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Regulation of Enzyme Activity by Enzyme Orientation: A Hypothesis

H. BROCKERHOFF

Neurochemistry Department, New York State Institute for Basic Research in Mental Retardation. 1050 Forest Hill Road, Staten Island, New York 10314

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In addition to substrate binding sites, many enzymes must possess supersubstrate binding sites that regulate attachment and orientation of the enzyme toward the matrix (micelle, membrane) in which the substrate molecules are embedded, the supersubstrate.

The problem of metabolic regulation is at the heart of modern biochemistry: how can enzymic activity be generated when it is needed and be suppressed when it is not? During the last decade it has become apparent that, in addition to the control of enzyme concentrations by synthesis and degradation, many enzymes can, in fact, be switched on and off by reversible activation-inactivation mechanisms. The controlling agents may be modulators or regulatory subunits, or the enzyme may be chemically modified by the action of other enzymes, e.g., kinases and phosphatasses. In each case, it is understood that "activation" means the generation of the reactive site of the enzyme, brought about by unblocking or by conformational changes of the protein (*16*, *18*).

In this article I propound the hypothesis that an alternative mechanism of activation may be provided by the control of the spatial orientation of enzymes towards their substrates.

It is, of course, a commonplace assumption that the enzymes of cellular organelles or of multienzyme complexes, such as fatty acid synthetase, are not haphazardly jumbled together but arranged in an orderly fashion, and that such order is prerequisite to enzymic activity. I am not, at present, concerned with such enzymes and their orientation, but with the orientation of isolated soluble enzyme molecules that act on substrates which are part of multimolecular structures, e.g., biological membranes; the extrapolation of my hypotheses to enzymes which are themselves part of such structures will be left open. The experimental evidence for orientation as a means of intracellular metabolic control may still be lacking, but there is already substantial evidence of the importance of orientation for a group of extracellular hydrolytic enzymes: lipases and phospholipases. The concepts developed here are, in part, derived from studies on pancreatic lipase, and a discussion of this enzyme and of phospholipases will serve as an introduction and background to my tenets.

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BACKGROUND: LIPOLYTIC ENZYMES

Pancreatic lipase hydrolyzes triglycerides and other esters of long-chain fatty acids. These substrates are insoluble in water, and the enzyme must therefore act at an oil–water interface. Its activity, under these conditions, is very great ($k_{cat} = 8 \times 10^3$ sec^{-1}), in contrast to the small activity displayed against water-soluble substrates (*10*). The enzyme obviously has a high affinity for the interface, and this is not surprising because it has to compete, in the intestinal fluid, with an enormous multitude of other proteins which also tend to flock toward interfaces. From *in vitro* experiments (*3*), it can be estimated that the interfacial affinity of the lipase is at least one-thousand-fold greater than that of bovine serum albumin.

It might seem, then, that the lipase shows a strong and specific affinity toward its "substrate." Paradoxically, however, there are strong indications of the absence of "lipophilic" binding, i.e., hydrophobic binding of the fatty acid chains, in the enzyme-

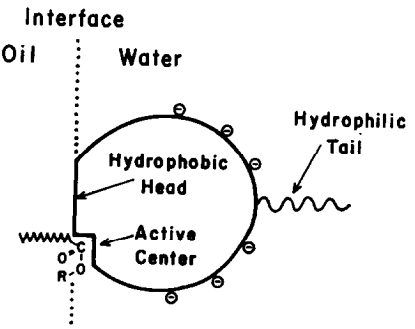

FIG. 1. Model of the attachment and orientation of pancreatic lipase at an oil–water interface. Substrate binding site ("active center") and supersubstrate binding site ("hydrophobic head") are adjacent but separate. The dimensions of these sites and of the "hydrophilic tail" are conjectural.

substrate complex (*4*), the most persuasive evidence being the rapidity with which formate esters (which are devoid of aliphatic chains) are hydrolyzed (*2*). The paradox is resolved in a model (*4*) in which the lipase is equipped with an interfacial attachment site, the "hydrophobic head," which is located on the enzyme surface near to the reactive site but *separate* from it (Fig. 1). The resulting favorable orientation of the enzyme is further stabilized by a hydrophilic "tail" consisting of polar amino acid and carbohydrate residues.

