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Structural and Functional Aspects of Enzyme Catalysis

Edited by
H. Eggerer and R. Huber

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With 116 Figures

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Preface[1]

Enzymes perform the executive role in growth, energy conversion, and repair of a living organism. Their activity is adjusted to their environment within the cell, being turned off, switched on, or finely tuned by specific metabolites according to demands at the physiological level.

Each enzyme discovered in the long history of enzymology has revealed its own individuality. Even closely related members of a family differ in specificity, stability or regulatory properties. Despite these, at first sight overwhelming aspects of individuality, common factors of enzymic reactions have been recognized.

Enzymes are stereospecific catalysts even when a nonspecific process would yield the same product. Knowledge of the detailed stereochemistry of an enzymic reaction helps to deduce reaction mechanisms and to obtain insight into the specific binding of substrates at the active site. This binding close to catalytically competent groups is related to the enormous speed of enzyme-catalyzed reactions. The physical basis of rate-enhancement is understood in principle and further exploited in the design of small organic receptor molecules as model enzymes. These aspects of enzyme catalysis are discussed in Session 1.

Session 2 emphasizes the dynamic aspects of enzyme substrate interaction. Substrate must diffuse from solution space to the enzyme's surface. This process is influenced and can be greatly facilitated by certain electrostatic propterties of enzymes. The dynamic events during catalysis are studied by relaxation kinetics or NMR techniques. The use of low temperatures to stabilize intermediates for structural studies has added substantially to our understanding of enzymatic reactions.

Metals as cofactors often play an important role at the catalytic center of enzymes. The trace element selenium occurs as selenocysteine or its oxidation products at the catalytic site of a number of enzymes. The stability of the higher oxidation states of selenium compared to sulfur may be advantageous. Metals are also powerful polarizing elements and can provide convenient spectroscopic probes to study the reaction pathway. Functions of metals in enzymes are the subject of Session 3.

Session 4 deals with modifications of proteins which alter their functional properties. Enzymes in thermophilic microorganisms are closely related in structure and function to the homologous enzymes from organisms adapted to less extreme conditions. Thermostability and optimal function at elevated temperatures are brought about by a few amino acid exchanges. Some common structural principles of thermostable proteins are recognized.

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[1] This volume is dedicated to the memory of Feodor Lynen (1911-1979), who would have celebrated his 70th birthday at the time the meeting was held

The specificity of enzymic reactions is used to advantage in the design of suicide inhibitors, which are transformed on the target enzyme from harmless substrate analogs into powerful irreversible inhibitors.

In some protein families nature provides enormous numbers of variants whose structural and functional properties can be studied and correlated with the amino acid substitutions. In the ovomucoid family it has been possible to map the influence of single residue exchanges at many sites of the molecule.

Enzyme interconversion by phosphorylation and dephosphorylation plays a dominant role in the regulation of glycogen metabolism and hormonal stimulation.

The last session represents a vivid account of enzymes viewed at the laboratory bench. Nature experimented in substituting a complicated cofactor for a much simpler compound. The elucidation of how this functional group is generated represents a fascinating chapter of enzymology. That pursuing a simple chemical question of substrate chemistry can yield amazing new insights into enzyme action is demonstrated in another example. Transport catalysis finally is shown to be related to enzyme catalysis.

Mosbach Colloquia are intended to acquaint the participants with the current state of knowledge within a field of biological chemistry. Enzymology has many facets, ranging from physiology to quantum chemistry. Almost any method or tool of modern biochemistry or biophysics may be used or has been used to elucidate structural and functional properties of enzymes.

We therefore faced the problem of finding a compromise between a scientific program emphasizing a particular, narrow aspect of enzymology or presenting a broad general survey of methods and results.

We hope we have found the appropriate mixture of presentations reflecting the constant refinement of our understanding of established fields and some of the fascinating new developments in enzymology. The cooperation of speakers and chairmen in this respect is gratefully acknowledged.

