Exchange of Valine-Oxygen during the Biosynthesis of δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-valine

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Incubation of [$^{18}O_2$]valine with purified ACV synthetase from *Cephalosporium acremonium* gave exclusive incorporation of a single ^{18}O label into δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV), consistent with the formation of a covalent valinoyl-ACV synthetase complex.

The first step in the biosynthesis of penicillins and cephalosporins involves the enzymatic condensation of L-α-aminoadipic acid, L-cysteine and L-valine with concomitant stereochemical inversion of the valine α-centre, generating the tripeptide δ-(L-α-aminoadipolyl)-L-cysteinyl-D-valine (ACV).[1] This ATP-driven process is catalysed by ACV synthetase, a member of the non-ribosomal peptide synthetase class of enzymes.[2] Such enzymes have been proposed to operate *via* a thiol-template mechanism with a pantotheine 'swinging arm' transferring activated amino acids to sites on the enzyme capable of catalysing peptide bond formation.[3] Amino acid activation involves a two-step process with initial aminoacyl adenylate formation followed by transfer of the amino-acyl moiety onto the enzyme, probably in the form of a thioester link to a cysteine residue.[3,4] Whilst no bond is ultimately formed to the valine carboxy group of ACV, there is evidence for activation of this amino acid as an amino-acyl adenylate, and subsequent valinoylation of ACV synthetase.[4] It is possible that such activation is required to facilitate epimerization of the valine α-centre, probably at the putative thioester stage.[3,5]

In vivo studies have shown the exchange of one and both valine oxyen atoms during penicillin[5] and ACV[6] biosynthesis through feeding experiments with [$^{18}O_2$]valine. However, the interpretation of data from such experiments was hampered

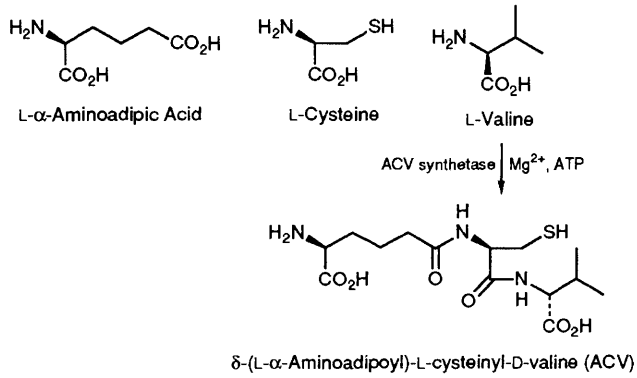

Fig. 1

Table 1 Electrospray mass spectrometry results for ACV and recovered valine from the incubation of D,L-[$^{18}O_2$]valine with ACV synthetase from *C. acremonium* in the presence of aminoadipic acid and cysteine

Compound								
	m/z	117	118	119	120	121	122	123
1. Synthetic	% obs.	—	5	—	—	10	100	7
D,L-[$^{18}O_2$]valine			$^{16}O_2$		$^{16}O^{18}O$		$^{18}O_2$	
	m/z	363	364	365	366	367	368	369
2. ACV formed[a]	% obs.	15	7	10	100	22	8	4
			$^{16}O_2$		$^{16}O^{18}O$		$^{18}O_2$	
	m/z	117	118	119	120	121	122	123
3. Recovered valine	% obs.	1	2	2	3	—	100	6
			$^{16}O_2$		$^{16}O^{18}O$		$^{18}O_2$	

[a] ACV was analysed as its free thiol form following DTT reduction.

Table 2 Electrospray mass spectrometry result for recovered valine from the incubation of D,L-[$^{18}O_2$]valine with ACV synthetase from *C. acremonium* in the absence of aminoadipic acid and cysteine

Compound								
	m/z	117	118	119	120	121	122	123
1. Recovered valine	% obs.	—	1	—	7	—	100	6
			$^{16}O_2$		$^{16}O^{18}O$		$^{18}O_2$	

by the possibility of valine carboxyl-oxygen exchange processes unrelated to the β-lactam biosynthetic pathway. We now report *in vitro* studies that demonstrate clearly the exchange of a single carboxyl-oxygen of valine during the biosynthesis of ACV. These studies have become possible due to the availability of partially purified ACV synthetase of high specific activity and the advent of electrospray mass spectrometry, which has allowed the isotopic analysis of underivatized ACV on a small scale (<10 μg). Previously, chemical derivatization of ACV, which is low yielding on a small scale, has been required to obtain chemical ionization mass spectra.

ACV synthetase was partially purified form *C. acremonium* using a modification† of the literature procedure[7]. Following gel filtration chromatography, the enzyme preparation typically had a specific activity in excess of 700 pkat g^{-1}.† Incubations with racemic [$^{18}O_2$]valine typically contained: $MgCl_2$ (32 mmol dm^{-3}), L-α-aminoadipic acid (2.5 mmol dm^{-3}), L-cysteine (2.5 mmol dm^{-3}), racemic [$^{18}O_2$]valine (5 mmol dm^{-3}), DTT (3 mmol dm^{-3}), ATP (20 mmol dm^{-3}), and ACV synthetase (approximately 1.0 pkat total activity) in a total volume of 3 ml. Incubations were conducted at 28 °C for at least 4 h. ACV formed (*i*) and unreacted valine (*ii*) were purified from the reaction mixture by reverse phase HPLC [octadecylsilane column, (*i*) MeOH: 25 mmol dm^{-3} NH_4HCO_3 (1:4); (*ii*) 25 mmol dm^{-3} NH_4HCO_3 as eluents].[6] Sufficient tripeptide was formed in this manner (approximately 1 mg, 40% conversion) to enable the first positive identification of ACV by ^{1}H NMR (500 MHz) spectroscopy from an *in vitro* experiment. The purified ACV and valine were analysed by electrospray mass spectrometry to detemine the isotopic concentration of ^{18}O in the samples (Table 1). The data obtained indicates, within experimental error, exclusive loss of a single ^{18}O label during ACV formation *in vitro* and little or no exchange of label in the recovered valine. In addition, incubation of [$^{18}O_2$]valine with ACV synthetase in the absence of aminoadipic acid and cysteine also resulted in little or no loss of ^{18}O label (Table 2).

† Full experimental details of the modified enxyme preparation will be published elsewhere. Specific activity units[7] are quoted as pmol ACV formed s^{-1} g^{-1} protein.

Thus, our earlier *in vivo* studies in which exchange of one and both valine oxygens was observed in the formation of ACV are probably best rationalised by the operation of exchange processes unrelated to ACV synthetase.

The *in vitro* exchange of ^{18}O label from [$^{18}O_2$]valine observed in this study is indicative of the effectively *non-reversible* formation of a reactive covalent intermediate between the valine carboxy group and ACV synthetase during ACV formation. This contrasts with the work of Vater *et al.*[8] who reported that for the peptide-forming Gramicidin synthetase, formation of amino-acylenzyme thioesters is a *reversible* process, thioester formation being reversed by the addition of AMP and pyrophosphate.

This report details the first *in vitro* mechanistic studies on ACV synthetase using labelled amino acids. Further studies to investigate the validity of the proposed thiol-template mechanism for this enzyme are in progress.

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