Kinetic mechanism of NADH-enoyl-ACP reductase from *Brassica napus*

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Abstract Enoyl-ACP reductase, a component of fatty acid synthase, is a target for anti-microbial agents and herbicides. Here we demonstrate the kinetic mechanism to be a compulsory-order ternary complex with NADH binding before the acyl substrate. Matrix-assisted laser desorption ionisation mass spectrometry analysis of enzymatically and synthesised crotonyl-ACP substrate showed the former to contain a single acyl group, whereas the latter contained up to four additional crotonylations. The use of authentic crotonyl-ACP will be important in future kinetic and crystallographic studies.

Key words: Enoyl reductase; Crotonyl-ACP; Crotonyl-coenzyme A; Matrix-assisted laser desorption ionisation mass spectrometry; *Brassica napus*

1. Introduction

The synthesis of fatty acids in plants and most prokaryotes is carried out by a series of soluble enzymes, each of which encodes a single catalytic activity. During fatty acid biosynthesis, the growing carbon chain is attached to acyl carrier protein (ACP); the carbon chain is elongated by repeated condensation of a two-carbon unit from the donor malonyl-ACP. The resulting β-keto group is sequentially reduced to a hydroxyl, enoyl and methylene group before a further condensation reaction takes place.

Enoyl-ACP reductase (ENR), which catalyses the NADH-specific reduction of a *trans* carbon–carbon double bond to produce saturated acyl-ACP has been purified from spinach leaves [1], avocado mesocarp [2] and *Brassica napus* seeds [3]. There has been extensive interest in ENR from both plant and bacterial sources as a potential target for development of new anti-microbial agents and herbicides. ENR is a target for isoniazid inhibition of the growth of *Mycobacterium tuberculosis*, the causative agent of tuberculosis [4,5], as well as the action of diazaborines that inhibit the growth of *Escherichia coli* [6,7]. The three dimensional (3D) structures of the ENR from *B. napus* [8], *M. tuberculosis* [9] and *E. coli* [10] have all been determined. Additionally it has been shown that the mode of action of the broad anti-microbial agent triclosan, a widely used additive in the detergent industry, is specifically directed at ENR [11,12]. Despite the solution of the 3D structure of ENR, and its importance as a target for rational drug design, no analysis has been performed on the kinetic mechanism of the enzyme.

The natural substrate for *B. napus* ENR is enoyl-ACP; the enzyme will also use enoyl-coenzyme A (CoA) derivatives as substrate analogues, but with higher K_m values. Crotonyl-CoA (cro-CoA) has most often been used in studies with this enzyme, as it is readily available commercially.

Here we report that use of the substrate analogue cro-CoA, in kinetic experiments, and crotonyl-ACP (cro-ACP), in substrate binding experiments, to investigate the kinetic mechanism of ENR. Enzymatically synthesised cro-ACP is shown to contain a single crotonyl substitution, whereas chemically synthesised cro-ACP contains up to four additional crotonyl groups. The use of authentic substrate will be important in future ENR–substrate co-crystallisation trials.

2. Materials and methods

2.1. ENR assay

The standard assay for detection of ENR activity contained 10 mM sodium phosphate buffer (pH 6.2), 120 μM cro-CoA, 140 μM NADH and a suitable amount of enzyme; the initial velocity of NADH oxidation was recorded at 340 nm. Assays carried out to determine the order of binding contained various concentrations of cofactor and substrate as described under Section 3.

2.2. Synthesis of analysis of ACPs

Recombinant *E. coli* apo-ACP was produced from a pET11d-based expression plasmid containing a 600 bp *E. coli* genomic DNA insert which includes the open reading frame for *E. coli* ACP (gift of A.R. Stuitje). Cro-ACP was synthesised by direct crotonyl-phosphopantetheine transfer from cro-CoA to apo-ACP catalysed by holo-ACP synthetase, or by chemical modification of holo-ACP purified from *E. coli* [13]. The enzymatic reaction contained 50 mM potassium phosphate buffer, 333 μM apo-ACP, 666 μM cro-CoA, 2 mM EDTA, 10 mM $MgCl_2$, 3.3 μM holo-ACP synthase (pH 7.3) [14]. The reaction was carried out for 3 h at 37°C, buffer exchanged using a PD10 column (Pharmacia) equilibrated in milli Q water and the product eluted in the same prior to freeze-drying and storage at −80°C.

