FLAVANONE SYNTHASE CATALYZES CO_2 EXCHANGE AND DECARBOXYLATION OF MALONYL-CoA

Fritz KREUZALER, Robley J. LIGHT* and Klaus HAHLBROCK+
Biologisches Institut II der Universität, Schänzlestr. 9–11, D-7800 Freiburg, FRG

Recieved 4 August 1978

1. Introduction

Flavanone synthase catalyzes the formation of the aromatic ring A of naringenin (5,7,4′-trihydroxyflavanone) from acetate units. The mechanism involves the sequential condensation of 3 molecules of malonyl-CoA with 1 molecule of 4-coumaroyl-CoA [1–3]. The condensation reaction is inhibited by cerulenin [1], and should also in other respects bear a resemblance to the condensation reactions of fatty acid synthase [4] and 6-methylsalicylic acid synthase [5]. We have now obtained evidence for the occurrence of two characteristic side reactions, CO_2 exchange and decarboxylation of malonyl-CoA, with purified flavanone synthase from irradiated cell suspension cultures of parsley (*Petroselinum hortense* Hoff.). A modified, hypothetic mechanism for the flavanone synthase reaction is presented on the basis of these results.

2. Materials and methods

2.1. *Labelled substrates*

[2-^{14}C]Malonyl-CoA (20.5 Ci/mol) was obtained from New England Nuclear (Dreieichenhain); $NaH^{14}CO_3$ (> 40 Ci/mol) was from Amersham-Buchler (Braunschweig).

2.2. *Enzymes*

An extensively purified flavanone synthase preparation from irradiated cell suspension cultures of *P. hortense* Hoff. [1] was separated from some residual, contaminating proteins by chromatography on Sephadex G-200. The experiments reported here utilized either the preparation after Sephadex G-200 (enzyme solution S), shown by disc-gel electrophoresis to be free of contaminating protein, or the preparation prior to Sephadex G-200 obtained by hydroxyapatite chromatography (enzyme solution H) [1].

A crude preparation of acetyl-CoA carboxylase from *P. hortense* cells was a gift from Dr J. Ebel, Freiburg.

2.3. *Enzyme assays*

Flavanone synthase activity was measured by the standard assay procedure as in [1].

Decarboxylase activity of flavanone synthase was determined by incubating 15 μl enzyme solution S (6–15 μg), 5 μl aqueous solution of 0.49 nmol [2-^{14}C]malonyl-CoA (0.05 μCi), and 85 μl 0.1 mol/l potassium phosphate, pH 6.0 or pH 8.0, containing 1.4 mmol/l 2-mercaptoethanol, for up to 30 min at 30°C. The reaction was stopped by adding 20 μl glacial acetic acid. The acidified mixture was chromatographed on Whatman 1 MM paper in isobutyric acid/conc. aqueous ammonia/water, 66:1:30. The portion of the chromatogram corresponding to the R_F of acetyl-CoA (0.45–0.5) was cut out and assayed for radioactivity.

For product identification, the acetyl-CoA was eluted from the paper with dilute acetic acid (pH 3) and freeze-dried. About 2/3rds of the isolated material (80 000 dpm), dissolved in 0.1 ml 0.1 mol/l Tris–HCl, pH 7.5, were incubated for 20 min at 30°C

+ Address correspondence to: Prof. Dr. K. Hahlbrock

* Permanent address: Department of Chemistry, Florida State University, Tallahassee, FL 32306, USA

with 0.1 ml crude acetyl-CoA carboxylase preparation containing 1 μmol/l $MgCl_2$, 0.5 μmol ATP, and 0.6 μmol $NaH^{14}CO_3$ (0.3 μCi). The reaction was stopped by adding 40 μl glacial acetic acid, and the mixture was chromatographed and the product identified as malonyl-CoA in the same manner as described above (malonyl-CoA R_F 0.25–0.3).

The CO_2 exchange reaction was studied with enzyme solution H. Although this preparation contained minor amounts of contaminating protein, the exchange reaction was inhibited by antibody prepared against purified flavanone synthase (F.K., unpublished) at an identical titer required to inhibit naringenin synthesis. For the assay, incubations at 30°C contained 6.6 μg flavanone synthase, 2.5 μmol potassium phosphate, pH 7.8, 125 nmol mercaptoethanol, 20 nmol malonyl-CoA, and 0.6 nmol $NaH^{14}CO_3$ (0.3 μCi) in a total 75 μl. After the indicated time, the reaction was stopped by the addition of 50 μl acetic acid, and the reaction mixture was evaporated to dryness on 3 X 10 cm filter paper strips, which were assayed for non-volatile radioactivity.