We wish to thank also the Gesellschaft für Biologische Chemie and its President, Prof. H.G. Wittmann, for giving us the opportunity to organize this colloquium, and Professors Auhagen and Gibian and their coworkers who had the burden of the technical organization of this meeting. The assistance of the Springer-Verlag, which allows publication of the proceedings of the Mosbach Colloquium 1981, is gratefully acknowledged.

October, 1981

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Contributors

You will find the addresses at the beginning of the respective contribution

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Mechanism of Enzyme Action

Stereochemistry in Relation to Enzyme Mechanism

J. W. Cornforth[1]

Stereochemistry was a part of enzymology long before anything was known about the chemical nature of enzymes. Stereochemistry was born in 1874, enzymes were christened in 1877; and Emil Fischer, born in 1852, was of an age to take notice of both events. When Fischer studied the action of crude ferments from seeds on derivatives of the sugars, he did not even know the correct structures of his substrates, let alone of his enzymes; but he knew which of his substrates were stereoisomers, and he could formulate his lock-and-key model of enzyme-substrate interaction as a purely stereochemical hypothesis. It is important to recognize that this hypothesis was confined to the problem of substrate specificity in enzymes. At that time (1894) there was already a secure stereochemical theory of the *structures* of organic molecules, a theory to which X-ray crystallography brought confirmation rather than correction; but the stereochemistry of chemical *reactions* was largely unknown territory. Paul Walden was doing his work on stereochemical inversion, but for four decades it was considered a curiosity outside the main stream of chemistry. Alexander McKenzie had started his work on "asymmetric syntheses", but the stereochemical basis was supplied by Prelog more than half a century later.

The picture began to change in the 1930's. Then, recognition of the Walden inversion as the normal process of bimolecular nucleophilic displacement drew much attention to the stereochemistry of chemical reactions in general. It happened also about that time that the steroids became objects of intensive research because of the biological activities of some of them. Many transformations in this semi-rigid skeleton of carbon atoms were effected, and stereochemical control was often imperative. This work led eventually in Barton's hands to the theory of conformational analysis, which is just as much a theory of reactivity as of structure. The importance of geometrical arrangement in accelerating chemical reactions was emphasized.

On the biochemical side, the late 1920's and early 1930's saw the first crystallizations of enzymes and the demonstration that the catalytic activity resided in a protein molecule. In the same period, the separation of deuterium by the late Harold Urey began to liberate biochemists from the dilemma that to study a living system you must often begin by killing it.

Chemists were not slow to use isotopes to investigate reaction mechanisms, and indeed the most elegant demonstration of the Walden inversion used radioactive iodide in combination with polarimetry to show that every exchange of iodine between 2-iodo-octane and iodide ion was an act of stereochemical inversion. Biochemists at first tended to use isotopes incorporated in simple chemical compounds to trace biosyntheses in living organisms, and this technique is still much

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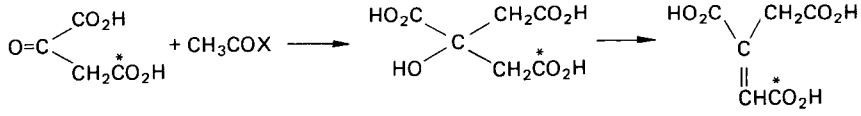
used today. But application of isotopic techniques to isolated organs, tissue slices and homogenates, cell-free systems and purified enzymes was not long delayed; and when this happened it soon produced an apparent paradox. The resolution of this paradox made stereochemistry of central importance to the study of enzyme mechanism.

The phenomenon now known as the Ogston effect could have been predicted from existing information long before 1948, and indeed before isotopes were discovered. For this omission the blame must be laid squarely on organic chemists, and it is instructive to ask why they not only failed to anticipate the phenomenon but were slow to explain it.