The model of Fig. 1 has been derived to account for the substrate specificity of the lipase, but it is eminently plausible for *a priori* reasons. The substrates of a lipase, being insoluble in water, cannot leave their interfacial matrix to diffuse to the enzyme; the enzyme must approach the substrate. It is not sufficient that the lipase be adsorbed to the interface, it must be adsorbed in the right orientation, because the mass of the enzyme is 100 times larger than that of the average single substrate molecule and its reactive site is concentrated in only a small part of the enzymic surface. Once at the interface, the lipase remains relatively immobile because of its size. The substrate molecules file past the reactive site by diffusion in the surface of the oil droplet; the high catalytic rate suggests that this diffusion may, in fact, be the rate-limiting process.

The spatial separation of hydrophobic head and reactive site is plausible for many reasons. First, it will allow the enzyme to adhere to surfaces that are not composed entirely of substrate molecules, e.g., mixtures of triglycerides with monoglycerides, phospholipids, or paraffins. Second, identity of the two sites would encumber the reactive site with the responsibility of providing interfacial attachment and spatial orientation; this additional burden could not help being detrimental to catalytic efficiency. Third, the attachment to an oil droplet is best achieved by hydrophobic, i.e., anhydrous, binding; but the reactive site must be accessible to the water that is needed to hydrolyze the intermediate acyl-enzyme. Fourth, the necessity for the lipase to maintain three different surface regions—catalytic, head, and tail—may explain why its molecular size (48 000) is two and four times larger than that of the digestive proteinases and nucleases, although these enzymes probably have more complicated catalytic sites: a more elaborate infrastructure is required to keep the three sites separate.

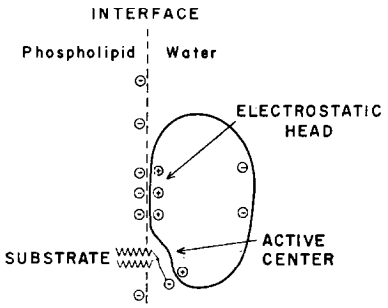

FIG. 2. Model of supersubstrate binding and orientation of pancreatic phospholipase. The model assumes electrostatic binding, but hydrophobic binding and hydrogen bonding may also be involved.

The case for the separate identity of reactive site and interfacial binding and orientation site is even more conclusive for the extracellular phospholipases. It is well known, mainly through the work of R. M. C. Dawson (*8*), that many of these enzymes are sensitive to the surface potential of the substrate micelles. This sensitivity of the enzymes is independent of their substrate specificity. For example, the phospholipase A_2 of porcine pancreas can hydrolyze the fatty acid ester in position 2 of the electrically neutral, zwitter-ionic phosphatidylcholine (lecithin), but only if the micelles of the substrate include amphiphilic anions such as bile salts (*9*). More important even, this activation can take place at a pH higher than the isoelectric point of the phospholipase, i.e., under conditions where the enzyme itself carries a negative net charge. Obviously, there can be only a limited region of positive potential on the surface of the protein; this region must face the micellar surface and the bile anions; and the site of substrate binding and catalytic activity must be next to it but distinct from it since the bile anions would necessarily interfere with substrate-enzyme binding, which involves the acidic group of the phospholipid (Fig. 2). Other phospholipases attack the same substrate, phosphatidylcholine, but require a positive surface potential. Dawson (*7*) has already suggested that the micellar surface charges regulate the orientation of the enzymes.

The extracellular lipolytic enzymes, then, can be regarded as hydrolases that have developed mechanisms of affinity and orientation toward the matrix in which their

substrates occur, the "supersubstrate"; and these enzymes cannot be the only ones that are so equipped. It stands to reason that all enzymes that find their substrates in matrices—in fat droplets, micelles, membranes, or organelles—must possess similar affinitive and orientative mechanisms. Accepting this conclusion, we can logically proceed to the hypothesis that these mechanisms may be used as metabolic control devices; but before elaborating this proposal, I shall try to define the principal concepts even more thoroughly.