The radioactive reaction product co-chromatographed with authentic malonyl-CoA in the solvent system described above (R_F 0.27). Another sample of reaction product was first hydrolyzed for 0.5 h with 1 N NaOH, acidified with 1 N HCl, and evaporated to dryness. The residue was triturated with ether and the ether extract chromatographed in butanol/formic acid/water, 10:2:15, upper phase. About 80% of the radioactive material co-chromatographed with authentic malonic acid. A second labeled compound, containing ~20% of the radioactivity, was observed but was not further identified.

Protein was measured by the biuret method (crude preparations) or by the direct spectrophotometric method at 260 and 280 nm [6]. Radioactivity was measured by scintillation spectrometry with a solution of 5 g PPO in 1 l toluene (50% counting efficiency).

3. Results

3.1. *Decarboxylation*

When flavanone synthase, purified to apparent homogeneity, was incubated with [2-^{14}C]malonyl-CoA in the absence of 4-coumaroyl-CoA, about 8% of the labelled substrate was converted to acetyl-CoA within 25 min. The rate of acetyl-CoA formation decreased progressively during the incubation period and was the same, regardless of whether the reaction was carried out at pH 6.0 or pH 8.0 (fig.1A). The initial rate of acetyl-CoA formation was ~ 4 pmol/min/μg protein. The two different pH values were chosen, because they were optimal, respectively, for

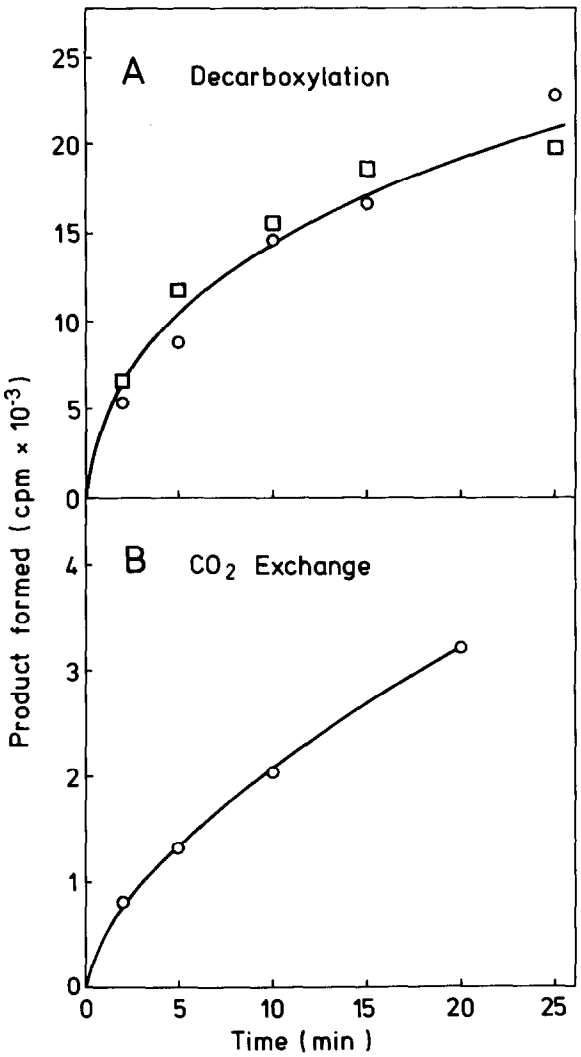

Fig.1. Dependence of the rates of decarboxylation (A) at pH 6.0 (□) and pH 8.0 (○) and of CO_2 exchange (B) on the time of incubation of purified flavanone synthase with malonyl-CoA. The formation of labelled acetyl-CoA and malonyl-CoA, respectively, was measured as described in section 2.

the rate of naringenin formation in the standard flavanone synthase assay (pH 8.0) and for the rate of eriodictyol (5,7,3′,4′-tetrahydroxyflavanone) formation with caffeoyl-CoA instead of 4-coumaroyl-CoA as substrate (pH 6.0) [7].

3.2. *CO_2 exchange*

A time course similar to that observed for the decarboxylation reaction was found for the incorporation of radioactivity into malonyl-CoA, when the purified synthase was incubated at pH 8.0 with malonyl-CoA in the presence of $H^{14}CO_3^-$ (fig.1B). About 0.9 nmol $^{14}CO_2$/μg protein were incorporated into malonyl-CoA within 20 min under the assay conditions for the exchange reaction. The initial rate of formation of labelled malonyl-CoA was ~ 80 pmol/min/μg protein.

