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Kinetic Probes for the Stoichiometry and Reactivity of Thiol Groups in Enzymes

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The two salient features of enzymes are their ability to recognize specific substrates and the special reactivity of selected groups within the microenvironment of the catalytic site. These properties are likely to be connected, since the catalytic site is not complete until the reactive enzyme-substrate complex has been formed in response to substrate binding. The study of the reactivities of thiol groups of model compounds and enzymes with known three-dimensional structures offers the possibility of correlating reactivity with environment.

It will be illustrated with results from studies on papain, aldolase, haemoglobin, triose phosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase that, in addition to the usual distinction of reactive and non-reactive thiol groups, there is a very wide range of reactivities. The role of different types of thiol groups and the information that can be obtained from precise stoichiometry will be discussed in terms of enzyme structure and catalysis.

Thiol-blocking reagents that react at different rates with a particular group on a protein will be compared. The advantage of using the very rapidly reacting chromophoric mercurials (McMurray & Trentham, 1969) with stopped-flow spectrophotometric techniques will be illustrated.

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The Thiol Groups of Aldolase

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The cysteine residues of rabbit muscle aldolase (EC 4.1.2.13) have been reported to be distributed unequally between two types of polypeptide chains (Chan, Morse & Horecker, 1967). However, recent evidence suggests that the subunits of aldolase are identical with respect to cysteine content (Eagles,

Johnson, Joynson, McMurray & Gutfreund, 1969; Anderson, Gibbons & Perham, 1969). The role of the cysteine residues in aldolase is also uncertain (Morse & Horecker, 1968).

Seven unique carboxymethylcysteine-containing peptides have now been isolated from tryptic digests of rabbit muscle aldolase carboxymethylated with iodo[2-¹⁴C]acetic acid in 8M-urea. These peptides have been characterized by amino acid and end-group analysis and their location within the polypeptide fragments produced by cyanogen bromide cleavage of the enzyme has been determined. The reaction of native aldolase with 5,5'-dithiobis-(2-nitrobenzoic acid), iodoacetamide and N-ethylmaleimide showed that a total of three cysteine residues per subunit of mol.wt. 40 000 were reactive towards these reagents, and that the modification of these three residues was accompanied by a loss in enzymic activity. Chemical analysis of the modified enzymes demonstrated that the same three thiol groups are involved in the reaction with all these reagents, but that the observed reactivity of a given thiol group varies with the reagent used. Thus one group reacted rapidly and two slowly with 5,5'-dithiobis-(2-nitrobenzoic acid), whereas two groups reacted rapidly and one slowly with iodoacetamide.

One reactive thiol group per subunit could be protected when the modification of the enzyme was carried out in the presence of substrate, fructose 1,6-diphosphate, under which conditions enzymic activity was retained. This thiol group has been identified chemically and, presumably, is at or near the active site. Similarly, limitation of the exposure of the native enzyme to iodoacetamide served to restrict alkylation to two thiol groups and left the enzymic activity unimpaired. The thiol group left unmodified is the same as that protected by substrate during more rigorous alkylation. However, it is now more reactive towards 5,5'-dithiobis-(2-nitrobenzoic acid) than in the native enzyme, suggesting some small conformational change has occurred in the modified protein. It is worth noting that preincubation of the enzyme with fructose 1,6-diphosphate, which was subsequently removed by dialysis, caused an irreversible fall in thiol-group reactivity measured in the 5,5'-dithiobis-(2-nitrobenzoic acid) reaction.

We conclude that the aldolase tetramer contains at least 28 cysteine residues. Each subunit appears to be identical with respect to number, location and reactivity of thiol groups.