The identity of ACPs was confirmed and quantified by visualisation of the mobility on 18% native polyacrylamide gels containing 0.5 M urea [15]. Matrix-assisted laser desorption ionisation (MALDI-TOF) mass spectrometry analyses of ACP and cro-ACP were carried out using a Voyager DE-STR BioSpectrometry workstation (PerSeptive Biosystems, Framingham, MA, USA) in linear positive ion mode with delayed extraction. The samples were diluted in 50% acetonitrile, 0.1% trifluoroacetic acid to a final concentration of approximately 1.0 pmol/μl and then loaded together (1:1) with a 3,5-dimethoxy-4-

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Abbreviations: ACP, acyl carrier protein; cro-ACP, crotonyl-ACP; cro-CoA, crotonyl-coenzyme A; ENR, enoyl-ACP reductase; enzyme, enoyl-ACP reductase NADH-specific (EC 1.3.1.9)

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hydroxycinnamic acid (Sigma) matrix (10 μg/μl in 50% acetonitrile, 0.1% trifluoroacetic acid) directly onto a sample grid. The grid was allowed to air dry before being loaded into the mass spectrometer. A laser intensity of 2500 was used to obtain optimum resolution of the ACP spectra and a low mass gate of 4000 *m/z* was applied to suppress interference from matrix ion peaks. A calibration mixture containing bovine insulin (5734.59 Da), *E. coli* thioredoxin (11 674.48 Da), and horse myoglobin (16 952.56 Da) was used to determine masses.

2.3. Ultrafiltration binding assays

Assays were carried out to assess the binding of each substrate to the enzyme in the absence of the other substrate. Ultrafree-MC 30 000 NMWL centrifugal micro-concentrators (Millipore, UK) were used to retain the enzyme, and any bound substrate molecules, above a 30 kDa cut-off filter. Duplicate assays were arranged so that substrate was mixed with pure protein, above the filter, in a molar ratio of 1 ENR tetramer (400 pmol):10 substrate (4 nmol). The enzyme–substrate mixture was allowed to equilibrate for 10 min at room temperature prior to filtration, which was carried out in a centrifuge at $5000\times g$ for 10 min at 20°C. The quantity of substrate present in the filtrate was determined from molar extinction coefficients. The molar extinction coefficient of *trans*-2-unsaturated thioesters is 6.7×10^3 M^{-1} m^{-1} at 263 nm [1] and that of NADH is 6.22×10^3 M^{-1} cm^{-1}.

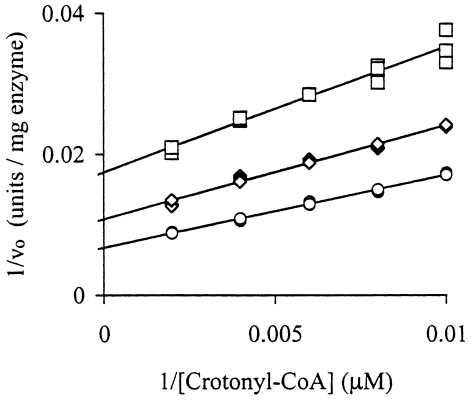

Fig. 1. Determination of kinetic mechanism as sequential or substitution. Assays were carried out using varying cro-CoA concentrations at fixed, non-saturating concentrations of NADH (3.44 μM (□), 9.09 μM (◇), and 14.20 μM (○)). Assays were performed in triplicate and data analysed by linear regression; the family of lines produced on the double reciprocal plot converged, indicating a sequential mechanism.

