RAPID INCORPORATION OF PRECURSORS INTO CANDICIDIN BY RESTING CELLS OF *STREPTOMYCES GRISEUS*

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Labeled acetate, propionate and *p*-aminobenzoic acid were efficiently incorporated into candicidin by phosphate-limited resting cells of *Streptomyces griseus*. The efficiency of incorporation in short-term experiments using phosphate-limited resting cells was similar to that achieved previously in long-term experiments using growing cells. (2-^{14}C)Propionate was more efficiently incorporated than (1-^{14}C)propionate, (U-^{14}C)propionate, or (U-^{14}C)-acetate. *p*-Aminobenzoic acid incorporation is linear over a 10-hour period while those of acetate or propionate reach a constant level after approximately 4 hours of incubation. Double-labeled candicidin of high specific activity was prepared by supplementing the resting cell system with (^{3}H)acetate and (^{14}C)*p*-aminobenzoic acid.

Candicidin is a polyene antifungal antibiotic composed of a macrolide ring with a heptaene chromophore, the aminosugar mycosamine (3-amino-3, 6-dideoxy-D-mannose) and an aldolically-linked *p*-aminoacetophenone group.

The macrolide ring seems to be synthesized by a head-to-tail condensation of acetate and propionate (as malonyl-CoA and methyl malonyl-CoA, respectively) in a process similar to that of fatty acid biosynthesis[1]. Although direct evidence is lacking, this type of condensation is supported by the fact that cerulenin, a specific inhibitor of the condensation of malonyl-CoA subunits in fatty acid synthesis, also inhibits the biosynthesis of candicidin[2]. Biosynthesis of the *p*-aminoacetophenone moiety proceeds through the shikimate pathway *via p*-aminobenzoic acid (PABA) in common with the biosynthesis of the aromatic amino acids[3]. Mycosamine, which is formed from glucose, appears to be attached posteriorly to the macrolide ring, since early formation of non-glycosylated components has been observed[4]. Previous studies on incorporation of precursors in long-term batch processes were marred by randomization of the label from the precursors as well as degradation of the antibiotic formed[5]. We have described previously a phosphate-limited resting cell system in which antibiotic synthesis occurs in short-term experiments without growth[6]. In this study, we use this system to establish that both acetate and propionate are precursors of the macrolide ring and that PABA is a precursor of the aromatic moiety of candicidin, as previously indicated[3].

Materials and Methods

Incorporation of labeled precursors and purification of candicidin

(^{14}C)Acetate (2 μCi/ml), (^{14}C) propionate (2 μCi/ml) or *p*-amino (^{14}C)-benzoic acid (1 μCi/ml) were added to 10 ml of resting cells phosphate-starved for 12 hours, and incubation was carried out for 4~10 additional hours. Samples (250 μl) were pipetted into 25 μl of 0.1 N sulfuric acid and the pellet precipitate which contained mycelium and particle candicidin was washed twice with distilled water at pH 4. After washing, the pellet was extracted with 250 μl of chloroform-methanol (1 : 1, v/v). Duplicate 20 μl aliquots were applied to 250 μm precoated thin-layer silica gel plates (Analtech Inc.) scored

in 1-cm wide bands. The plates were developed in the lower phase of chloroform-methanol-20% ammonium hydroxide (2: 2: 1, v/v) along with a standard of candicidin[7]. Candicidin-containing bands were identified under UV light, scraped into scintillation vials and counted in a Packard Tricarb 3320 scintillation counter using naphthalene - dioxane - omnifluor (10: 100: 0.8, v/v/w) as scintillation fluid. Color quenching due to candicidin was corrected using the channel-ratio method.

To recover labeled candicidin, the silica gel bands containing candicidin were extracted with the lower phase of chloroform - methanol - borate buffer pH 8.2 (2: 2: 1, v/v). The extracts were concentrated under vacuum, washed with distilled water, precipitated in acetone and finally dried with diethyl ether as described for purification of candihexin[8].

