No kinetic isotope effect was found in studies with deuterated malonyl-CoA as substrate (K. I. Arnstadt, unpublished work), indicating that CO₂ and the thiol (HSR) are eliminated in a concerted process.

The effects of thiol-blocking agents on the enzyme system involved in fatty acid synthesis will be discussed with particular emphasis to the participation of neighbouring groups of the enzyme in the condensation reaction.

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Selective Reactivity of Thiol Groups in Proteins

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The reactivity of the same functional groups in different proteins is known to vary to a great extent. The rates of alkylation of essential thiol groups in several enzymes range over five orders of magnitude (Eisele, 1967). In addition to the fact that groups can be masked by parts of the protein chains, these enormous differences may be due to activating or deactivating effects of neighbouring functional groups in suitable steric positions.

A simple model of a thiol group influenced by a neighbouring group is cysteine. The thermodynamic and kinetic parameters characterizing dissociation behaviour and nucleophilicity suggest a strong interaction between the thiol and amino groups of cysteine. Such a chemical system may be called a combined functional group (Wallenfels & Streffer, 1966).

An even higher order of combined functionality may be obtained with at least two groups differently influencing the reaction rate of a thiol group. In this case a centre of chirality with respect to the chemical reactions of the thiol group is formed. Protein thiol groups that are strongly influenced by neighbouring groups show high stereoselective preference for asymmetric reagents (Eisele, 1967; Wallenfels & Eisele, 1968; Eisele & Wallenfels, 1968a,b).

The thiol group of papain reacts with L(-)- α -iodopropionic acid 12 times as fast as with the D(+)-isomer. The reaction with α -iodopropionic acid is about 1000-fold faster with papain than with the thiol group of cysteine. The reaction rate with α -iodopropionamide with papain is only about 10 times that with cysteine. The stereochemistry of the reaction with the amide is opposite to that of the acid: D(+)- α -iodopropionamide reacts 4 times as fast as the L(-)-isomer. The alkylation data and their pH-dependence led to the hypothesis that a histidyl and a carboxyl residue influence the thiol group. As the absolute configuration of the asymmetric reagents is known, the chiral arrangement of the groups in the transition state of the alkylation reactions could be reconstructed (Wallenfels & Eisele, 1968). Independent X-ray work by Drenth, Jansonius, Koekoek, Swen & Wolters (1968) confirmed the proposed structure.

If the steric arrangement of groups in the neighbourhood of an active-centre thiol group is altered by a conformational change, the reactivity as well as the stereoselectivity of the thiol group will change. Conformational changes in the area of the active centre may be monitored by observation of changes in reactivity and/or stereoselectivity.

Glyceraldehyde phosphate dehydrogenase from yeast and from rabbit muscle are known to undergo conformational changes on addition of the ligand NAD⁺ (Kirschner, Eigen, Bittmann & Voigt, 1966; Conway & Koshland, 1968). The apoenzymes from both origins react faster with the D(+)-isomer of the free α -iodopropionic acid and the L(-)-isomer of the amide. On addition of NAD⁺ the stereoselectivity changes, now preferring the L(-)-isomer.

The results with the yeast enzyme could be interpreted in terms of the allosteric model of Monod, Wyman & Changeux (1965): the T state of the enzyme reacts most rapidly with the D(+)-isomers, whereas in the R state the L(-)-isomers of the reagents are preferred. The dependence of the reaction rates on the NAD⁺ concentration could be fitted to the allosteric function of state $\bar{R}$ (Eisele & Wallenfels, 1968b).

With the muscle enzyme the stereoselectivity for the L(-)-isomers is a discontinuous function of the NAD⁺ concentration: it is decreased by the first molecule of NAD⁺ bound to the tetrameric enzyme and then increases stepwise with the binding of three further NAD⁺ molecules. These results may easily be explained by sequential conformational changes induced by NAD⁺ binding as proposed by Conway & Koshland (1968).

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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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