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Imidazole and Proton Transfer in Catalysis

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The role of imidazole as a catalyst for group transfer by nucleophilic attack and proton transfer in chemical and enzymic reactions will be reviewed. There is strong evidence that a single imidazole group is involved in catalysis by some (but not all) 'serine' acylhydrolases, particularly chymotrypsin. The reasons for the special effectiveness of imidazole as a nucleophilic catalyst include its tertiary amine, cyclic structure and the possibility of resonance interaction between the imidazole ring and the carbonyl group in the transition state and product of nucleophilic attack; the acetylimidazole product is highly reactive because of the absence of normal amide resonance stabilization and the ease of protonation to the acetylimidazolium ion. Comparison with reactions of 1-acetyl-3-methylimidazolium ion demonstrates that most reactions of acetylimidazole with nucleophiles proceed through the *N*-protonated species. However, in spite of these attractive properties there is no firm evidence that imidazole is utilized by enzymes as a nucleophilic catalyst for acyl transfer; it is a nucleophilic catalyst for enzyme-catalysed phosphoryl transfer, but it does not show unusual reactivity as a non-enzymic catalyst for phosphoryl transfer.

Imidazole is also an effective catalyst for proton transfer in acyl transfer reactions, as shown most directly by its catalysis of hydrolysis and transfer reactions of acetylimidazole. Some progress towards understanding the detailed mechanism of this catalysis has been made by comparison with reactions of 1-acetyl-3-methylimidazolium ion catalysed by methylimidazole. The fact that catalysis of the hydrolysis of this compound is observed means that catalysis involves proton transfer to or from the carbonyl or water oxygen atoms. The absence of catalysis for the reaction with ammonia and the fact that catalysis does occur in the imidazole-catalysed reaction with acetyl-imidazole suggest that catalysis of the latter reaction involves proton donation by imidazolium ion to the leaving imidazole group (which is not possible in the acetylimidazolium ion reaction). The importance of proton transfer for these reactions is demonstrated by the very large sensitivity of the reactions of (unprotonated) acetylimidazole to the

basicity of the nucleophile and the fact that many reactions of acetylimidazole with nucleophiles are, in fact, general-base catalysis of hydrolysis rather than acyl transfer to the nucleophile. Solvent deuterium isotope effects and structure-reactivity correlations provide some evidence that imidazole acts as a catalyst for proton transfer in enzymic reactions. Teleologically it is not unreasonable that it should act as a general acid-base catalyst, rather than a nucleophilic catalyst, because its efficacy as a nucleophilic catalyst is low for normal substrates with poor leaving groups, whereas it is an effective general-base catalyst for such reactions.

The detailed mechanism of proton transfer in general acid-base catalysis of acyl- and carbonyl-group reactions is unknown. The proton transfer in most such reactions is in some sense 'concerted', but there are examples of apparent stepwise proton transfer, in which a diffusion-controlled proton-transfer step is rate-determining. Both mechanisms provide attractive methods for catalysis by enzymes, since enzymes cannot bring about local increases in H^+ or OH^- ion activity to facilitate required proton-transfer steps.

Imidazole Catalysis in Model Reactions for 'Serine' Peptidase and 'Cysteine' Peptidase Activities

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Acyl-group transfer from *N*-acetyl-*O*-formyl-serine amide to glycine amide is catalysed by imidazole. The transition state for the reaction is closely related to that for hydrolysis of a glycine peptide by a serine peptidase, such as chymotrypsin, and the catalytic activity of imidazole therein can be assigned without ambiguity.

Imidazole accelerates the transfer of acyl groups from thiol esters to amines, such as glycine ester, by means of both general-acid and general-base catalysis at physiological pH values. These intermolecular reactions are more complicated than those involving oxygen esters, since some of the proton-transfer steps approach in rate that of a diffusion-controlled reaction (Barnett & Jencks, 1969) and may become rate-limiting. Nevertheless it is possible to assign catalytic roles to imidazole in the transition states for acyl-group transfer from sulphur to nitrogen. These provide a chemical analogy for the mechanism of thiolysis of amides by cysteine peptidases, in which the catalysis is basically different from that projected for serine peptidases. It will be shown that similar juxtaposition of functional groups in the active sites of enzymes, as shown by X-ray crystallography, need