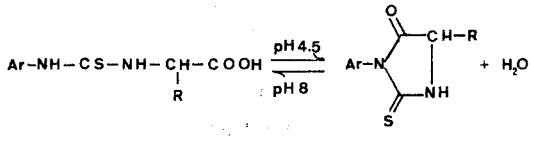

Fig. 4. Enzyme catalysed transformation of aryl-thiocarbamylaminoacids

derivative of *L*-Arg (the *D* enantiomer being inactive) while the cyclic reaction product recemizes rapidly. The cyclisation reaction is in fact parallel to the disappearence of the optical activity of the reaction mixture. The enzymatic hydrolysis of the cyclic derivative (taking place at *pH* 8) transforms only the *L* enantiomer, constantly reconstituted from the *D* enantiomer through a tautomeric equilibrium (Fig. 5). Since the linear product is sterically stable, the reaction is

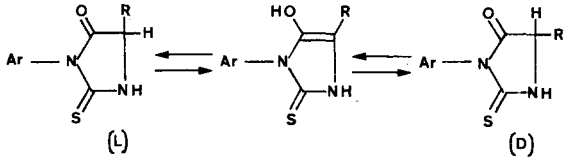

Fig. 5. Tautomerism of arylthiohydantoines

accompanied by the appearance of optical activity. Thus we can see that a certain structure of the substrate can produce *recemase* activity in a usually hydrolytic enzyme.

Fig. 6 shows a chymotrypsin-catalysed 100% hydrolysis of a racemic thiazolinone (structurally releated to aromatic aminoacids) into an optically active thio-acyl aminoacid, the unusual racemase activity of the enzyme being switched on by structural participation of the substrate[18]. *L*-Thiazolinones of aromatic aminoacids are in a

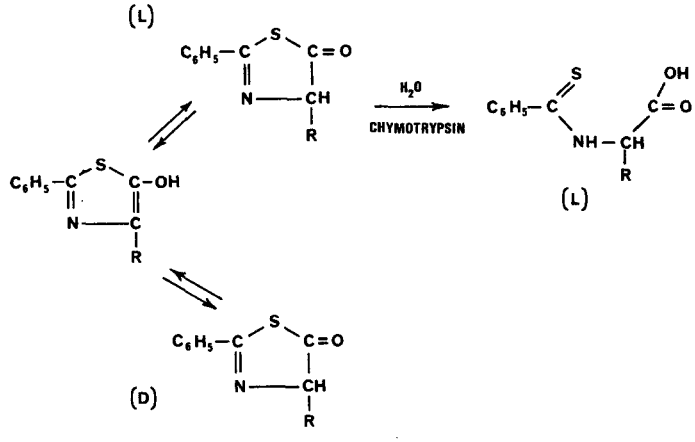

Fig. 6. Enzyme catalysed invertion of configuration

racemic equilibrium with the *D* enantiomer through a tautomeric enol intermediate and are specific substrates for chymotrypsin. By contrast their products of hydrolysis are sterically stable and this accounts for the *racemase* activity brought about by the substrate-enzyme co-operation.

The same happens with trypsin[19] and the hydrolysis of the racemic substrate entails the appearance of optical activity.

Another class of substrates capable of inducing *racemase* activity from chymotrypsin are beta-carbolines originated by cyclisation and dehydratation from acyl-Trp esters[20] described in Fig. 7.

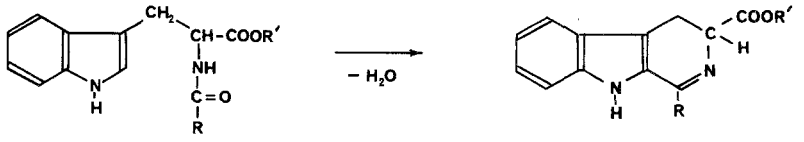

Fig. 7. 3,4-dihydro-β-carboline derivatives from parent N^{α} acyl tryptophan

A synthesis of carboxyl esters of 3,4-dihydro-β-carbolines which preserves the configuration of the tryptophan core has been described[21]. These are chymotrypsin substrates and the velocities of hydrolysis are higher when the *D* enantiomers are used. The reaction products are *D* enantiomer derivatives in any case even if *L* enantiomers are used as substrate. For the latter an additional step of invertion of configuration is thus necessary[18] (Fig. 8).

