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ALTERNATE PATHWAYS OF MALONYLCoA FORMATION IN *STREPTOMYCES AUREOFACIENS*

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Key Word Index—*Streptomyces aureofaciens*; biosynthesis; tetracyclines; formation of malonylCoA.

Abstract—Alternate metabolic pathways for the formation of malonylCoA in the actinomycete *Streptomyces aureofaciens* are examined. Comparison of the specific activities of pyruvate kinase, pyruvate dehydrogenase, and phosphoenol–pyruvate carboxylase during cultivation, the degree of incorporation of individual radioactive substrates into the tetracycline molecule, and the high randomization of acetate-[2-^{14}C], indicate that the malonylCoA used in tetracycline biosynthesis does not appear solely through the carboxylation of acetylCoA. The role of the phosphoenolpyruvate carboxylase and oxaloacetate dehydrogenase systems in the formation of malonylCoA is established, and using radio-GLC, a cell-free preparation of *S. aureofaciens* mycelium is shown to form malonate from oxaloacetate. The reaction requires HSCoA and NAD^+.

INTRODUCTION

The skeleton of the tetracycline (TC) molecule is formed according to the acetate rule by condensation of 8 molecules of malonylCoA with the terminal group formed by malonamoylCoA [1]. It is still generally accepted that during the biosynthesis of the TC skeleton, malonylCoA is formed by caboxylation of acetylCoA (acetylCoA carboxylase) [2]. The formation of the malonylCoA units used for the biosynthesis of lipids and of secondary metabolites of the oligoketide type is usually taken as proceeding via a single process, catalyzed by the same enzymes and forming a common pool of biosynthetic intermediates. Thus with *Streptomyces noursei* var. *polyfungini* the increased activity of acetylCoA carboxylase was shown to be correlated both with an increased formation of tetraene antibiotics and with lipogenesis [3].

In earlier work we followed the relationship between acetylCoA carboxylase activity and the biosynthesis of chlortetracycline (CTC) in *S. aureofaciens*. It was observed that the sp.act. of the enzyme was highest during the logarithmic phase of growth while during the stationary phase, i.e. during a period of intense biosynthesis of the antibiotic, only slight activity was present [4].

It was therefore uncertain how the relatively high amount of malonylCoA needed for TC biosynthesis is formed. We therefore studied enzyme systems involved in the two alternate metabolic pathways for malonylCoA formation from the common precursor, phosphoenolpyruvate (PEP): (a) pyruvate (Pyr) kinase and the Pyr dehydrogenase complex yielding acetylCoA (which is further carboxylated), and (b) PEP carboxylase and the enzyme system decarboxylating oxaloacetate (OA) to malonylCoA [5, 6]. The activity of these enzyme systems was followed in the course of cultivation both in the low-production strain of standard type (*S. aureofaciens* RIA 57) and in the production variant (*S. aureofaciens* 8425). Using incorporation of ^{14}C-labelled substrates, the proportion of the alternate biosynthetic pathways leading to malonylCoA in the total biosynthesis of TC was assessed.

RESULTS AND DISCUSSION

The OA dehydrogenase system and enzymes of PEP metabolism

Decarboxylation of OA was demonstrated in cell-free extract of stationary phase (72 hr) of *S. aureofaciens* 8425 by GC–RC. OA-[4-^{14}C] originated directly in the reaction mixture with the aid of the excess of PEP carboxylase present and added PEP and bicarbonate-[^{14}C].

After 20 min incubation CoASH and OA were added and the reaction mixture was kept another 20 min at 28°. Then it was cooled in ice, carrier malonic acid added, and the reaction product extracted, esterified with diazomethane, separated by GLC and detected as radioactive malonate.

The radioactivity of the malonate peak (1553 dpm) was significantly higher than the radioactivity of background (*ca* 80 dpm).

