SITE OF ACTIVATION OF *o*-SUCCINYLBENZOIC ACID DURING ITS CONVERSION TO MENAQUINONES (VITAMIN K_2)

R. KOLKMANN, G. KNAUEL, S. ARENDT and E. LEISTNER
Institut für Pharmazeutische Biologie und Phytochemie der Westfälischen Wilhelms-Universität, 4400 Münster, FRG

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1. Introduction

o-Succinylbenzoic acid (i.e., OSB or 4-(2′-carboxyphenyl)-4-oxobutanoic acid) (I) has been established as a precursor in the biosynthesis of menaquinones (V) and anthraquinones [1–3]. Its cell-free conversion to 1,4-dihydroxy-2-naphthoic acid (IV) is dependent on the presence of ATP and coenzyme A [4]. The reaction is catalyzed by 2 enzymes, viz. *o*-succinylbenzoyl-CoA acid synthetase and naphthoate synthase [5]. The overall reaction (I–IV) is unusual in that it represents a novel ring closure and aromatization reaction which has been proposed to be a Dieckmann reaction [6]. Such a reaction, however, requires activation at both carboxyl-groups whereas we have shown that only one carboxyl-group is activated [7]. A prerequisite for the elucidation of the mechanism of this unusual reaction is the identification of the site of activation of *o*-succinylbenzoic acid. In spite of difficulties [4,5] we were able to isolate the activated *o*-succinylbenzoic acid [7]. This has now made it possible to determine the site of activation. Evidence is presented here that the aromatic, rather than the aliphatic carboxyl-group is linked to coenzyme A in the activated intermediate.

2. Experimental

Material used, growth of *Mycobacterium phlei*, and extraction of the *o*-succinylbenzoyl-CoA acid synthetase were as in [7].

2.1. *Enzymic synthesis of the OSB-CoA ester*

The incubation mixture contained *o*-succinylbenzoyl-CoA acid synthetase preparation (1.5 mg), potassium phosphate (20 μmol, pH 6.5), dithiothreitol (0.04 μmol), $MgCl_2$ (4 μmol), ATP (2 μmol), CoASH (0.5 μmol), [1-^{14}C]OSB (I) (0.014 μmol, 0.1 μCi) in a final volume of 280 μl. Incubation was carried out at 30°C and terminated by cooling (0°C) and addition of formic acid (20 μl).

2.2. *Isolation, methylation and mild hydrolysis of the OSB-CoA ester*

The incubation mixture (section 2.2) was applied to cellulose thin-layer plates (cellulose MN 300 HR, Macherey and Nagel, Düren) and developed in *n*-butanol–acetic acid–water (5:2:3, by vol.). The coenzyme A ester was located by radio scanning (R_F 0.63) and eluted with water (1.9 ml, 0°C). Yield 66 400 dpm (i.e., 30% of I). The eluate was diluted with a 10-fold excess of distilled methanol and cooled to 0°C. Methylation was carried out for 1 min at 0°C by addition of an etheral diazomethane solution.

The diazomethane was removed by flushing N_2 through the solution. From the reaction mixture methanol was removed by evaporation under vacuum at room temperature and sodium hydroxide (50 μl, 2 M) was added. After 12 min at room temperature hydrochloric acid (500 μl, 1 M) was added and the aqueous solution was immediately extracted with ethyl acetate. The organic phase was dried (sodium sulfate), filtered and found to contain 37 900 dpm, whereas 1000 dpm remained in the aqueous phase. A mixture of the synthetic 'aliphatic' (VI) (2.117 mg) and 'aromatic' (VII) (2.033 mg) methyl ester of OSB (I) was added as carrier.