Double-label incorporation

(^{3}H) Acetate and (^{14}C) PABA were added together as in single label experiments. Samples were processed as above except that counting was done in a Beckman LS–230 scintillation counter. Standard (^{3}H) and (^{14}C) Beckman Isoset windows were used. Quenching corrections when required were done by the method of HENDLER[9].

Results

Incorporation of Labeled Precursors into Candicidin

Results of the incorporation of (^{14}C) acetate and (^{14}C) PABA into candicidin are summarized in Table 1. We found that propionate labeled uniformly or in either C–1 or C–2 is incorporated into candicidin with a 3∼5 fold higher specific activity than is obtained with acetate. (2–^{14}C) Propionate is more efficiently incorporated than is (1–^{14}C) or (U–^{14}C) propionate. While incorporation of PABA is linear over a 10-hour period (Fig. 1), incorporation of acetate and propionate are not. This is probably due to dilution of acetate, and to a lesser degree, propionate in amphibolic pathways, while PABA is incorporated into candicidin without significant diversion.

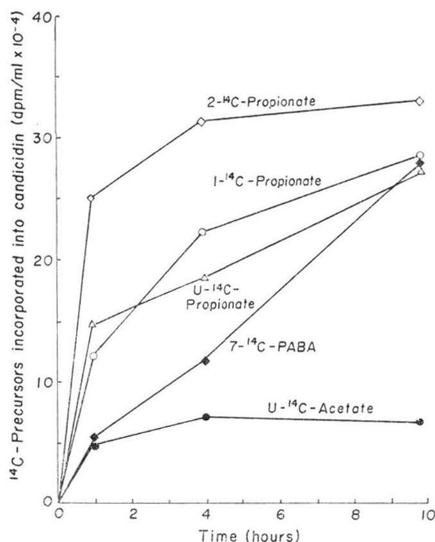

Fig. 1. Incorporation of labeled precursors into candicidin by phosphate-limited resting cells of *S. griseus*.

Precursors were added at time zero at the concentrations indicated in Materials and Methods. Radioactive candicidin was extracted and purified as indicated.

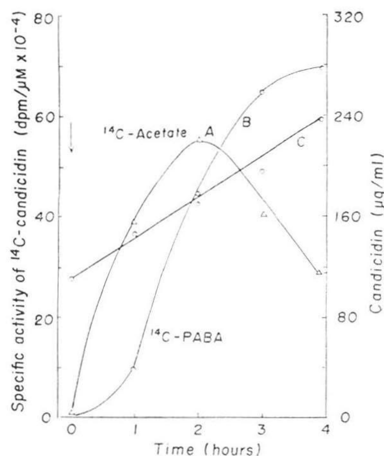

Fig. 2. Specific activity of ^{14}C-candicidin following precursor addition.

Precursors were added at the concentrations indicated in Methods at the times indicated by the arrow. (A) Specific activity of candicidin labeled with acetate; (B) specific activity of candicidin labeled with PABA; (C) total candicidin.

Table 1. Incorporation of labeled precursors into candicidin by phosphate-limited resting cells of *S. griseus*

Precursor	μCi added	Candicidin		Efficiency of incorporation** (%)
		Label incorporation (dpm/ml)	Specific activity* (dpm/μmol)	
(U-^{14}C) Acetate	20	72×10^3	5.6×10^5	1.51
(1-^{14}C) Propionate	20	226×10^3	17.5×10^5	6.15
(2-^{14}C) Propionate	20	313×10^3	24.2×10^5	7.41
(U-^{14}C) Propionate	20	188×10^3	14.5×10^5	6.43
(7-^{14}C) PABA	20	101×10^3	7.7×10^5	6.22

* Specific activity after 4 hours of incubation in presence of labeled precursors.

** Efficiency of incorporation after 10 hours of incubation with labeled precursors.

A molecular weight of 1200 was assumed for candicidin.[14]

Labeled precursors were added to resting cells preincubated overnight in phosphate-limited (5×10^{-4} M phosphate) synthetic medium. Candicidin was extracted, purified by thin-layer chromatography and counted as indicated in Materials and Methods.