I think that the trouble lay, and still lies, in the ways chemists use to impart information about molecules. How liberated I should feel if I were able to show three-dimensional moving representations of my molecular subjects in such a way that the reader could take in their chemical and stereochemical nature and behaviour at the same time. But this would at present be prohibitively costly, and when this chapter is published I shall have to deal with my molecular representations as chemists have done for a century and more: that is, hammer them flat and show the result from one side only. This true on paper, on the blackboard, or on a projected slide. It is an expensive business to impart even the semblance of three dimensions to a two-dimensional projection, and it is a slow business to construct and transform three-dimensional models. A good visual imagination can help to remedy these defects, but the sad truth is that we still learn most of our chemistry in Flatland, to the detriment of our science. Adolf von Baeyer's strain theory, which predicted that rings became progressively less stable as they departed in either direction from a five-membered ideal, was a classic example of Flatland thinking.

So a 1948 organic chemist, making a two-dimensional representation of oxaloacetate and its enzymic reaction with "active acetate", as it was known before 1948, to form citrate, then dehydrated by a second enzyme to aconitate, would write something like this, without the asterisks:



And if a biochemist then told him that this reaction, when carried out with oxaloacetate isotopically labelled at *C, gives citrate in which the acetic acid chain carrying the label participates in the enzymic dehydration to the complete exclusion of the other chain, his first reaction would be disbelief.

The picture in three dimensions is quite different. Making no assumptions about the mechanism of citrate synthesis, we still know that a new C-C bond is formed between the carbonyl carbon of oxaloacetate and the methyl carbon of "active acetate". No matter what chemical mechanism is chosen for the condensation, there are two different ways of writing it, depending on the relative orientations of the two substrates. In Fig. 1a the new bond is formed on what is now called the re face of the oxaloacetate carbonyl: that is, the face showing oxygen, carboxyl and carboxymethyl groups in clockwise order around the carbon. In the alternative, (Fig. 1b), now known to be correct, the order is anticlockwise. The point is that these two arrangements are spatially not the same. In a statistically symmetrical medium like liquid water, there is no reason why reaction should occur more readily by

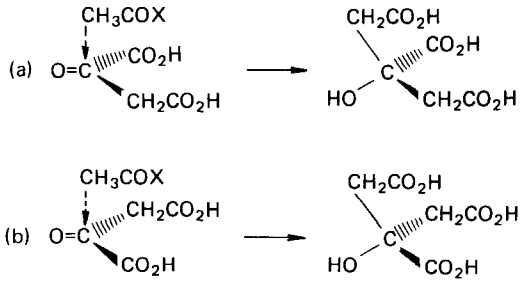


Fig. 1a,b. Topology in carbon-carbon bond formation on citrate synthetase

one stereochemical path rather than the other (if we neglect a small influence that might be exercised by asymmetry in the group X). But this reaction occurs at the active site of an enzyme, and an enzyme is an irregular assembly of chiral amino acids. There is no reason a priori to suppose that it can accommodate the catalysis equally as well when the stereochemical arrangement is (a) as when it is (b). And if it imposes one of these arrangements, then the carboxymethyl group derived from oxaloacetate has in citric acid a particular spatial position that is the same for every molecule and different from that of the carboxymethyl group derived from "active acetate".

In the dehydration to aconitate, the lost water comes from the tertiary hydroxyl group and one hydrogen from one of the acetic acid chains. Once again, if the enzyme imposes a particular spatial arrangement of the citric acid molecule for catalysis of this reaction, there is no possibility that the two acetic acid chains will be used indifferently: only one of them, spatially the same one in every molecule transformed, will be used, and that one happens to be the chain which, because of the stereospecificity of citrate synthase, originates from oxaloacetate.

These two specificities by no means exhaust the stereochemistry of the enzymic synthesis of aconitate from oxaloacetate via citrate. Aconitate formed in this way has a specific cis geometry of the double bond, as shown. The generation of this double bond is a stereospecific anti elimination which means that the pro-S hydrogen is specifically removed. Finally, in the formation of citrate it is known that the C-C bond is formed with inversion of configuration at the methyl group. The whole process is shown in Fig. 2.

