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Stereospecificity of Hydride Transfer in the Flavin Mono-oxygenase Orcinol Hydroxylase

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Orcinol hydroxylase, a flavoprotein mono-oxygenase, catalyses the oxidation of orcinol to 2,3,5-trihydroxytoluene. It has been purified to homogeneity and crystallized from a strain of *Pseudomonas putida* grown on orcinol (Ohta & Ribbons, 1970). The enzyme catalyses a typical mixed-function oxidation, using NADH, NADPH or reduced acetylpyridine as electron donor, and is active with several analogues of orcinol, including resorcinol and *m*-cresol. *m*-Cresol, however, is not hydroxylated, equimolar amounts of reduced nucleotide and O₂ being consumed while equimolar quantities of H₂O₂ are formed. Resorcinol is partly hydroxylated to hydroxyquinol, but there is some concomitant uncoupling of electron transfer from the hydroxylation reaction, and H₂O₂ is also formed (Ribbons & Ohta, 1970; Ribbons *et al.*, 1971).

4S-[4-³H]NADH was prepared from [4-³H]NAD⁺ by reduction with ethanol and yeast alcohol dehydrogenase. The opposite isomer (4R-[4-³H]₁NADH) was prepared in analogous experiments with NAD⁺ and [1-³H₂]ethanol, and their authenticity was confirmed by using pyruvate and rabbit muscle lactate dehydrogenase.

The specifically labelled nucleotides were incubated with orcinol hydroxylase in duplicate reaction mixtures for each isomer and each aromatic substrate. When orcinol was substrate, 3.1% of the tritium was recovered in water, from the 4S species of NADH. Similar results were obtained when the 4S-[4-³H]NADH was oxidized in the presence of resorcinol or *m*-cresol as effector; only slightly more radioactivity appeared in the water. The complementary experiments with 4R-[4-³H]NADH confirmed the stereospecificity of the transfer of hydride when orcinol was the substrate, 83% of the tritium being found in the water. However, when resorcinol was the aromatic substrate, there was a large isotope effect with 4R-[4-³H]NADH, and this was more pronounced when *m*-cresol was substrate; only 42.3 and 27.1% of the radioactivity supplied was

recovered in the water respectively. At higher temperature (55°C) with orcinol as substrate there was also an isotope effect with the 4R species of NADH; uncoupling of hydroxylation from electron flow also occurred at 55°C. The recovery of radioactivity in the water and that retained in the NAD⁺ formed was at least 90% in all experiments.

These findings will be discussed in relation to the mechanism of the enzymic reaction.

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The Modification of Bongkreikic Acid Inhibition of Mitochondrial Adenine Nucleotide Translocation by Coenzyme A

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The tricarboxylic bongkreikic acid is a potent inhibitor of the translocation of adenine nucleotides across mitochondrial membranes, but a time-lag occurs between the addition of bongkreikic acid and the appearance of inhibition (Henderson & Lardy, 1970; Klingenberg *et al.*, 1970; Henderson *et al.*, 1970). The lag period can be shortened by prior incubation with adenine nucleotides (Kemp *et al.*, 1970) and is much longer at lower temperatures (Henderson *et al.*, 1970). Shug *et al.* (1971) demonstrated that an inhibition of adenine nucleotide transport by fatty acids is in fact dependent on their conversion into the CoA derivative, and this suggested to us that CoA may also be involved in the inhibition by bongkreikic acid.

A direct assay of the adenine nucleotide exchange activity has been utilized (Henderson *et al.*, 1970) under conditions where inhibition by bongkreikic acid appeared after 7–10 min and was complete in a further 2 min. The presence of 200 μM-CoA shortened the lag period to ½–1½ min. Carnitine did not substitute for CoA, nor did it prevent the CoA effect, and neither carnitine nor CoA inhibited translocation in the absence of bongkreikic acid. Uncoupling agents did not prevent the CoA 'activation', and CoA did not appear to affect inhibition of the adenine nucleotide translocation by atractyloside.

A time-lag was also apparent when the inhibition

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