EVIDENCE FOR ENZYME-SUBSTRATE INTERMEDIATES IN THE ARYL-ALDEHYDE: NADP OXIDOREDUCTASE CATALYSED REDUCTION OF SALICYLATE

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1. Introduction

During *in vitro* studies on the reduction of aromatic acids an enzyme from mycelia of *Neurospora crassa* could be characterised which catalyses the formation of differently substituted benzoic and cinnamic aldehydes from the corresponding acids [1, 2]. In this reaction, besides of the transfer of hydrogen from NADPH to the acid, ATP was shown to be split into AMP and pyrophosphate [3] and not as formerly assumed into ADP and orthophosphate. This fact demonstrates that ATP serves as the energy donator in this endergonic process, suggesting the occurrence of an activated intermediate. There was no evidence for a free acyl-phosphate or acyl-nucleotide involved in this reaction and interest was focused on possibly protein-bound intermediates. The results presented in this paper give strong evidence for the occurrence of an enzyme-nucleotide and an enzyme-acyl intermediate in the course of this reaction.

2. Materials and methods

Aryl-aldehyde: NADP oxidoreductase was purified as described previously [2]. ATP-[-8-^{14}C] and ATP-[γ-^{32}P] as well as auxiliary enzymes and coenzymes were purchased from C.F.Boehringer, Mannheim. ATP-[β-^{32}P] was prepared according to Ives [4]. Salicylate-[-7-^{14}C] was purchased from The Radiochemical Center, Amersham. Enzyme-substrate complexes were separated from the low molecular compounds of the reaction mixture by gel-filtration at 4° on 1.5 X 20 cm columns of Sephadex G-50 Fine (Pharmacia) equilibrated in 50 mM Tris-HCl, pH 8.0, containing 2 mM 2-mercaptoethanol, 1 mM EDTA, and 5% glycerol. The protein peak, marked by the addition of Blue Dextran (Pharmacia) to the reaction mixture, was collected in 1 ml samples. From aliquots of each fraction radioactivity was measured by liquid-scintillation counting in Bray's solution [5] and the amount of enzyme was estimated as described previously [2].

3. Results

3.1. *Formation of an enzyme-salicylate complex*

The first experiment was on whether an intermediate enzyme-salicylate is formed during the

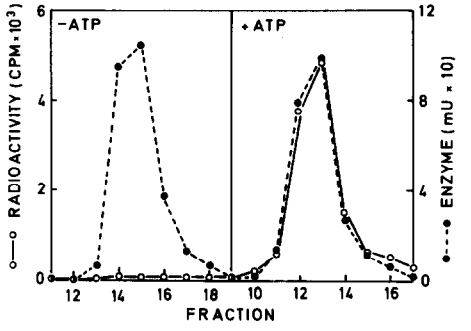

Fig. 1. Formation of enzyme-salicylate. The reaction mixture contained in a final volume of 1 ml in μmoles: 100 Tris-HCl, pH 8.0, 10 $MgCl_2$, 2 dithiothreitol, 0.14 salicylate (2 μCi), 2 ATP (omitted as indicated), and 0.62 mg enzyme (210 mU). After incubation at 30° for 10 min the reaction mixture was chilled, applied to a Sephadex G-50 column, and analysed as described under methods.

Table 1
Formation of salicylic aldehyde from enzyme-salicylate.

Enzyme-salicylate added (dpm)	NADPH	Salicylic aldehyde formed (dpm)
19,700	-	2,700
19,700	+	18,500

To 5 ml of enzyme-salicylate-[7-^{14}C] (19,700 dpm) were added in μmoles: 5 dithiothreitol, 50 $MgCl_2$, and 1.7 NADPH. The reaction mixture was brought to pH 6.8 with 0.5 ml 1 M potassium phosphate buffer, pH 6.5, giving a final volume of 5.8 ml. After incubation at 30° for 20 min 0.5 μmole of unlabelled salicylic aldehyde was added. The protein was denatured by the addition of 0.4 ml 3 M perchloric acid. The solution was neutralized with 0.4 ml 3 M KOH, buffered with 1 ml 1 M phosphate buffer, pH 8.0 and extracted twice with 4 ml benzene-ether (1 : 1; v/v). The organic phase was washed with additional 3 ml of 0.1 M phosphate buffer, pH 8.0, and analysed for salicylic aldehyde.

