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A spectrophotometric assay for deacetoxycephalosporin C synthase

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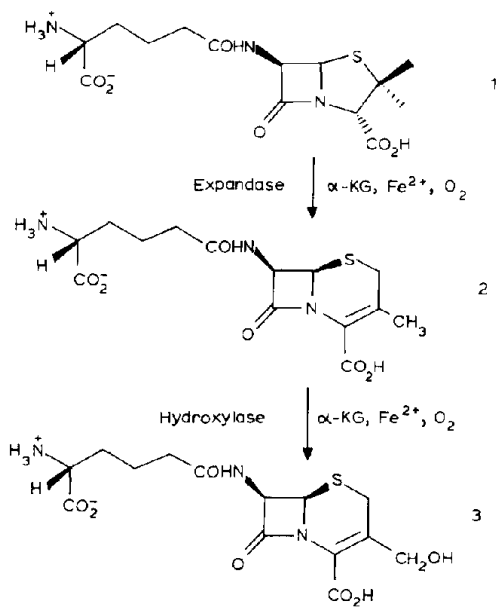
A continuous direct spectrophotometric assay for deacetoxycephalosporin C synthase was developed, based on the absorption at 260 nm characteristic of the dihydrothiazine moiety of cephalosporins. K_m values of 0.18 mM for penicillin N and 0.16 mM for α-ketoglutarate were determined. A coupled assay using succinate thiokinase, pyruvate kinase and lactate dehydrogenase showed that succinate was a product of both deacetoxycephalosporin C synthase and hydroxylase reactions. The expandase reaction exhibited a 1:1.06 stoichiometry for deacetoxycephalosporin C and succinate.

Cephalosporin; Expandase; Hydroxylase; Succinate; Enzyme kinetics

1. INTRODUCTION

Deacetoxycephalosporin C synthase catalyses the oxidative expansion of the five-membered thiazolidine ring of penicillin N (Pen N) [1] to a six-membered cephem (deacetoxy-cephalosporin C; DAOC; [2]). The cephem is then hydroxylated to deacetylcephalosporin C (DAC) [3] before further metabolism to cephalosporin C ([1,2] reviews). In the eukaryote *Cephalosporin acremonium* (*Acremonium chrysogenum*) expandase and hydroxylase activities appear to be properties of a single 40 kDa monomeric protein [3–5] in contrast to the prokaryote *Streptomyces clavuligerus*, where the activities have been separated on DEAE-Trisacryl chromatography [6].

Studies on DAOC synthase have been based on the hole plate bioassay [7], TLC, HPLC or NMR analysis of products after enzyme incubations. All these methods are discontinuous and therefore unsatisfactory for initial rate determinations. The bioassay is sensitive but lacks specificity, while the other methods show greater specificity but decreased sensitivity.



Scheme 1.

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We therefore developed a continuous, direct spectrophotometric assay for DAOC synthase activity based on the generation of increased UV absorption at λ_{max} 260 nm characteristic of the dihydrothiazine moiety of cephalosporins. In addition, we have shown that succinate was a product of both expandase and hydroxylase activities in *Cephalosporium*, by coupling succinate production to succinate thiokinase, pyruvate kinase and lactate dehydrogenase. Succinate was produced stoichiometrically with DAOC (1.06:1) in the expandase reaction.

2. MATERIALS AND METHODS

2.1. *Materials*

Succinate thiokinase, pyruvate kinase (rabbit muscle, type VII), lactate dehydrogenase (porcine heart, type XXXV), NAD^+ and GTP (Li salt, type IX) were obtained from Sigma, London. All other materials were as described [3,8].

2.2. *Enzyme preparation*

Partially purified DAOC synthase/hydroxylase (about 50% pure) was prepared by chromatography on Sephadex G-75 and Matrex Gel Red A (Procion Red HE3B) from *Cephalosporium acremonium* CO 728 essentially as described [3,8].

2.3. *Enzyme assay*

2.3.1. Hole plate bioassay

This was performed as described [3,7] using supersensitive *E. coli* ESS 21/30. In this procedure DAC as well as DAOC was determined in the assay, but the relative amount of the former was very low under the conditions used.

2.3.2. Direct spectrophotometric assay

Enzyme assays were carried out at 30°C, unless otherwise stated, in non-reflecting semi-micro quartz cuvettes, using a Pye Unicam PU 8800 UV-VIS double beam recording spectrophotometer. Assays contained α-ketoglutarate (0.8 mM), DTT (10 mM), $(NH_4)_2SO_4$ (80 mM), $FeSO_4$ (0.04 mM), L-ascorbate (0.001 mM), penicillin N (1 mM) and enzyme (60 μl; 100 nM final concentration) in Tris-HCl buffer, pH 7.4 (50 mM), containing 0.015% NaN_3, final volume 600 μl. Blanks contained all reagents except Pen N. Blanks containing Pen N but without enzyme were also used. Assays were started by the addition of enzyme, and the reaction monitored at 260 nm. 1 IU of enzyme is defined as that amount catalysing the production of 1 μmol of product $\cdot$ min^{-1} under the conditions of the assay. As with the hole plate bioassay, this assay also measures production of other cephalosporins, although their relative amounts will be very low under the conditions of the assay.

2.3.3. Coupled spectrophotometric assay for succinate production

In the preliminary experiments, measurement of succinate production was coupled to (i) mitochondrial succinate dehydrogenase and assayed by phenazine methosulphate and dichlorophenolindophenol [9], and (ii) NADH depletion via the reverse reaction of succinic semialdehyde dehydrogenase were investigated. In the former case, the high blank rate made the assay untenable, while the equilibrium constant for the latter reaction lay well towards succinate production at the pH optimum of the expandase reaction.