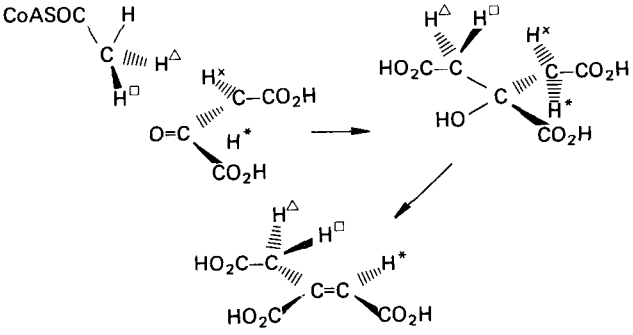


Fig. 2. Detailed stereochemistry of citrate and aconitate formation

The stereochemical information embodied in Fig. 2 was obtained in a number of ways, but in general they depend on the principle of prochirality. The molecule of citric acid, for example, is not chiral, but if one of its two carboxymethyl groups is altered in any way at all, a chiral molecule is formed. Usually the alteration is made by replacing one or more of the atoms of this group by an isotopic species. If the atom in question happens to be one of the hydrogens forming one of the two methylene groups, then this group becomes a centre of chirality in addition to the central carbon atom: four different stereoisomers can be formed in this way.

This artificial chirality is introduced so that an observer can follow the fate of the isotope when the labelled molecule is a substrate in a subsequent enzymic reaction. The chirally labelled molecules are occasionally produced by total chemical synthesis using reactions of known specificity; but as information about the stereochemical course of more and more enzymic reactions accumulates it has become more customary to use such reactions for producing substrates of known chirality. Often, the chirality-generating enzymic reaction forms part of a chemical synthesis by which the labelled substrate is finally produced.

For tracing the chemical and stereochemical fate of the labelling isotope, after the enzymic reaction being studied, several methods are available. If the reaction is an elimination which may or may not remove the isotopic atom, any method that can show the isotope's presence or absence in a product will suffice. If the chirality of an isotopically labelled product must be determined, one may similarly use known stereospecific enzymic (or chemical) reactions in which the isotope is eliminated or retained according to its chirality. The classical method of correlating absolute configuration with optical rotations has been very useful for deuterium-labelled compounds: we, and then others, have used the enantiomers of monodeuteriosuccinic acid (Fig. 3), in conjunction with sensitive spectropolarimeters, to identify the absolute configuration at any monodeuteriated methylene group that can

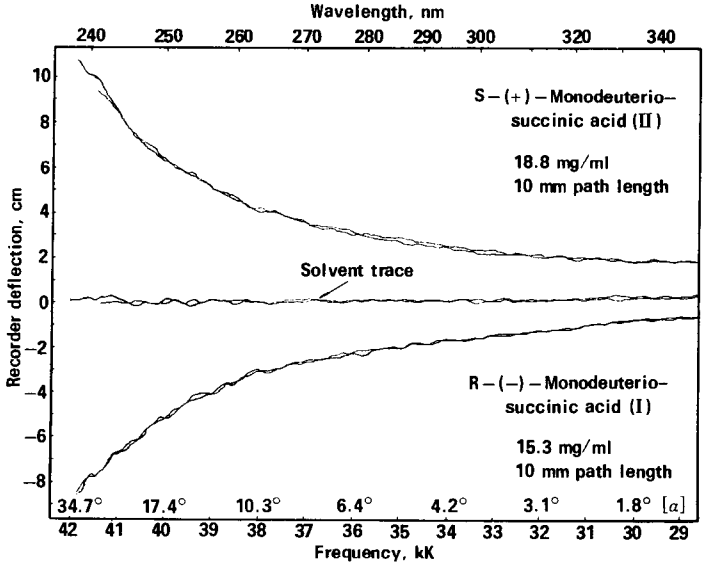


Fig. 3. Optical rotatory dispersion of (2R)- and (2S)deuteriosuccinic acids

be brought into this chemical form. Another method available for determination of this type of chirality is neutron diffraction crystallography utilizing the Bijvoet effect; so far, only one structure, monodeuterioglycollic acid, has been solved in this way but I think there will be more. With advances in the power and sensitivity of NMR spectrometers and the development of NMR techniques for inspecting ^{2}H and ^{3}H nuclei in a molecular environment using diluted samples of reasonable size, stereochemical problems involving these isotopic species can sometimes be solved very easily.