SUBSTRATE AND SUPERSUBSTRATE

Many requirements must be met before an enzyme can react with its substrate: the enzyme must be in an active form, cofactors and cosubstrates must be present, the substrate must in be the proper ionic state, and so on. The most fundamental of requirements is usually not quoted; this is the mutual accessibility of enzyme and substrate. If the substrate is not accessible, for instance, buried in a superstructure or a cell compartment into which the enzyme cannot penetrate, no reaction can take place. If the substrate is accessible, its combination with the enzyme proceeds by diffusion, followed by binding at the substrate binding site which is part of the reactive site.

If a substrate is embedded in a large matrix, as in a biological membrane, it may still be accessible from a spacial viewpoint, even if it can no longer freely diffuse in three dimensions. For example, the polar head groups of phospholipids extend from the lipid phase into the aqueous environment, and the approach of enzymes that act on these groups is entirely possible. However, before enzyme-substate binding can take place, the enzyme must come to terms with the matrix of the substrate, i.e., the multimolecular structure of which the substrate molecule is only one part; this structure I call supersubstrate.

The supersubstrate presents a surface with certain physicochemical properties: it has a net charge, hydrophilic-hydrophobic properties, and often also hydrogen bonding sites. The enzyme molecule is similarly equipped, but because of its stable architecture the sites are not randomly distributed on the surface of the protein; there exist regions of electrostatic and hydrophobic polarity. These regions have to be matched with the supersubstrate before there can be an approach of substrate and reactive site. For example, an esterolytic enzyme, like any protein, will be attracted to a triglyceride–water interface, but its reactive site is small compared to its total surface, and hydrophobic binding (van den Waals binding) to the oil is likely to occur in the wrong places; only the specialized esterases called lipases possess a correctly placed and stable hydrophobic region which brings the reactive site toward the substrate. For another example, if a supersubstrate has a net negative charge, an enzyme with a negative head and a positive tail will attach itself in the wrong orientation, and even though the substrate molecules imbedded in the supersubstrate may have the correct charge for binding to the reactive site, they will not be able to reach it.

Enzymic supersubstrate-binding is a logical consequence of the large size of enzymes compared to their substrates: there are large areas of "useless" surface on every enzyme molecule that *must* interact with the environment of the substrate. The argument can be turned around: enzymes must be large to stabilize the structure not only of the

reactive site but also of the supersubstrate binding and orientation sites. This argument has already been used in support of the lipase model.

Given the logical necessity of affinity and orientation effects as it follows from the known properties of interfaces and proteins, it would be astonishing if these effects were not integrated into cellular metabolism; in other words, if they were not used for the regulation of enzymic activities. In the next section I give some hypothetical examples of how such regulation might be achieved.

HYPOTHETICAL MECHANISMS

Hormone-sensitive enzymes. The hormone-sensitive lipase of adipose tissues is activated by phosphorylation (*19*). It is unlikely that the new phosphate group is directly involved in the catalysis of triglyceride hydrolysis. A conventional explanation for its activating effect would invoke a conformational change of the catalytic site, induced by the remote phosphate group by relay through the protein molecule. However, there is at present no evidence for a rearrangement of the catalytic site, and I suggest that two other mechanisms are equally probable. First, the phosphate, by inducing conformational changes, might activate a supersubstrate binding site rather than the catalytic site, or deactivate an unproductive supersubstrate binding site, perhaps by sterically blocking a hydrophobic site that kept the enzyme in the wrong orientation toward the oil droplet. Second, the phosphate group might itself be the orienting agent. The oil droplet in an adipose cell is very probably surface-loaded with phospholipids and therefore negatively charged. By electrostatic repulsion, the strongly negative phosphate group of the active enzyme might trigger the reorientation that is necessary to bring the reactive site to the oil-cytosol interface.