2.3. *Purification of the 'aliphatic' (VI) and 'aromatic' (VII) methyl esters of I*

The methyl esters were separated on a precoated

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Table 1
Purification of the 'aliphatic' (VI) and the 'aromatic' (VII) monomethyl ester of *o*-succinylbenzoic acid (I) using 3 different chromatography systems

Purification step	OSB-monomethyl ester	Amount (μmol)	Radioact. (dpm)	Spec. radioact. (dpm/μmol)
1	(a) Aliphatic (VI)	4.3	12 390	2885
	(b) Aromatic (VII)	3.5	251	71
2	(a) Aliphatic (VI)	2.6	7060	2715
	(b) Aromatic (VII)	2.5	54	21
3	(a) Aliphatic (VI)	0.8	2480	3096
	(b) Aromatic (VII)	1.0	0	0

Carrier material of both monomethyl esters was added to a mixture which had been obtained by mild hydrolysis of enzymically synthesized and subsequently methylated coenzyme A ester of *o*-[1-^{14}C]succinylbenzoic acid

silica gel plate (Sil G UV_{254}, Merck, Darmstadt) with chloroform–ethyl acetate–formic acid (45:6.6:0.5, by vol.) as a solvent. 'Aliphatic' ester R_F 0.30, 'aromatic' ester R_F 0.26. Both esters were eluted (ethyl ether) and rechromatographed separately in two different solvent systems (silica gel, toluene–acetic acid (9:1, by vol.), 'aliphatic' ester R_F 0.14, 'aromatic' ester R_F 0.24; *n*-hexane–ethylmethylketone (7:3, by vol.), 'aliphatic' ester R_F 0.23, 'aromatic' ester R_F 0.20). After each purification the eluate was evaporated, the residue dissolved in methanol and the specific radioactivity determined (table 1).

2.4. *Synthesis of the 'aliphatic' (VI) and the 'aromatic' (VII) monomethyl ester of I*

The dimethyl ester of I (200 mg) was dissolved in ethyl ether (100 ml) containing HCl (6 N, 8 ml) and refluxed for 2.5 h. Water was added to the solution and the organic layer collected. Extraction of the aqueous phase was repeated and the combined ether extracts were washed with water. The dried ether fraction was concentrated and applied to preparative thick-layer plates (Sil G UV_{254}, Merck, Darmstadt), which were developed 3 times in chloroform–ethyl acetate–formic acid (45:6.6:0.5, by vol.). OSB, residual amounts of the dimethyl ester and 2 bands (R_F 0.29 and R_F 0.25) appeared which were eluted, treated with charcoal and identified as follows (section 2.6).

2.5. *Identification of the monomethyl esters (VI, VII) of OSB*

Both compounds which appeared at R_F 0.29 and R_F 0.25 were identified as monomethyl esters VI and VII on the basis of their mass and ^{1}H NMR spectra as well as their R_F-values relative to OSB (R_F 0.1) and OSB dimethyl ester (R_F 0.8) in the solvent system mentioned above (section 2.5). On mass spectrometry both compounds (at R_F 0.29 and R_F 0.25) gave the same molecular ion (M^+ 236) but due to the phthalide cation different base peaks at m/e^+ 149 or m/e^+ 163 (163, i.e., 149 + CH_2), respectively. These observations indicated that the compound at R_F 0.29 is the 'aliphatic' ester (VI) whereas the compound at R_F 0.25 is the 'aromatic' ester (VII). This was confirmed by ^{1}H NMR spectrometry. As expected the compound with R_F 0.29 gave a methyl signal at 3.71 ppm whereas the compound with R_F 0.25 gave a signal at 3.88 ppm relative to TMS. The dimethyl ester of OSB gave clearly distinguishable signals at 3.71 ppm and 3.88 ppm. Thus the compound at R_F 0.29 is the 'aliphatic' ester (VI) whereas the compound at R_F 0.25 is the 'aromatic' ester (VII).

3. Results and discussion

It has been shown by a double labelling experiment [7] that the activated *o*-succinylbenzoic acid is a *mono*-coenzyme A derivative, presumably a coenzyme A thioester. Since *o*-succinylbenzoic acid (I) has 2 carboxyl groups activation at either the 'aromatic' or 'aliphatic' carboxyl group can be envisaged (fig.1). Isolation of the activated *o*-succinylbenzoic acid [7] has now made it possible to determine the site of activation. Enzymatically prepared *o*-succinylbenzoic acid