3. Results and discussion

3.1. Characterisation of recombinant plant ENR

B. napus ENR was expressed in *E. coli* BL21 (DE3) from the T7-based plasmid pEAR2 [16] and purified essentially by the procedure used to isolate the enzyme from rape seed [3]. During cloning procedures used to introduce plant ENR cDNA into the *E. coli* expression vector, the N-terminal amino acid sequence was changed from SESSES to MAESSES [16]. Protein sequence analysis of the expressed recombinant protein revealed the sequence AESSES, indicating removal of the initiating formyl-methionine residue.

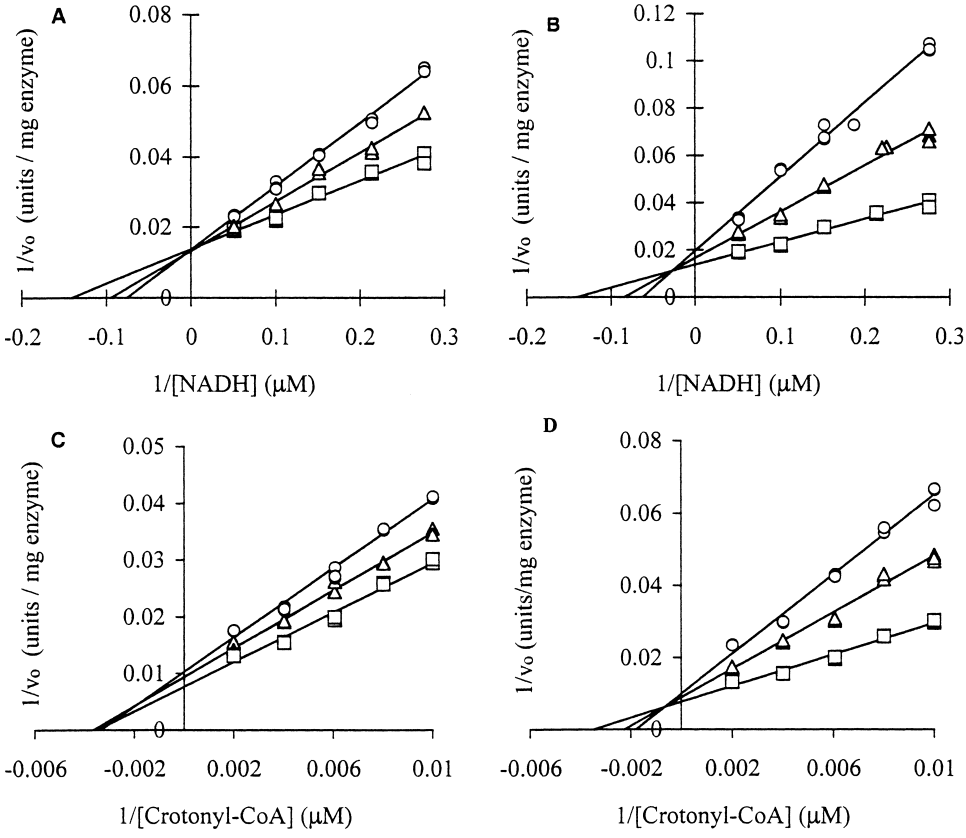

Fig. 2. Product inhibitor studies to determine the order of substrate binding. Assays were performed using varying concentrations of one substrate at fixed, non-saturating concentrations of the second substrate in the absence of product (□) and in the presence of 0.5 mM (△) and 1.0 mM (○) product. Double reciprocal plots using: (A) varying NADH concentrations at 120 μM cro-CoA and different fixed concentrations of NAD^+; (B) varying NADH concentrations at 120 μM cro-CoA and different fixed concentrations of butyryl-CoA; (C) varying cro-CoA concentrations at 10 μM NADH and different fixed concentrations of NAD^+; (D) varying cro-CoA concentrations at 10 μM NADH and different fixed concentrations of butyryl-CoA.