The nonenzymic decarboxylation of OA was excluded by the incubation of the reaction mixture in which the enzyme was omitted or by keeping the reaction mixture with the enzyme in an ice bath after addition of CoASH and OA. The radioactivity of the malonate peak did not exceed the values of the background. In the case when the nonradioactive OA carrier was omitted in the reaction mixture the radioactivity of the malonate peak was equal to the values of the background. This provides supporting evidence that the radioactivity of malonate originates from OA which as a substrate for the enzyme reaction must be present in a satisfactory concentration. No radioactivity

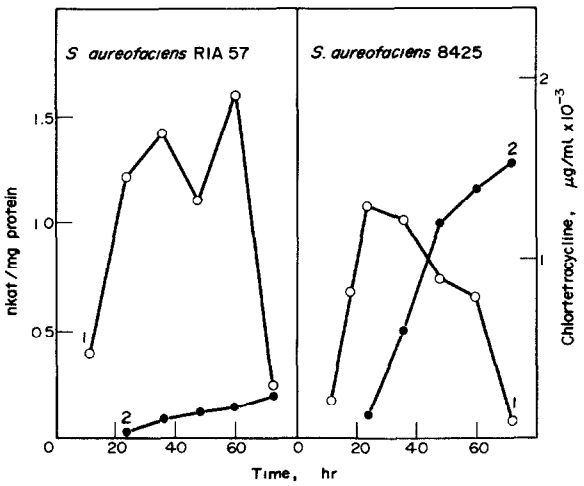

Fig. 1. Specific activity of pyruvate kinase (curve 1) and chlortetracycline production (curve 2).

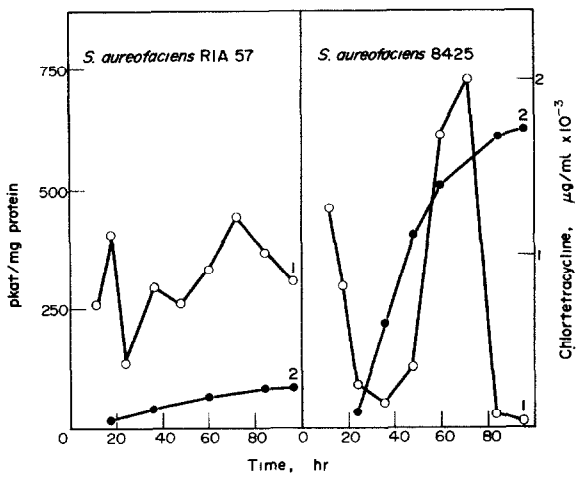

Fig. 3. Specific activity of phosphoenolpyruvate carboxylase (curve 1) and chlortetracycline production (curve 2).

was found in the malonate peak when the reaction mixture was extracted after incubation without the addition of nonradioactive malonate carrier. This indicates that the radioactivity of the malonate peak represents the radioactivity of malonate.

The synthesis of malonate proceeded at a lower rate in the absence of added HSCoA and NAD^+ because crude extracts were used, in which these compounds were presumably present already. By addition of HSCoA and NAD^+ to the reaction mixture the activity of the reaction increased roughly 2×.

This alternate pathway of malonylCoA biosynthesis is present in the cell together with the 'classical' system generating malonylCoA by carboxylation of acetylCoA. The latter pathway is particularly active during the exponential phase, when the activities of Pyr kinase, (Fig. 1), of the Pyr dehydrogenase complex (Fig. 2) and of acetylCoA carboxylase [4] are all maximal. The low activity of these enzymes during the stationary phase is a characteristic feature of the production variant. However, the activity of PEP carboxylase rises during the stationary phase, particularly in the production strain where its rise is associated with an increased rate of CTC biosynthesis (Fig. 3).

At an activity of PEP carboxylase equal to about 500 pkat/mg protein, one can expect a synthesis of about 600 nmol malonylCoA per hr per ml fermentation broth. This corresponds to a rate of synthesis of *ca* 25 µg CTC per hr per ml medium. This is more than the actual production rates usually observed (the rate of CTC synthesis equals 15 µg per hr in stationary phase) to justify the conclusion that the PEP carboxylase route can account for most if not all of the malonylCoA required for CTC biosynthesis.

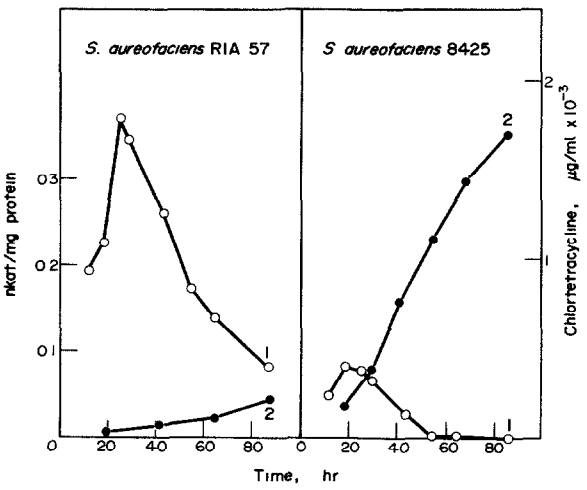

Fig. 2. Specific activity of pyruvate dehydrogenase complex (curve 1) and chlortetracycline production (curve 2).

