Hydroxylation: the activation of oxygen by peroxidase

Peroxidase ordinarily catalyses electron transfer from donors to hydrogen peroxide. The donors oxidized in this manner undergo free radical and related reactions, but hydroxylation of aromatic nuclei does not take place (see, for example[1,2,3]). We wish to report now a new type of peroxidase-catalysed reaction in which aerobic, non-specific aromatic hydroxylation occurs.

In solutions containing dihydroxyfumarate (Emol. = 8890, 292 mμ in H_2O; = 8350, 308 mμ in ether; 200 μmole), aromatic co-substrate (50–200 μmole) and horseradish peroxidase (0.2 mμ-mole)* dissolved in acetate or phosphate buffers (5.0 ml, pH 4.7–7.0, 0.06 *M* in deionized distilled water) and vigorously oxygenated at 25°, electrophilic hydroxylation is the principal reaction observed. Thus, tyrosine is formed from phenylalanine, 3,4-dihydroxyphenylalanine is formed from tyrosine and *m*-tyrosine, 4-methylcatechol is formed from *p*-cresol, *m*-hydroxybenzoic acid and gentisic acid are formed from benzoic acid, and gentisic acid is formed from *m*-hydroxybenzoic acid and salicylic acid. These products were identified by paper chromatography, using butanol, acetic acid and water (4:1:5), benzene, propionic acid and water (2:2:1), and appropriate identifying reactions (*cf.* [4]). Ascorbate cannot replace dihydroxyfumarate. Peroxidase cannot be effectively replaced by cytochrome *c*, by beef liver catalase, or by stroma-free horse red-cell hemolysate, but slow hydroxylation occurs in the absence of enzyme, presumably owing to heavy metal contaminant (*cf.* [5]). However, on a molar basis, peroxidase is at least 1000 times as effective as ferrous iron or hemin, and the kinetics of the rapid peroxidase-catalysed reaction are much different from those of the slow control hydroxylations. The enzymic reaction is inhibited by Mn^{++} (3 μmoles) and by catalase (0.4–3.1 mμmoles).

This hydroxylating system has been studied using salicylic acid as substrate and determining gentisic acid formed under varying conditions by a modification of the method of MITOMA[6] and by a spectrometric method employing differences in extinction coefficients of gentisic acid and salicylic acid at 280 mμ and at 340 mμ**. The initial rate of hydroxylation is high under the conditions described, 23% conversion occurring in the first 5 min. The yield increases slowly thereafter at pH 6, optimum for the reaction, but can then be raised by addition of more dihydroxyfumarate although not by addition of peroxidase. The rate of hydroxylation increases with increasing initial dihydroxyfumaric acid concentration, but increase of peroxidase concentration beyond that specified above results in a decrease. The rate is dependent upon oxygen partial pressure and upon thoroughness of oxygenation. It is about 5 times higher in pure oxygen than in air.

All peroxidases catalyse aerobic oxidation of dihydroxyfumarate[7], one molecule of oxygen being consumed per molecule of dihydroxyfumarate[8]. At temperatures higher than 4°, in the absence of Mn^{++}, Complex III of peroxidase predominates in the dihydroxyfumarate–oxygen system ([8]; *cf.* [9]), and the enzyme is inhibited by carbon monoxide, displaying the spectrum of carbon monoxide ferroperoxidase ([9]; *cf.* [8]). It is therefore reasonable that Complex III has an oxyhemoglobin type of structure[10,11] and that oxyferroperoxidase is involved in the enzymic reaction of dihydroxyfumarate with oxygen.

Our results suggest that a peroxidase complex, catalytic in the aerobic oxidation of dihydroxyfumarate, is also catalytic in coupled aerobic, non-specific hydroxylation of aromatic compounds. Since the hydroxylative attack is strongly directed towards electronegative positions, we propose that a positively-charged oxygen complex of peroxidase is the active intermediate. Oxyferroperoxidase or ferryl peroxidase ($Fe_p^{++}O$) formed from oxyferroperoxidase by a two-equivalent reduction, may react with aromatic nuclei in the following manner, dihydroxyfumarate being the electron donor:

$$Fe_p^{++}O_2 + ArH = Fe_p^{++}O + ArOH$$

or:

$$Fe_p^{++}O_2 + 2e = Fe_p^{++}O + O^{--}$$

$$Fe_p^{++}O + ArH = Fe_p^{++} + ArOH$$

A model for cytochrome oxidase action is thus provided:

$$Fe_p^{++} + O_2 = Fe_p^{++}O_2$$

$$Fe_p^{++}O_2 + 2e = Fe_p^{++}O + O^{--}$$

$$Fe_p^{++}O + 2e = Fe_p^{++} + O^{--}$$

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** The melting point and spectrum of gentisic acid isolated from these reactions are consistent with the properties of pure gentisic acid, but chromatograms indicate other hydroxylation products.

This hypothesis is now under test, using oxygen-18 as a tracer*.

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Intracellular peptides of *Pseudomonas hydrophila**

Although intracellular peptides have been isolated from a wide variety of cells and tissues[1], there is no assurance in many of these reports that the peptides were not formed by proteolysis. Numerous metabolically active peptides were obtained by TURBA AND ESSER[2] from extracts of *Torula utilis* grown on 1-^{14}C-acetate as the sole carbon source. The same authors were unable to isolate measurable quantities of peptides from extracts of *Leuconostoc mesenteroides*[3]. If peptides do represent intermediates in protein synthesis[4] they should be detectable in rapidly growing bacteria. The present communication reports the separation of intracellular peptides from log-phase cultures of *Pseudomonas hydrophila* grown on glucose as the sole carbon source.

A large number of such peptides has been obtained from these cultures. Alcoholic extracts of washed cells were concentrated, extracted with ether and dialyzed. After fractionation of the permeates by high voltage paper electrophoresis[5], five to seven ninhydrin-positive acidic bands appeared, one of which corresponded to the position of glutamic acid. A band remaining near the origin contained most of the neutral amino acids, and at least one neutral peptide. As many as seven ninhydrin-positive bands have appeared on the basic side, although their number and position have varied in different preparations. Photometric analyses[6] on eluates before and after acid hydrolysis indicated the presence of numerous basic peptides.

The peptides and amino acids on the acidic side were further separated by a combination of paper chromatography and repeated high-voltage paper electrophoresis. Three of the original ninhydrin-positive bands have so far been resolved into a total of eleven peptides, containing from five to eleven constituent amino acids. Whether these peptides have been separated as individual chemical species, or as groups of related compounds remains to be determined.

Evidence for the metabolic activity of the peptides was obtained from measurements of total and specific radioactivity. Dialyzates from ether-extracted alcoholic extracts of samples, taken at 1, 5, 12, 30, 60 and 120 min after the addition of uniformly-labelled ^{14}C-glucose to a log-phase culture, were radio-autographed after fractionation by high-voltage paper electrophoresis. All ninhydrin-positive bands were radioactive in less than 1 min, and no new bands appeared in the 2 h period. Elution of acidic, neutral and basic fractions as three separate groups was followed by estimation of the weight[7] and radioactivity[8,9] of carboxyl-C before and after acid hydrolysis. Residual cell protein had the highest total radioactivity even in the 1 min sample, with lower activity in the combined amino acid *plus* peptide fraction. Specific radioactivities plotted against time gave parallel hyperbolic curves, with peptides below the curve for amino acids, and with cell protein still lower and almost linear. This evidence suggests rapid synthesis of peptides from amino acids. Work is in progress on the structure and function of these compounds.

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