The enzymes of glycogen metabolism are also controlled by phosphorylation and dephosphorylation (*17*). The surface of a glycogen granule, viewed as a supersubstrate, offers numerous hydrogen bonding sites, and the enzymes may make use of them to attach and orient themselves, with the mediation of the phosphate groups.

Triphosphoinositide metabolism. This phospholipid (phosphatidylinositol-4,5-diphosphate) is a typical component of the myelin sheath of nerves. It can be dephosphorylated to phosphatidylinositol, which can be rephosphorylated (*13*). The phosphatases and phosphorylases involved are "soluble" enzymes, and they are activated or inactivated by Ca^{2+} or Na^{+} and K^{+} ions. Since Ca^{2+} is very tightly complexed by the phosphate groups, but the alkali ions are not, it is clear that the surface potential of the supersubstrate, the myelin, must vary in dependence on the concentration of these ions. The ions may thus activate or deactivate the enzymes by controlling electrostatic supersubstrate binding and enzyme orientation, without being involved in the actual catalytic reactions.

Decarboxylation of serine. Serine is incorporated into phospholipids by exchange against the basic group of the lipid. The resulting phosphatidylserine can be decarboxylated to phosphatidylethanolamine (*1*). Both lipids are common components of biological membranes, and since phosphatidylserine is more strongly electronegative than phosphatidylethanolamine, their relative proportion will help define the magnitude of the surface potential. Homeostasis is achieved, I suggest, by the potential of the super-

substrate: if it becomes too negative, the decarboxylating enzyme is turned toward the lipid surface; if too positive, it is turned away, and the serine-introducing mechanism is switched on by orientation of the appropriate enzyme. The example makes it clear that the supersubstrate rather than the substrate must be involved in the homeostatic mechanism. If the substrate, phosphatidylserine, as such, would bind to the decarboxylase without superior control, the enzyme would proceed to digest all the available substrate molecules. Instead, it is switched off by electrostatic reorientation as soon as the phosphatidylserine/phosphatidylethanolamine ratio decreases to a critical level, and homeostasis is thus achieved.

With this example I have ventured into a metabolic region where the enzymes are not soluble, but are themselves bound to subcellular particles. Enzymes and substrates are probably parts of the same organelle; however, the lipids are doubtlessly aggregated in micellar or lamellar structures, and the anchorage of the enzymes in a superstructure of their own should not hinder their approach and orientation toward the lipid supersubstrate.

Cholesterol esterase. The enzyme from pancreas needs bile salts for activation; the enzymes from brain need bile salts or Triton (*20*). The hydrolysis of cholesterol esters is dramatically accelerated if the substrate is incorporated into phospholipid micelles. Pure cholesterol esters are only very slowly hydrolyzed by any of the enzymes. It is possible that the activators act as spacers in the interface and make the ester group of the substrate accessible to the enzyme. It is equally possible, however, that they create a supersubstrate that is equipped to attract and orient the enzyme by electrostatic or hydrogen bonding; or they may prevent the van der Waal's attraction that binds the enzyme to the non-activated substrate, but in the wrong orientation.

Cholesterol esters are found in the immature brain, but they almost disappear with the onset of myelination. The concentration of cholesterol esterase increases at this time (*5*); this may entail an increased turnover of the ester, but hardly its total disappearance. It seems more likely that during myelination the substrate is incorporated into the matrix of the newly formed polar lipids and can then be approached and attacked by the enzyme.

Experts will easily find flaws and inconsistencies in the models I have presented; but to criticize the models on this account would be to miss their purpose. They are not intended to explain areas of cellular metabolism; they are hypothetical illustrations of the possibility of metabolic regulation by enzyme orientation and supersubstrate binding.

