STOICHIOMETRIC STUDIES ON ARYL-ALDEHYDE: NADP OXIDOREDUCTASE FROM *NEUROSPRA CRASSA*

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1. Introduction

From mycelia of *Neurospora crassa* the enzyme aryl-aldehyde: NADP oxidoreductase (AAR) can be extracted which catalyzes the reduction of aromatic acids to the corresponding aldehydes. In previous publications [1, 2] it was reported that the energy necessary for this conversion is provided by cleavage of ATP into ADP and orthophosphate. Recently, this reaction was reexamined using ^{14}C-labelled ATP; in this case, only the formation of AMP was observed [3]. Furthermore, the occurrence of an enzyme-AMP intermediate [4] made it more likely that AMP, not ADP, is the true nucleotide arising from ATP under the catalysis of AAR.

To clarify this discrepancy, the stoichiometry of the reaction was reinvestigated using an analytically homogenous preparation of AAR. The results presented in this communication clearly establish the formation of AMP and pyrophosphate from ATP.

2. Materials and methods

AAR was purified by a modification of the method previously given [2]. Additionally, preparative polyacrylamide gel-electrophoresis was employed as final step of the purification. The enzyme was found homogenous according to the criteria of analytical disc-electrophoresis. Details of the applied procedures will be published elsewhere. Auxiliary enzymes and coenzymes were purchased from C.F. Boehringer, Mannheim, Germany. ATP-[-8-^{14}C] and benzoate-[-7-^{14}C] were obtained from the Radiochemical Centre, Amersham, England.

Analysis of nucleotide formation was carried out by applying aliquots of the reaction mixture to Whatman 3 MM paper together with 0.1 μmole of unlabelled ATP, ADP, and AMP, respectively, as carriers. Chromatography was performed in isobutyric acid:conc. NH_4OH:H_2O:0.1 M EDTA (100:4:60:1.6; v/v) or isopropanol:H_2O:20 percent trichloroacetic acid:conc. NH_4OH:0.1 M EDTA (70:10:20:0.3:1; v/v). For determinations of radioactivity, the spots were located by means of UV-light, cut out, and counted by liquid scintillation.

Orthophosphate and pyrophosphate (the latter after cleavage with inorganic pyrophosphatase) were quantitatively determined according to Fiske and SubbaRow [5]. The oxidation of NADPH or reduction of NADP were followed spectrophotometrically.

The formation of aldehyde was assayed as previously published [2].

3. Results

3.1. *Kinetic analysis of ATP-cleavage*

When a reaction mixture containing ^{14}C-labelled ATP and limiting amounts of NADPH is incubated with homogenous AAR, the only nucleotide formed

Abbreviations:
AAR, aromatic acid reductase, or aryl-aldehyde: NADP oxidoreductase (EC 1.2.1)
PP_i, inorganic pyrophosphate.

Table 1
Stoichiometry of the AAR-catalyzed reduction of benzoate.

	(μmole)
Benzaldehyde, formed	1.12
NADPH, consumed	0.94
ATP, consumed	0.93
ADP, formed	0
AMP, formed	1.01
Pyrophosphate, formed	0.98
Orthophosphate, formed	0

The reaction mixture contained in a final volume of 1.0 ml 100 μmole Tris-HCl, pH 8.0, 10 μmole $MgCl_2$, 2 μmole dithiothreitol, 2 μmole (0.1 μCi) benzoate (omitted in the blank), 1.2 μmole NADPH, 1.8 μmole (0.5 μCi) ATP, and 220 μg AAR. After completion of the reaction, the samples were deproteinized by the addition of 0.2 ml 3 M perchloric acid, neutralized with 0.2 ml 3 M KOH, and centrifuged. The supernatants were analyzed as described under methods.

from ATP is AMP. The quantities of ATP-consumption and AMP-formation agree well. There is no evidence for the production of ADP even after prolonged incubation. Traces of ADP showing no turnover in both sample and blank are due to impurities of the radioactive ATP used in the assays. However, in accordance with earlier experiments [2], when NADPH is replaced by NADP-reducing auxiliary system (including isocitrate and isocitrate dehydrogenase) appreciable amounts of ADP can be observed. Thus, the formerly reported finding of ADP may be due to myokinase activity present in the auxiliary enzyme system.

3.2. *Stoichiometry of the reaction*

The next experiments were conducted to obtain exact data on the stoichiometric relations of each substrate involved in the reaction. To facilitate the photometric estimation of NADPH, benzoate and not the standard substrate salicylate was chosen as H^+-acceptor; thus interference of the yellow chelate arising from salicylaldehyde [6] was avoided. The results (cf. table 1) show that all substrates react in equimolar ratios and that ATP is exclusively cleaved into AMP and pyrophosphate. Again the formation neither of ADP nor orthophosphate occurred.

From these data it is now possible to formulate the reaction catalyzed by AAR as follows:

$$R\text{–}CO_2H + ATP + NADPH + H^+ \rightleftharpoons$$

$$R\text{–}CHO + AMP + PP_i + NADP^+ + H_2O.$$

3.3. *Equilibrium constant of the reaction*

On the basis of the known reaction equation it was possible to study the equilibrium of the reaction. Reactions in assay mixtures containing differing, but exactly known concentrations of substrates were followed by measuring the absorbance of NADPH until stand of the reaction was reached. From the total change in absorbance thus determined, the equilibrium concentrations of all the reacting substrates were calculated. The exact concentration of H^+ was determined from the pH-value of the reaction mixture, and the concentration of water was assumed as 55.3 M.

By means of this sensitive assay, a slight reverse reaction towards the formation of acid could be detected when aldehyde, AMP, pyrophosphate, and NADP were incubated with AAR. This finding also supports the validity of the determinations concerning the products of the ATP-cleavage reported in the preceding sections.

Determining the equilibrium constant at pH 8 the following value, obtained as a mean from forward and reverse reactions, was calculated:

$$K = \frac{(\text{benzaldehyde})\,(\text{AMP})\,(PP_i)\,(NADP^+)\,(H_2O)}{(\text{benzoate})\,(\text{ATP})\,(\text{NADPH})\,(H^+)} =$$

$$1.5 \times 10^9;$$

i.e., the equilibrium of the reaction highly favours the formation of aldehyde from the corresponding acid.

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References

[1] G.G. Gross, K.H. Bolkart and M.H. Zenk, Biochem. Biophys. Res. Commun. 32 (1968) 173.
[2] G.G. Gross and M.H. Zenk, European J. Biochem. 8 (1969) 413.
[3] M.H. Zenk and G.G. Gross, in: Recent Advances in Phytochemistry, Vol. 4, ed. V.C. Runeckles (Appleton Century Crofts, New York) in press.
[4] G.G. Gross, FEBS Letters 5 (1969) 177.
[5] C.H. Fiske and Y. SubbaRow, J. Biol. Chem. 66 (1925) 375.
[6] F. Feigl, Spot Tests in Organic Analysis (Elsevier Publ. Comp., Amsterdam, London, New York, 1966) p. 342.