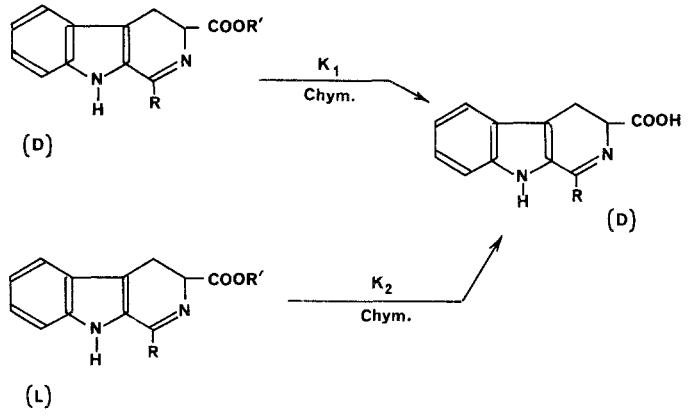

Fig. 8. Hydrolase and racemase activities of α-chymotrypsin

3,4-dihydro-β-carboline esters show a tendency to racemize in solution but the velocity of enzymatic hydrolysis of the *L* enantiomer derivative, which is proportional to the concentration of chymotrypsin, largely overcomes the spontaneous racemisation. The inversion of the optical configuration occurs within the enzyme-substrate complex and, in this case, chymotrypsin acts as a true *racemase*. 3,4-dihydro-β-carboline esters can therefore be considered as intramolecular *Schiff* bases responsible for the unmasking of unusual racemase activity of seryl proteases. The esters are sterically unstable because of the ester and imine groups neighbouring the chiral center, whereas their carboxylate analogues are perfectly stable.

However, the preference of the enzyme for the *D*-isomers is not restricted to the β-carboline derivatives. When the *D*-isomers of aromatic amino acids and glycine esters react with chymotrypsin in the presence of aromatic aldehydes, such as pyridoxal, their hydrolysis increases with the increasing concentrations of the aldehyde. Actually the *D* enantiomers of amino acid esters become substrates for the enzyme when they have reacted to become *Schiff* bases; the *L* enantiomers still behave as substrates for the enzyme while their *Schiff* bases are unreactive and thus pyridoxal decreases the overall rate of enzymatic hydrolysis. We have therefore a case where an artificial cofactor molecule can modify an enzyme process by interacting with the substrate[22].

Conclusion

The main purpose of the present article was to suggest an approach to the interpretation of enzyme activity which would stress the role of enzyme partners in such activity. After the enzyme—substrate interaction several properties can appear which are not exhibited by the partners on their own. These range from the unmasking of new catalytic functions of the enzyme molecule itself to localizing the active catalytic center of the reaction in the substrate molecule, suitably marshalled for the purpose by the enzyme. The accomplishment of so-called "suicidal" reactions[23], linked to the transformation of an inert substrate into an enzyme inhibitor, is another example of an unmasking process which this time occurs after the enzyme-substrate interaction and involves the substrate.

It has been pointed out that the catalytic sites of the hydrolytic enzymes are apolar regions and that the transition states of the enzymatic reactions mostly involve apolar interactions. Multifunctional catalysis by hydrolytic enzymes may therefore occur in a cyclic fashion in the apolar regions of the active site, as suggested,

among others, by examples of covalent and non-covalent catalysis carried out by polymeric catalysts and cycloamylose model[24]. The idea that more than one catalytic apparatus can function within the active site of a given hydrolytic enzyme also has some precursors in the literature since it has been found that the introduction of a coenzyme analogue on the periphery or at the active site of a hydrolytic enzyme can switch on unexpected oxido-reductase activity[25]. From the evolutionary standpoint it has been remarked that the embedding of catalytic groups in hydrophobic regions of a protein and a stepwise modification of such a structure are plausible events in the progressive specialization of the enzyme function[26].

The present paper illustrates the cases of proteolytic enzymes, such as trypsin and chymotrypsin which, in addition to the esterase and protease activities usually exhibited, are endowed with cryptic catalytic functions, e.g. racemase and dehydratase, which appear when certain types of substrates are used. These examples suggest that the memory of evolutionary changes may have been retained in the enzyme molecules and it may be postulated that suitable substrate structures or environmental changes could help in retrieval of this memory. The possibility of modulating or even changing the catalytic activity of an enzyme, by choosing suitable substrates, also suggests that a number of chemical reactions of possible biotechnological importance might benefit from enzymatic catalysis since some enzymes can show *in vitro* properties they usually do not exploit *in vivo*.

Acknowledgements

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