No inhibitory effect on the exchange reaction was observed for iodoacetate (10 mmol/l), iodoacetamide (20 mmol/l), or CoASH (up to 1.2 mmol/l). By contrast, 2 mmol/l acetyl-CoA inhibited the exchange reaction to a similar degree (33% inhibition) as found for the synthase reaction (50% inhibition at 2 mmol/l acetyl-CoA [1]). No formation of labelled malonyl-CoA was observed, when malonyl-CoA was replaced by acetyl-CoA (0.7 or 2 mmol/l) in the assay for CO_2 exchange. The rate of CO_2 exchange was not significantly reduced in the presence of up to 40 μmol/l 4-coumaroyl-CoA. Like the overall synthase activity, the CO_2 exchange activity of flavanone synthase was relatively unstable on storage at 4°C. About 80% of the exchange activity was lost in 8 days.

4. Discussion

The present results demonstrate that purified flavanone synthase catalyzes CO_2 exchange and decarboxylation of malonyl-CoA both in the presence and in the absence of 4-coumaroyl-CoA. Under appropriate conditions, decarboxylation of the malonate residue is also catalyzed by fatty acid synthases from various sources [8] and by 6-methylsalicylic acid synthase from *Penicillium patulum* [5]. CO_2 exchange has been observed with several fatty acid synthases [9]. The common occurrence of these reactions further indicates the large similarity of the mechanisms of chain elongation in the synthesis of the various acetogenins.

However, one remarkable difference between flavanone synthase and the other synthases is the nature of the malonyl thioester. Prior to the condensation reactions in fatty acid and 6-methylsalicylic acid synthesis, the malonyl residue is transferred from CoA either to an acyl carrier protein (ACP) or to a 4′-phosphopantetheinyl residue which is directly bound to the enzyme. By contrast, the formation of naringenin proceeded in the absence of ACP, and no evidence was found for a 4′-phosphopantetheinyl residue of the enzyme (F.K., K.H., unpublished results). Decarboxylation occurred in the presence of sufficient iodoacetate or iodoacetamide to block not only enzyme sulfhydryl groups, but also free CoASH formed in the reaction mixture. Acetyl-CoA, the product of the decarboxylation, could not have been formed by a transfer of the malonyl group to the enzyme, followed by a transfer of the acetyl product

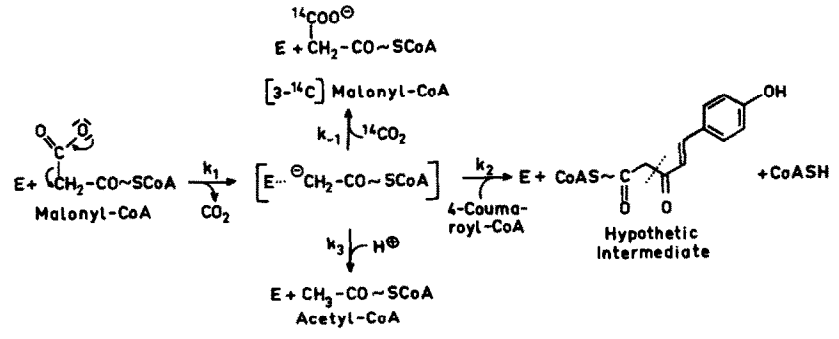

Fig.2. Hypothetical mechanism of malonyl-CoA decarboxylation (rate constant k_1) as the initial step catalyzed by flavanone synthase (E). Subsequent, possible reactions are: the reverse reaction (k_{-1}), chain elongation (k_2), or proton addition (k_3).

back to CoASH. Hence, malonyl-CoA seems to be the immediate substrate for flavanone synthase, rather than an enzyme-bound malonyl residue, as suggested [1,3]. It should be noted that the lack of stimulation of the rate of malonyl-CoA decarboxylation by iodoacetamide in the case of flavanone synthase is also in contrast to the situation with fatty acid and 6-methylsalicylic acid synthases [5,8].

Figure 2 shows a hypothetical mechanism which would explain the occurrence of all three reactions of malonyl-CoA catalyzed by flavanone synthase: decarboxylation, CO_2 exchange, and condensation with 4-coumaroyl-CoA (or with a subsequent intermediate of chain elongation). All three reactions can be explained by assuming an initial formation of an acetyl-CoA carbanion which, in the normal flavanone synthase reaction, condenses with 4-coumaroyl-CoA to form the first intermediate of chain elongation. (The acetyl carbanion could, of course, have some enolate character, and it would undoubtedly have to be stabilized by some neighboring group on the enzyme.) At least under the artificial assay conditions with the purified enzyme, the putative carbanion could alternatively react either with a proton to form acetyl-CoA (decarboxylation) or with CO_2 to regenerate malonyl-CoA (CO_2 exchange). Thus, the mechanism of naringenin formation does not seem to involve a concerted reaction of decarboxylation and condensation, as proposed for fatty acid biosynthesis in yeast, and generally postulated for the synthesis of other 'polyacetate' compounds derived from malonyl-CoA [10].

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft and a Senior US Scientist Award to R.J.L.

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