One of my more drastic oversimplifications deserves comment; it concerns the electric properties of an interface. A negative surface charge of a micelle or a membrane is not an isolated phenomenon. Positive counter ions are attracted from the aqueous phase, and the layer next to the surface will, in fact, have a charge opposite to that of the surface. A reverse cationic gradient extends into the aqueous environment. The depth of the field depends on the ionic strength of the solution. The cationic gradient is accompanied by a pH gradient (*6*). Such gradients will influence the orientation of enzymes (*7*). The effect of salt concentrations on the gradients as well as on the activity of many enzymes suggests that these phenomena may often be interconnected, i.e., that salts influence supersubstrate binding rather than substrate binding or the catalytic process. The fact that the electric environment of interfaces is more complicated than

assumed in the models does not, of course, argue against the concept of electrostatic enzyme–supersubstrate interaction.

PROOF AND PROSPECTS

How can supersubstrate binding and enzyme orientation be verified experimentally? The separate existence of substrate binding and supersubstrate binding can be investigated by kinetic measurements of enzymic reactions, most simply by measuring the Michaelis constant, K_m. This constant, though not usually identical with the enzyme–substrate dissociation constant, is nevertheless a measure of enzyme–substrate affinity: among structurally related substrates, those which are more tightly bound to the enzyme have the smaller K_m; binding strength depends on geometrical and electrostatic fit. It has been realized for some time that the K_m of lipolytic reactions must have a different meaning. Dixon and Webb (11) suggested that this apparent "K_m" is the interface–enzyme dissociation constant. Verger, Mieras and de Haas (21) recently proposed that "K_m" is a complex constant containing both enzyme-interface and enzyme-substrate affinity factors. In any case, if it can be shown that "K_m" depends on the nature of the supersubstrate rather than on that of the substrate, enzyme–supersubstrate binding must be inferred. Experiments might show, for instance, that substrates with very different sterical structures but imbedded in membranes of equal physical properties have the same "K_m" in an enyzmic reaction. Conversely, the "K_m" for the same substrate might be changed by changing the nature of the matrix.

Lipolytic enzymes also have the ability to hydrolyze water-soluble esters, although at rather low rates, and for such substrates a "true" K_m can be determined. If enzymic affinity could be measured against such a substrate in monomolecular dispersion, and then against the same substrate imbedded in a surface, the supersubstrate character of the surface could be assessed. The "true" K_m of imbedded substrates that cannot be properly dispersed could perhaps also be obtained by extrapolation from the K_m of water-soluble analogues, just as the "true" dissociation constants of long-chain, water insoluble fatty acids can be estimated from those of their short-chain relatives.

When the three-dimensional structure of an enzyme is known it becomes possible to search for supersubstrate binding and orientation sites on its surface. However, the enzymes whose complete structures are presently known, mostly digestive enzymes, are not likely to make use of orientative mechanisms. The surface structures of lysozyme (*14*) and carboxypeptidase (*12*) which allow them to bind several residues of their polymeric substrates must not be confused with supersubstrate binding sites: they are substrate binding sites proper. The carbohydrate residues of digestive ribonucleases, which are remote from the active site (*15*), may be "tails" that serve the purpose of orientation but not regulation; it is not probable that on-off mechanisms would be employed during digestion. Regulatory attachment and orientation sites will more likely be discovered in larger, more complicated, intracellular enzymic proteins.

Eventually it may be possible to make orientation effects visible. An enzyme with a diameter of 50 Å would be large enough for electron microscopy to distinguish its "head" and "tail" if these sites could be properly fixed or labeled. If the different positions that the enzyme molecule assumes towards different supersubstrate surfaces

could be "frozen," the correlation between enzymic activity and orientation could be measured.

As for practical applications (which must be provided in these times), there is the possibility, remote but not improbable, of controlling metabolic processes—and metabolic diseases—by manipulating the orientative features of enzymes or the surface properties of supersubstrates. Enzymes could be modified by introducing charged or hydrophobic substituents, supersubstrates by incorporating drugs. As examples, control of fat digestion and mobilization, arrestation of demyelination, or attack of atherosclerotic deposits come to mind. Far away as such targets might seem, they would appear in closer reach than manipulations of the reactive or allosteric sites of enzymes.

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