reaction. Aryl-aldehyde: NADP oxidoreductase was incubated with salicylate-[-7-^{14}C] and the protein was isolated as described above. As shown in fig. 1, a complex between enzyme and substrate is formed only in the presence of ATP. To decide whether this compound is a true intermediate of the reaction, the salicylate-[-7-^{14}C] labelled complex isolated from the Sephadex column was further incubated with and without NADPH at pH 6.8. Any formed salicylic aldehyde was extracted from the reaction mixture and the nature and purity of the product was checked by thin-layer chromatography and preparation of the 2,4-dinitrophenylhydrazone. As shown in table 1, salicylic aldehyde is formed from enzyme-salicylate only in the presence of NADPH; ATP is not necessary in this reaction. Therefore, it is evident that the enzymic reduction of aromatic acids involves the intermediate formation of an enzyme-acyl compound.

3.2. *Formation of an enzyme-nucleotide intermediate*

The next experiments were conducted to study the role of ATP in the formation of enzyme-salicylate. The enzyme was incubated with either ATP-[-8-^{14}C], ATP-[-γ-^{32}P], or ATP-[-β-^{32}P] and in each case the protein was isolated again by Sephadex gel filtration. Labelled protein was obtained only in the case of ATP-[-8-^{14}C] (fig. 2), indicating the formation of an enzyme-nucleotide compound and excluding the

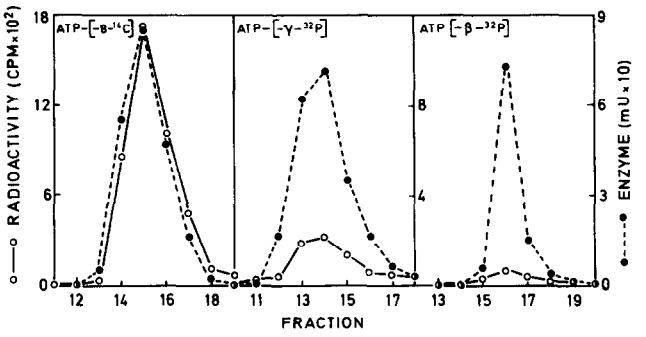

Fig. 2. Formation of enzyme-nucleotide. The reaction mixture contained in μmoles: 50 Tris-HCl, pH 8.0, 10 $MgCl_2$, 2 dithiothreitol, 0.1 ATP (2 μCi), and 0.6 mg enzyme (210 mU); in the case of ATP-[β-^{32}P] 1.6 mg enzyme (140 mU) was used. Other conditions were as described in fig. 1.

formation of an phosphorylated or pyrophosphorylated enzyme. This finding gives evidence that AMP and not ADP is attached upon the enzyme and thus may form an activated intermediate.

4. Discussion

There are examples for enzyme-AMP compounds probably serving as intermediates in enzyme reactions (cf [6, 7]). Attempts to prove that the enzyme-AMP reported in this paper takes part as an activated intermediate in the aryl-aldehyde oxidoreductase reaction have so far been unsuccessful. Preliminary experiments at different pH values and varied incubation conditions failed to demonstrate the formation of salicylic aldehyde from enzyme-AMP in the presence of salicylate and NADPH. This failure may be due to technical difficulties, or the enzyme-AMP undergoes structural alterations during its isolation. The presented findings suggest that the enzymic reduction of aromatic acids in *N. crassa* involves the formation of an enzyme-AMP and an enzyme acyl intermediate. However, we have no knowledge as to now about the existence of one common or two different binding sites for the nucleotide and the acid. Furthermore, it has to be analysed whether the acid is bound directly to the protein under the release of AMP. Further studies will lead to a better understanding of the mechanism of this reaction.

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