of activity by the product provides strong support for the crystallographic hypothesis of the activity of lysozyme.

Selective Photo-oxidation of Amino Acids in Proteins

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The photoactivation of oxygen, mediated by sensitizers, produces a reagent capable of modifying the methionyl, cysteinyl, histidyl, tyrosyl and tryptophyl side chains in proteins (Spikes & Straight, 1967). The photo-oxidative attack can be directed towards specific residues by careful control of the reaction conditions. This can be achieved by several approaches.

First, a high selectivity can be obtained by a suitable choice of the sensitizer. For example, in the presence of haematoporphyrin, methionine is specifically photo-oxidized to the sulphoxide both in neutral aqueous solution and in acetic acid solution (Jori, Galiazzo & Scoffone, 1969). On the other hand, proflavine can be made to act selectively on tryptophan by irradiation in aqueous formic acid (Benassi, Scoffone, Galiazzo & Jori, 1967; Galiazzo, Jori & Scoffone, 1968); the competing photo-oxidation of methionine can be overcome by chemical reduction of the sulphoxide with thiols (Jori, Galiazzo, Marzotto & Scoffone, 1968a,b). Finally, Crystal Violet sensitizes the selective photo-oxidation of cysteine to cysteic acid over the complete pH range and in acetic acid solution (Jori, Galiazzo & Scoffone, 1969b).

Secondly, the photo-oxidation can be rendered more selective by taking advantage of the shielding effect exerted by the protein conformation on some of the susceptible residues. A method for differentiating the exposed from the buried residues by means of dye-sensitized photo-oxidation was developed by Ray & Koshland (1962). Their procedure was refined by us in order to detect the presence of partially exposed residues (Jori, Galiazzo & Scoffone, 1970b). The method involves the induction of a multi-stage unfolding of protein molecules by the stepwise addition of a suitable denaturant: in this way a gradually increasing number of residues become susceptible of photo-oxidation, first the fully exposed ones, then the partially buried ones and finally the deeply buried ones. This approach was applied to the elucidation of the degree of exposure of the methionyl residues in lysozyme (Jori *et al.* 1969a; Galiazzo, Tamburro & Jori, 1970) and in ribonuclease A.

Finally, the photoreaction can be confined to predetermined regions of a protein molecule by using a protein-bound sensitizer. Owing to the

short lifetime of the intermediate photoexcited species, only those photo-oxidizable residues that are close to the sensitizer are modified. In this connexion, one can utilize the enhanced reactivity displayed by functional groups in the active site of many enzymes to label them in a specific fashion. Both some natural coenzymes, such as the porphyrins or pyridoxal phosphate, and many group-specific reagents, containing nitro or keto groups, are efficient sensitizers for the photo-oxidation of amino acids (Jori *et al.* 1970b). The validity of this procedure was successfully tested with ferricytochrome *c* (Jori, Galiazzo, Gennari & Scoffone, 1970a), as well as with 41-DNP-ribonuclease A (Scoffone, Galiazzo & Jori, 1970) and *S*-DNP-papain. Therefore this novel strategy appears to provide a valuable tool for mapping the topology of proteins in solution.

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Pulse Radiolysis: An Oxidative-Reductive Probe in General Biochemical and Enzyme Studies

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Pulse radiolysis, the high-energy analogue of flash photolysis, provides a versatile high-speed technique for the study of electron transfer and other redox processes in general biochemical and enzymic systems. As in flash photolysis, changes in the optical absorption properties of a system are

measured as a function of wavelength, time and other parameters. In solution the initial perturbation arises from the absorption of a single pulse of high-energy radiation, which induces a large transient change in the redox properties of the solvent. In water the effect is due mainly to the formation of the strongly oxidizing hydroxyl radical and to the strongly reducing hydrated electron (e_{aq}^-).

The individual effects or reactions of these species can be studied in isolation by use of appropriate selective radical scavengers, i.e. (a) nitrous oxide, which converts e_{aq}^- into OH radicals, and (b) 2-methylpropan-2-ol, which reacts with OH to form a relatively unreactive β -hydroxyl radical.

Both e_{aq}^- and OH react rapidly with most amino acids, enzymes, nucleotides and various biochemical agents at, or near, diffusion-controlled rates. However, specificity of reaction of each species can be introduced by the use of certain solutes that convert e_{aq}^- or OH into radicals that then react only with certain molecular structures. Pulse radiolysis can therefore be used as a selective redox probe to locate sites of damage in biomolecules or multi-component systems. Further, the data, when combined with results from the appropriate bio-assay, can be used to obtain information on the importance or otherwise of such sites to biological activity.

Electron attachment to simple disulphides, e.g. cystine and GSSG, produces a transient species possessing a strong characteristic absorption maximum at 420nm (Adams, 1967). This is the radical ion $[RSSR]^-$ where the odd electron is located on the sulphur bridge. Reduction of sulphur bridges is observed after pulse radiolysis of lysozyme (Adams, Aldrich, Cundall & Willson, 1969; Davies, Ebert & Shalek, 1968), and similar data for ribonuclease, papain and α -chymotrypsin will be presented. Specificity of one-electron reduction of disulphide bridges with respect to these enzymes, as shown by the formation of $[RSSR]^-$, can be achieved by use of the reducing radical CO_2^- , which transfers an electron to lysozyme and papain but not to chymotrypsin.

Hydroxyl radicals attack enzymes at various sites, although the ring-sulphur-containing amino acids are particularly reactive. For lysozyme, though not necessarily for other enzymes, oxidative, rather than reductive radical attack at the sulphur bridges, appears to be the major mode of inactivation. Tryptophan residues are certainly involved. Specificity of reaction can be achieved by conversion of the OH radical into other oxidizing species, e.g. the halide or pseudo-halide radical ion X_2^- . Thiocyanate ion reacts with OH to form the radical anion CNS_2^- . This species reacts specifically with tryptophan residues in lysozyme, and this process

results in enzyme inactivation (Aldrich, Cundall, Adams & Willson, 1969). Since the radical absorbs strongly in the visible the reactions can be followed directly by pulse radiolysis. Other applications of this technique will be discussed.

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Elucidation of the Fine Structure of Large Molecules by Nuclear Magnetic Resonance

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Cobalt as a Functional Group in Enzymes

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The cobalt atom occurs as a functional group in enzymes that require derivatives of vitamin B₁₂, but it can also act as a functional group in two other types of enzyme by substituting cobalt for a required metal, e.g. in zinc-containing enzymes and in certain haem-containing systems.

In the vitamin B₁₂ derivatives cobalt is a low-spin ion and interest centres on the making and breaking of cobalt-carbon bonds. We shall present evidence that the intermediate in the reaction is the cobalt(II) state, which suggests that the mechanism of action of vitamin B₁₂ systems involves a radical step. The radical is detected by absorption spectroscopy and not by electron paramagnetic resonance, indicating that relaxation effects are dominant.

Replacement of zinc by high-spin cobalt(II) (an isomorphous replacement) in numerous enzymes where the metal acts as an acid catalyst makes the functional group a direct probe of its own environment. The application of this method by using parallel model studies will be discussed. An alternative replacement, that of haem by cobalt porphyrin, has corresponding advantages and we shall illustrate these by reference to studies of inter-molecular complexes.

The Contribution of Studies of the Reactivity of Protein Functional Groups to an Understanding of Enzymic Mechanisms

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