The specific activity of candicidin labeled from (^{14}C) acetate decreases after 2 hours (Fig. 2) due to exhaustion of the labeled acetate and dilution of the radioactive candicidin by unlabeled antibiotic formed thereafter. The specific activity of candicidin labeled with propionate increases over a 4-hour period, but decreases at 10-hour (not shown), and that of candicidin labeled with PABA increases during at least 4-hour after addition of the precursor (Fig. 2).

Double Labeling of Candicidin

Double-labeling studies are useful to define the biosynthetic origin and assembly of the moieties of the antibiotic. The specific incorporation of PABA and acetate into the aromatic moiety and the macrolide ring of the molecule of candicidin respectively, provided a model to prepare double labeled candicidin. Experimental results are summarized in Table 2. Tritiated acetate and (^{14}C) PABA are effectively incorporated into candicidin. The kinetics of incorporation are similar to that observed when each individual labeled precursor is added separately.

Discussion

The efficiency of incorporation of labeled acetate and propionate and the specific activities of candicidin achieved in short-term experiments (4~10 hours) using phosphate-limited resting cells are

Table 2. Kinetics of incorporation of radioactive precursors into candicidin during preparation of double-labeled candicin by phosphate-limited cells of *S. griseus*.

Time (hours)	(^{3}H)-Acetate incorporated into candicidin (dpm/ml of culture)*	(^{14}C)-PABA incorporated into candicidin (dpm/ml of culture)*	Candicidin (μg/ml)
1	17.9×10^3	16.3×10^3	142
2	24.4×10^3	29.9×10^3	170
3	33.8×10^3	56.1×10^3	198
4	52.1×10^3	83.3×10^3	237

2 μCi/ml of (^{3}H)-acetate and 1 μCi/ml of (^{14}C) PABA were added at time 0 to a resting cell culture preincubated overnight in synthetic medium with 5×10^{-4} M phosphate added. Candicidin was extracted and purified by thin-layer chromatography and counting of radioactivity and quenching corrections were made as indicated in Materials and Methods.

* Average of three determinations.

similar to those achieved by other authors in long-term experiments (5~8 days) using growing cells.[3,10,11] The higher rate of label incorporation into the antibiotic in the phosphate-limited system is due to the lack of growth-related metabolism which competes for precursors. The short duration of the experiments is of additional importance because of the thermolability and time-dependent degradation of polyene antibiotics. The slow linear incorporation of PABA into candicidin probably is related to the slow uptake of this precursor by the cell[2]. Propionate and acetate, on the other hand, are taken up by the cells very rapidly before dilution occurs[2].

Propionate appears to be a more suitable precursor than acetate for labeling of the macrolide ring of candicidin. The high degree of incorporation of propionate agrees with results on the biosynthesis of nystatin[12], amphotericin B[10,11] and lucensomycin[13]. Since propionate is incorporated as three carbon units without decarboxylation to acetate[13], these results indicate that propionyl-CoA (probably as methylmalonyl-CoA) is used together with acetyl-CoA (as malonyl-CoA) in the biosynthesis of candicidin. The aglycone macrolide of candicidin is thus a "heterogeneous" macrolide derived from at least two different types of building unit.

The high efficiency of incorporation and specific activity obtained with 2-(^{14}C) propionate as compared with other types of labeled propionate was also noted by LINKE *et al.*[11] in the formation of amphotericin B. It seems probable that this is due to the loss by decarboxylation of radioactivity from the C-1 carbon of 1-(^{14}C) propionate and U-(^{14}C) propionate during the condensation step.

Supplementation of the resting cell system with both tritiated acetate and (^{14}C) PABA provided an easy method for preparation of specifically double-labelled candicidin. Preliminary studies indicate that there is no randomization of acetate-derived tritium radioactivity in the aromatic moiety or (^{14}C) PABA-derived label into the macrolide ring.

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