ENR purified from developing rape seed had apparent K_m values of 7.6 μM for NADH and 178 μM for cro-CoA [3]; the recombinant protein showed similar Michaelis constants of 9.5 μM for NADH and 253 μM for cro-CoA when assayed under identical conditions. Analysis of the recombinant enzyme revealed a subunit mass of approximately 32 kDa on SDS–PAGE and a native molecular mass of 130 kDa by gel filtration. These criteria indicate that the plant ENR cDNA is expressed in *E. coli* and the purified protein is correctly assembled and fully functional (data not shown).

The enzyme was able to reduce cro-ACP, cro-CoA and decenoyl-CoA with NADH as cofactor, but was unable to act on any of these substrates in the presence of NADPH. These data confirm that the *B. napus* cDNA encodes an NADH-specific ENR and in this respect it is unlike the *E. coli* enzyme that is able to utilise both NADH and NADPH as cofactor [17].

3.2. Molar extinction coefficient for ENR

Comparison of amino acid analysis data with the known amino acid sequence of ENR, deduced from the cDNA, showed that the analysis was accurate to ±6% for the amino acids: glycine, arginine, phenylalanine, leucine and lysine which are not subject to modifications during analysis [18]. The same preparation of recombinant ENR was also used to determine the spectral properties of the enzyme. Data from these two experiments were used to calculate the molar extinction coefficient at 278 nm of 2.95×10^5 M^{-1} cm^{-1}.

3.3. Stabilisation of the catalytic activity of diluted enzyme

It was necessary to carry out enzyme assays over an extended period of time, using the same dilution of enzyme; therefore conditions were sought to minimise time-dependent changes in catalytic activity. Dilution in 37.5 mM sodium phosphate, 1 mg/ml bovine serum albumin (BSA), 1 mM dithiothreitol, 1 mM EDTA and 20% w/v glycerol (pH 6.2), maintained full activity over a 5 h period; 98% of the activity, present immediately after dilution, was observed more than 24 h later. The presence of BSA did not reduce the concentration of cro-CoA in the assay mixture, as determined by initial velocity measurements of freshly prepared enzyme dilutions in the presence and absence of BSA.

3.4. Determination of reaction mechanism; sequential or substitution?

An investigation to differentiate between sequential and substitution reaction mechanisms was carried out using cro-CoA as a substrate analogue. In a series of assays using fixed, non-saturating levels of NADH (3.44 μM, 9.09 μM and 14.20 μM) and varying concentrations of cro-CoA (100–500 μM), the family of lines produced on a double reciprocal plot converged (Fig. 1). A similar converging pattern of lines was observed when fixed, non-saturating levels of cro-CoA and varying NADH concentrations were employed (data not show). These patterns are characteristic of a sequential or ternary complex reaction mechanism.

3.5. Product inhibition studies

The products of the ENR reaction, butyryl-CoA and NAD^+, were utilised as inhibitors in order to determine whether the reaction proceeded via a random or compulsory-order ternary-complex mechanism using the rules of Cleland [19]. Analysis of the results in the form of double reciprocal plots indicated competitive inhibition when NADH was varied in the presence of NAD^+ (Fig. 2A). When NADH was varied in the presence of butyryl-CoA (Fig. 2B), cro-CoA varied in the presence of NAD^+ (Fig. 2C) and cro-CoA varied in the presence of butyryl-CoA (Fig. 2D), mixed patterns of inhibition were observed. This combination of inhibition patterns is indicative of a compulsory-order ternary-complex mechanism with NADH binding first.