When the enzymic reaction being studied results in the transformation of a methyl group, the problem of chiral isotopic labelling is more complex. No methyl group is prochiral, but all methyl groups are pro-prochiral: if two of the hydrogen atoms are replaced by two different isotopic species a chiral methyl group (Fig. 4) is generated, and fortunately hydrogen has three isotopes. The problem of preparing chiral methyl groups of known absolute configuration conveniently incorporated in molecules of acetic acid, was solved some 13 years ago. It can be done by purely chemical means or by a combination of chemical and enzymic methods. Because of the practical difficulties in preparing and handling undiluted tritium, these specimens contain very small proportions of chiral acetic acid along with a much larger proportion of monodeuteriated acetic acid. The practical problems were to ensure that all tritium-containing molecules in a specimen had the same chirality, and to devise an assay of chirality that should be sensitive to the very small proportion of chiral molecules present. These problems were surmounted and it is now even possible, thanks to Professor Eggerer, to determine, with fair accuracy, the optical purity of any chiral methyl group that has been brought into the form of acetic acid.

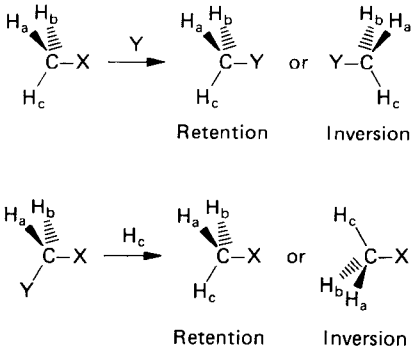


Fig. 4. Transmethylation of chiral methyl group. Generation of chiral methyl from chiral methylene

When a methyl group is transferred intact to another molecule, or to another position in the same molecule, by a stereospecific enzymic process, or when a methyl group is generated in a stereospecific enzymic reaction from a methylene group, isotopic labelling can be arranged so that a chiral methyl group is produced; and if this group can be brought without racemization into the chemical form of acetic acid the chirality (rectus or sinister) and optical purity can be determined by assay. The stereochemistry of the enzymic process is then known absolutely. When a methyl group is enzymically transformed, by replacement of one of its hydrogens, into a methylene group (Fig. 5) the situation is different. The enzymic process may be stereospecific and usually it is, in the sense that there is a definite relationship between the hydrogen atom that is displaced and the atom or group replacing it.

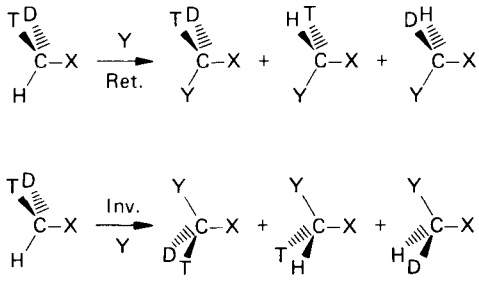


Fig. 5. Transformations of a chiral methyl group with replacement of one hydrogen

But if the enzymic reaction is not reversible, the relationship can be shown only by studying the reaction with a substrate having a chiral methyl group of known absolute configuration. The hydrogen removed from this methyl group may then be protium, deuterium or tritium, and the relative proportions of each isotopic species removed depend on the kinetic isotope effects for that particular enzymic reaction. In practice a mixture of methylene compounds is always produced, but in a stereospecific reaction only two molecular species contain tritium. One of these also contains deuterium, the other protium; and the configuration of the tritium atom is always different in the two species. The ratio of the abundances of these two species is equal to the kinetic intramolecular deuterium isotope effect for the reaction. The configuration in the two species is determined by the stereochemistry of the reaction.

The reaction of this type which was originally used for assay of chirality in methyl groups, and which has been used ever since, is the irreversible condensation of glyoxylate with acetyl-coenzyme A on malate synthase (Fig. 6). This produces the species (Fig. 6a) and (Fig. 6b) a ratio between three and four to one, so that when the total product, S-malate, is submitted to the action of fumarate hydratase, an enzyme which specifically exchanges the 3R hydrogen of malate with the medium by reversible dehydration to fumarate, 76%-80% of the tritium in malate is lost if the precursor was 3S-acetate and 76%-80% is retained if the precursor was 3R-acetate.