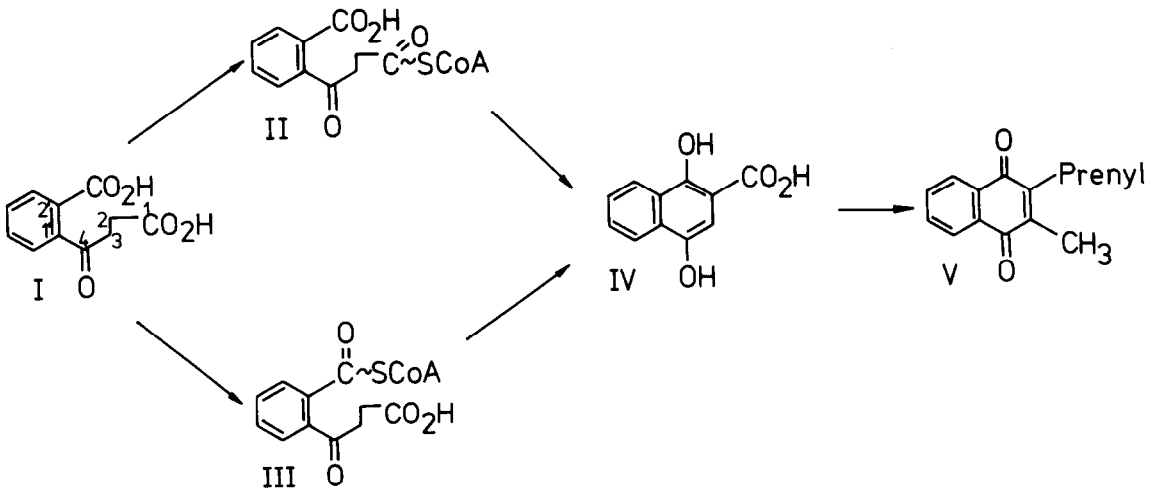
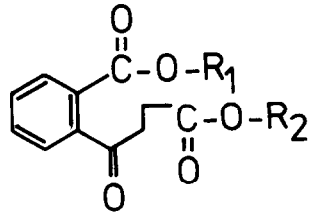


Fig.1. Activation of *o*-succinylbenzoic acid (I) at the 'aromatic' or 'aliphatic' carboxyl group and conversion of the resultant coenzyme A ester (II or III) to 1,4-dihydroxy-2-naphthoic acid (IV) and menaquinones (V). The intermediacy of II is disproven on the basis of results reported herein.

coenzyme A ester (either II or III, fig.1) was isolated and treated with diazomethane under very mild conditions which would ensure methylation of the free carboxyl group. The resultant diester was hydrolyzed under conditions which would preferentially cleave the thioester group. The first chromatography of the methyl ester fraction showed that 80–100% of the activity coincided with the 'aliphatic' monomethyl ester (VI) of *o*-succinylbenzoic acid (I), whereas 0–20% cochromatographed with the free acid indicating partial hydrolysis also of the methyl ester group. No other radioactive product (*o*-succinylbenzoic acid spirodilactone, a decomposition product of the thioester [7], or the diester of *o*-succinylbenzoic acid) was detectable. Both the authentic 'aliphatic' (VI) and the 'aromatic' ester (VII) which had been added to the ethyl acetate fraction were taken through three subsequent purification steps and their specific activity determined. It was found that the specific radioactivity of the 'aliphatic' ester (VI) remained constant whereas the 'aromatic' ester (VII) turned out to be inactive (table 1). Association of radioactivity with the 'aliphatic' monomethyl ester (VI) rather than the 'aromatic' monomethyl ester (VII) proves that the coenzyme A is bound to the 'aromatic' (see III), rather than the 'aliphatic' (see II) carboxyl group of *o*-succinylbenzoic acid. Thus the previously made predictions [4–6] turned out to be correct. These experiments are also consistent with the finding [7] that only 1 carboxyl group of *o*-succinylbenzoic acid (I) is activated because a monomethyl ester is obtained rather than free *o*-succinylbenzoic acid alone. We have also shown [7] that the coenzyme A ester is converted to 1,4-dihydroxy-2-naphthoic acid (IV) without any cofactor requirement. This means that the coenzyme A ester (III) is subject to a Dieckmann condensation which is unusual because only 1 carboxyl group is activated. This novel ring closure reaction is likely to be distributed in nature as widely as are vitamin K_2 (menaquinones) and vitamin K_1 (phylloquinone).



VI R_1=H, R_2=Me

VII R_1=Me, R_2=H

Fig.2. Structure of the 'aliphatic' (VI) and 'aromatic' (VII) monomethyl esters of OSB.

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