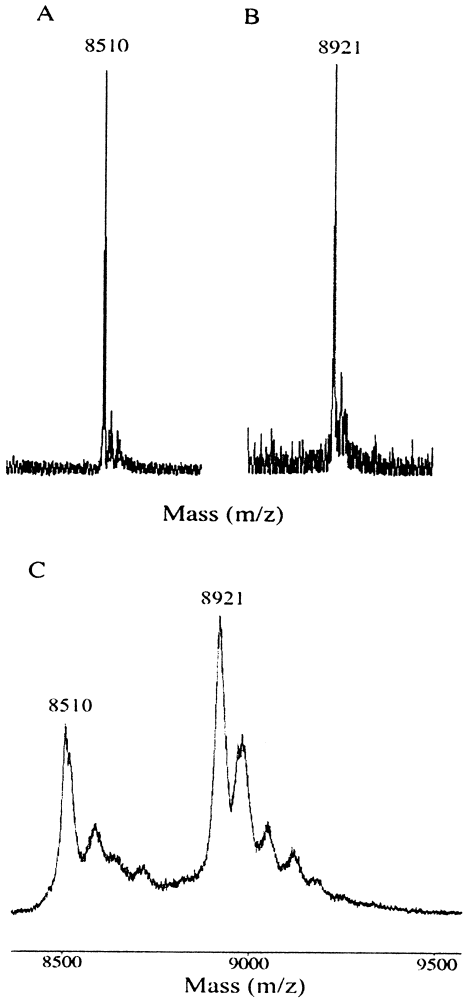

Fig. 3. MALDI-TOF mass spectrometry analysis of ACPs. (A) *E. coli* apo-ACP, (B) enzymatically synthesised cro-ACP and (C) chemically synthesised cro-ACP.

3.6. Substrate binding assays

Evidence was sought to verify the reaction mechanism using substrate binding assays and ACP substrates. The authenticity of apo-ACP and enzymatically synthesised cro-ACP was verified by mass spectrometry (Fig. 3A,B) and visualisation on 18% native gels containing 0.5 M urea. The mass recorded for cro-ACP of 8921 Da indicated the presence of a single crotonyl group.

The cofactor, NADH, bound to ENR in a ratio of 1 mol of enzyme to approximately 4 mol of NADH (1:3.6 and 1:4.3 in duplicate assays). The native ENR enzyme is a tetramer with each subunit having one NADH binding site, capable of binding NADH in the absence of the other substrate. This corre-

lates with our results of product inhibition kinetic experiments and also with crystallographic data [20].

There was no measurable interaction between cro-ACP and ENR in substrate binding assays. Therefore we conclude that cro-ACP does not bind to ENR in the absence of NADH and confirms that the reaction mechanism must proceed via an ordered ternary complex with NADH binding first. A model has been proposed from recent crystallographic studies that suggested the acyl chain of enoyl-ACP would lie above the nicotinamide ring of the cofactor in the substrate binding site, allowing the correct geometry for hydride attack of the enoyl substrate [21]. Such a model would require the NADH cofactor to be bound to the enzyme before the enoyl substrate. Similarly, there was no interaction between apo-ACP and ENR, indicating no interactions occur between amino acids of the enzyme and the carrier protein in the absence of NADH.

3.7. Implications for future studies

Cro-CoA is a convenient substrate analogue for measurement of enzyme activity. However, crystals of ENR grown in the presence of cro-CoA showed binding at the site where the adenine ring and associated ribose of NADH bind in an ENR–NAD binary complex [20]. Therefore cro-CoA has limited utility in ENR–acyl substrate co-crystals and future work will require the use of the true ACP-based substrates.

Previous syntheses of cro-ACP have largely been conducted using holo-ACP, purified from *E. coli*, chemically modified with crotonic anhydride [13]. Fig. 3C shows a MALDI-TOF spectrum of chemically crotonylated *E. coli* ACP. The peak at 8510 Da is apo-ACP (a minor component of the starting material), which does not contain the phosphopantetheine prosthetic group and therefore was not acylated to form cro-ACP. Cro-ACP has a mass of 8921 Da and is the major product of the reaction. Several additional peaks follow those at 8510 Da and 8921 Da, with a periodicity of approximately 71 Da, indicating up to four additional crotonylations of the carrier protein by this method. Cro-ACP synthesised chemically can be used as an assay substrate [13], but would complicate attempts at structural determination of an ENR–cro-ACP complex because of the additional crotonylations of the carrier protein. Using the enzymatic synthesis described in this work, crotonylation of the 4′ phosphopantenthenyl thiol group is uniquely and specifically achieved (Fig. 3B). The native substrate will be required for detailed kinetic and crystallographic studies.

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