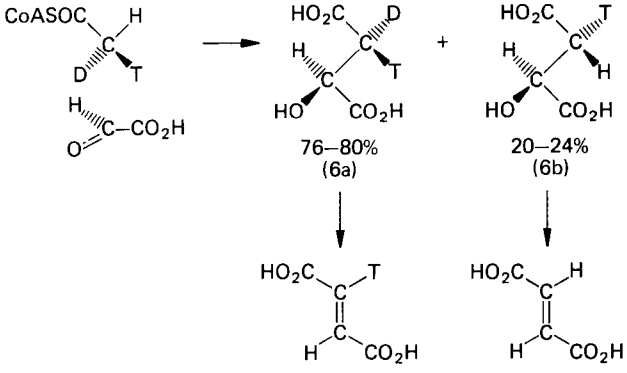


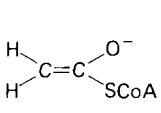
Fig. 6. Condensation on malate synthetase of (R)-acetyl-coenzyme A

This experiment was originally carried out with acetates of known absolute configuration and as an assay of configuration it does not de-

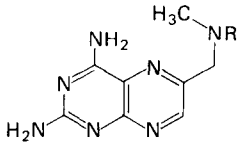
pend on any assumptions about what is happening on the enzymes. Given the known stereochemistry of the fumarate hydratase reaction, one can deduce from the experiment that the reaction on malate synthetase proceeds with inversion of configuration at the methyl group *provided that the intramolecular* k_H/k_D *for this reaction is 3-4*. Because it was later possible in this case to apply to the malate an enzymic sequence of known stereochemistry regenerating chiral acetate, which was assayed in its turn, we know that there is a normal isotope effect of this magnitude. But the logical proviso remains for every enzyme investigated by this type of experiment. More recently I have devised a general procedure to make the deduction of stereochemistry independent of the kinetic isotope effect on the enzyme, by indicating which of the two tritiated species (Fig. 6a and b) contains deuterium as well as tritium.

The chiral methyl group has been used to investigate the substrate stereochemistry of dozens of enzymes [1] and dozens more have been examined with the help of chiral methylene groups. What is the significance of the information gained? As an example, we may look again at the reaction on malate synthetase. Three events are noticeable in this condensation: removal of a proton from the methyl group, formation of a new C-C bond, and hydrolysis of thiolester. A concerted reaction combining the first two steps would be an improbable event in the absence of enzyme: formally, it would be an unprecedented bimolecular electrophilic substitution with inversion of configuration. Chemically, the condensation is of the Claisen type, where a hydrogen α to a carbonyl group is ionized to form an enol or enolate anion which then participates in nucleophilic attack on a second carbonyl group. In non-enzymic chemistry these two processes are separate.

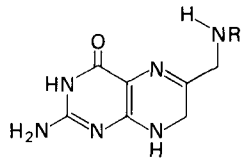
Are they separate on the enzyme? This is quite possible: an enolate (Fig. 7a) formed from acetyl-coenzyme A has two topologically different sides to the new carbon-carbon double bond and it could react stereospecifically with glyoxylate to give the observed stereochemical result even after movement on the active site. On the other hand it is quite possible that a single conformational change of the enzyme-substrate complex could facilitate both the removal of the proton and the approach of the developing sp_2 carbon to the carbonyl group of glyoxylate: this would be virtually a concerted reaction. The stereochemical information does not distinguish between these alternatives: it is a statement about the relative geometry of proton abstraction and carbon-carbon bond formation. But this information does impose a limitation on the number of mechanisms that can be constructed.



(7a)



(7b)



(7c)

R = $-C_6H_4-CONHCHCH_2CH_2CO_2H$
CO_2H

Fig. 7a-c

An interesting example is provided by work on the enzyme dihydrofolate reductase from *L. casei* (Fig. 7). The crystal structure of a ternary complex of this enzyme with its natural cofactor NADPH and the inhib-