Incorporation of labelled substrates into tetracycline

Labelled substrates were added to the fermentation medium at the beginning of the stationary phase (24 hr). The results are present in Table 1.

From the ratio of incorporation of Pyr and acetate one may assume that the Pyr dehydrogenase complex is not the limiting enzyme system in the biosynthesis of TC skeleton because the incorporation of acetate does not exceed substantially the incorporation of Pyr. The role of acetylCoA carboxylase cannot be unambiguously solved because the incorporation of malonate is probably

Table 1. Incorporation of labelled substrates into tetracycline

Substrate	Incorporation into tetracycline (dpm/mg)	Incorporation into carbamoyl group (% of total activity of tetracycline)
Acetate-[2-^{14}C]	22600	2.95
Pyruvate-[3-^{14}C]	20000	2.73
Malonate-[2-^{14}C]	7500	2.78
Alanine-[U-^{14}C]	16500	7.15
Pyruvate-[1-^{14}C]	1240	39.20
Bicarbonate-[^{14}C]	1600	37.60
Propionate-[1-^{14}C]	860	6.35

much lower than the incorporation of acetate as a result of poorer permeation of dicarboxylic ions. The low incorporation of Pyr-[1-^{14}C] and bicarbonate-[^{14}C] and the high proportion of this radioactivity incorporated in the carbamoyl carbon of TC indicates that CO_2 originating either from bicarbonate or from the carboxyl of Pyr is utilized by *S. aureofaciens* for carboxylation of acetylCoA.

The radioactivity of the carbamoyl group (estimated by Hofmann degradation) is about equal after incorporation of acetate-[2-^{14}C], Pyr-[3-^{14}C] and malonate-[2-^{14}C], averaging 2.8%. This indicates a high degree of randomization of the acetate units, since the carbamoyl group is the original carboxyl group of malonate and should not be labelled at all if malonylCoA were formed by the 'classical' PEP–Pyr–acetylCoA–malonylCoA pathway. Assuming that the other carbon atoms in the TC skeleton originating from the carboxyl group of malonate (acetate) have the same radioactivity, they would together account for about 28% of the total radioactivity of TC. Catlin *et al.* [7] found a value of 15.8% from the degree of incorporation of acetate-[2-^{14}C] into the $C^{(6)}$ of oxytetracycline (which is a carboxyl carbon according to the acetate rule). When acetate-[2-^{14}C] is randomized passing through tricarboxylic acid cycle—the carbons of the four carbon acids originating from the carboxyl group of acetate have in the steady state half radioactivity than the carbons originating from the methyl group of acetate [8].

If malonylCoA originates from such a labelled acid then having taken all theoretical possibilities of its origin into consideration, the ratio between the radioactivity of carbons originating from methyl and from carboxyl of acetate will be between 2:1 and 1:1. If 10 carbons of the TC skeleton originating from the carboxyl group of acetate account for 28% of the total radioactivity then the fraction of TC formed by alternate metabolic pathways lies between 53–78%.

PropionylCoA is most probably not a direct precursor of malonylCoA since incorporation of both propionate-[1-^{14}C] and propionate-[3-^{14}C], [9] into TC apparently occurs only after complete randomization. Although the molecule of propionate was labelled only at $C^{-(1)}$ or at $C^{-(3)}$ the carbons of TC skeleton were labelled universally. A uniform distribution of radioactivity in the TC skeleton was also attained after incorporation of alanine-[U-^{14}C] as we may assume from the incorporation into the $CONH_2$ group shown in Table 1 (with equal distribution of the radioactivity in the TC skeleton each carbon atom acquires 5.26% of total activity). The high incorporation of alanine into TC indicates that the biosynthesis of the antibiotic also utilizes amino acids (alanine also via Pyr). It is likely that with high production of CTC (particularly at the end of the stationary phase) the malonate units are formed from carbon residues appearing during degradation of cell protein.

On the basis of these results the hypothesis can be advanced that the enzyme systems of primary metabolism and secondary biosynthesis, generating the same biosynthetic intermediate, need not be identical. These conclusions are related to results of Hošťálek *et al.* [10]. who found that during the exponential phase of *S. aureofaciens* hexoses are phosphorylated by ATP glucokinase, whereas during the stationary phase (particularly in the production variant) sugar dissimilation proceeds via polyphosphate glucokinase.

EXPERIMENTAL

A production variant *Streptomyces aureofaciens* 8425 (Research Institute of Antibiotics and Biotransfromations, Roztoky near Prague) and a low-production strain *S. aureofaciens* RIA 57 (Research Institute of Antibiotics, Moscow) were used. Maintenance and cultivation were described earlier [11].

Preparation of cell-free extract. After washing with H_2O, the mycelium was disintegrated in a bacterial press (X-press, Biox) and suspended in a homogenization buffer (0·1 M Tris–HCl, pH 7.4; 2 mM EDTA; 15% glycerol; 1 mM mercaptoethanol). The homogenate was centrifuged at 20000 *g* for 30 min and, after removing the low MW components on a column (1 × 10 cm) of Sephadex G-25, it was used for determination of enzyme activities.

Enzyme assay. The Pyr dehydrogenase complex (E.C. 1.2.4.1) was determined colorimetrically by measuring the amount of ferrocyanide liberated during oxidative decarboxylation of Pyr using ferricyanide as electron acceptor [12]. The reaction mixture contained in 1.4 ml (μmol); K-Pi buffer (pH6) 150, thiamine pyrophosphate 0.2, $MgCl_2$ 0.3, Na Pyr 50, K ferricyanide 25. Pyr kinase (ATP: Pyr phosphotransferase, E.C. 2.7.1.40) was estimated spectrometrically [13]. The reaction mixture contained in 3 ml (μmol); Tris–HCl (pH 7.4) 150, NADH 0.45, ADP 0.69, PEP 2.3, $MgSO_4$ 24, KCl 225, lactate dehydrogenase 4 units. PEP carboxylase (orthophosphate: OA carboxylyase (phosphorylating), E.C. 4.1.1.31) was estimated by an isotopic method, based on incorporation of labelled bicarbonate into OA which was converted to the 2,4-dinitrophenylhydrazone [14]. The reaction mixture contained in 0·7 ml (μmol): Tris–HCl (pH 7.4) 70, $MgCl_2$ 1.4, $KH^{14}CO_3$ 10 (10 μCi), PEP 4.

OA dehydrogenase system. OA-[4-^{14}C] was derived from PEP and bicarbonate-[^{14}C] present in the reaction mixture. This contained in 2 ml (μmol): Tris–HCl (pH 7.4) 140, $MnCl_2$ 2·8, PEP 8, $KH^{14}CO_3$ (100 μCi) 20, NAD^+ 3, protein 1.8 mg. After 20 min incubation HSCoA (1.5 μmol) and OA as a carrier (30 μmol) were added and the mixture was incubated for another 20 min. After termination of incubation it was cooled in ice, malonic acid (30 μmol) was added, and after acidification with $HClO_4$ and removing the radioactive CO_2, it was extracted with Et_2O), esterified with CH_2N_2 and chromatographed on GC–RC with FID at 80° to 250° at 4°/min on a column (183 cm × 4 mm) of Chromosorb W coated with 5% DEGA; N_2 at 40 ml/min. The eluted compounds were separated on a fraction collector provided with water cooling and trapped on glass wool. The radioactivity of the individual fractions was measured in a toluene scintillation soln. Enzyme activities were assayed at 28°.

Incorporation of labelled substrates. Cultivation was done in a medium where NaCl was replaced with NaBr (0.5%) [9]. This suppressed the formation of CTC, and TC resulted as the unique fermentation product. Labelled substrates, malonate-[2-^{14}C], Pyr-[3-^{14}C], Pyr-[1-^{14}C], propionate-[1-^{14}C], alanine-[U-14C] (Radiochemical Centre Amersham, Great Britain), acetate-[2-^{14}C] bicarbonate-[^{14}C] (Zentralinstitut für Kernforschung, Dresden G.D.R.) were added to a 24 hr culture of *S. aureofaciens* 8425 and incubated for a further 3 days. The amount of total and sp. act. and experimental conditions were the same for all substrates. TC was isolated according to ref. [15] and purified to constant radioactivity. Radioactivity was assayed by the liquid scintillation technique using an internal toluene-[^{14}C] standard or by the method of infinitely thin layer. Hofmann degradation of the carbamoyl group of TC was as described in ref. [9].

Other methods. CTC and TC were estimated spectrophotometrically, [16] and the protein content according to ref. [17].

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