We therefore measured succinate production by a coupled assay method using succinate thiokinase, pyruvate kinase and lactate dehydrogenase [10]:

$$\text{Succinate} + \text{Coenzyme A} + \text{GTP} \xrightarrow{\text{succinate thiokinase}} \text{CoA} + \text{GDP} + P_i \quad (1)$$

$$\text{GDP} + \text{phospho}\textit{enol}\text{pyruvate} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{GTP} \quad (2)$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + NAD^+ \quad (3)$$

Assays, at 30°C, in semi-micro non-reflecting quartz cuvettes contained all the components of the direct spectrophotometric assay with either Pen N or DAOC (1 mM) as substrates together with coenzyme A (1 mM), phospho*enol*pyruvate (1.5 mM), NADH (0.2 mM), GTP (0.2 mM), $MgCl_2$ (10 mM), KCl (100 mM), lactate dehydrogenase (5 IU), pyruvate kinase (4 IU) and succinic thiokinase (1.4 IU). The reaction was monitored at 340 nm using a Pye Unicam PU 8800 spectrophotometer. Blanks, containing all components except Pen N or DAOC, were used

throughout, and the assay system (in the absence of the expandase components) was checked using succinate.

Assays were linear for at least 20 min, and NADH depletion was only observed on the addition of Pen N or DAOC.

3. RESULTS AND DISCUSSION

Fig.1 shows the UV-absorption spectra of DAOC and Pen N. The molar extinction coefficient of DAOC (ϵ_{260}) at λ_{max} (260 nm) was calculated as $7.34 \pm 0.11 \times 10^3$. Similar ϵ_{260} values were calculated for DAC and cephalosporin C. Incubation of Pen N with enzyme and co-factors gave a linear increase in A_{260} for up to 2 h. There was a linear increase in $\Delta A_{260} \cdot \text{min}^{-1}$ over a six-fold range of enzyme concentration indicating no enzyme polymerisation over the concentration range studied. The pH profile of the enzyme using the spectrophotometric assay was very similar to

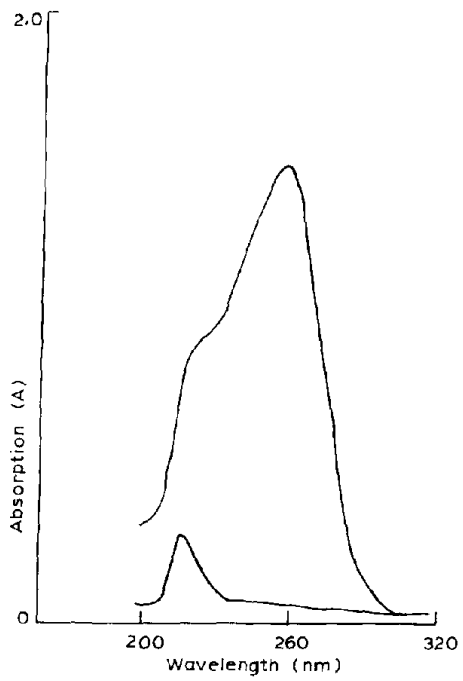

Fig.1. UV spectra of DAOC (0.2 mM, upper trace) and Pen N (0.2 mM, lower trace) in Tris-HCl buffer, pH 7.4 (50 mM). DAC and cephalosporin C had similar absorption spectra to DAOC. Spectra were recorded on a Pye Unicam PU 8800 spectrophotometer against blanks containing Tris-HCl buffer, pH 7.4 (50 mM).

that described previously using the hole plate bioassay [3] with a broad pH optimum of 7.4–8.0.

Fig.2a shows the effect of temperature on the enzyme assay, with an optimum at 35°C. The Arrhenius plot was linear over the range studied, with an activation energy of 140 kJ.

Fig.2b shows the effect of $(NH_4)_2SO_4$ concentration on the assay. The optimum concentration was 70–80 mM, and a graph of log v/v_o vs $\sqrt{I}$ gave a linear plot of positive slope 1.35 over most of the range studied.

Fig.2c shows double reciprocal plots for Pen N and α-ketoglutarate, with K_m values of 0.18 mM and 0.16 mM, respectively. These values are slightly higher than those quoted for α-ketoglutarate using the hole plate bioassay (0.04 mM [3]) and Pen N and α-ketoglutarate using HPLC assays (0.029 mM and 0.022 mM [4]).

Apart from the lack of specificity of the bioassay, and the indirect nature of the HPLC assay, one reason for the higher K_m values may be the lower ascorbate concentration required for the direct assay (0.001 mM as against 1 mM for the bioassay) owing to the UV absorption of ascorbate at 260 nm. As DTT and ascorbate may both be serving the same function, to keep the iron in a reduced form and/or to maintain protein thiols in their reduced state [3], this may not be a serious problem, and the DTT concentration in the direct assay has been increased to 10 mM (from 1 mM in the bioassay). The direct spectrophotometric method has been used successfully in determining the substrate specificity of DAOC synthase with a number of structural variants of Pen N [11].

The coupled assay using succinate thiokinase, pyruvate kinase and lactate dehydrogenase showed that succinate was a product of both expandase and hydroxylase activities (table 1). Similar estimates for expandase activities by bioassay and direct and coupled spectrophotometric methods were obtained, suggesting that the modification to ascorbate and DTT concentrations was not crucial for quantitative determinations. Lower initial velocity measurements were obtained for hydroxylase activity than for expandase, as suggested by the HPLC assay of both activities ([4] and J.B. Coates, M.J.C.C. and J.E.B., unpublished). In the expandase reaction, DAOC was produced stoichiometrically with succinate (1.0:1.06). No succinate was detected in expandase or hydrox-

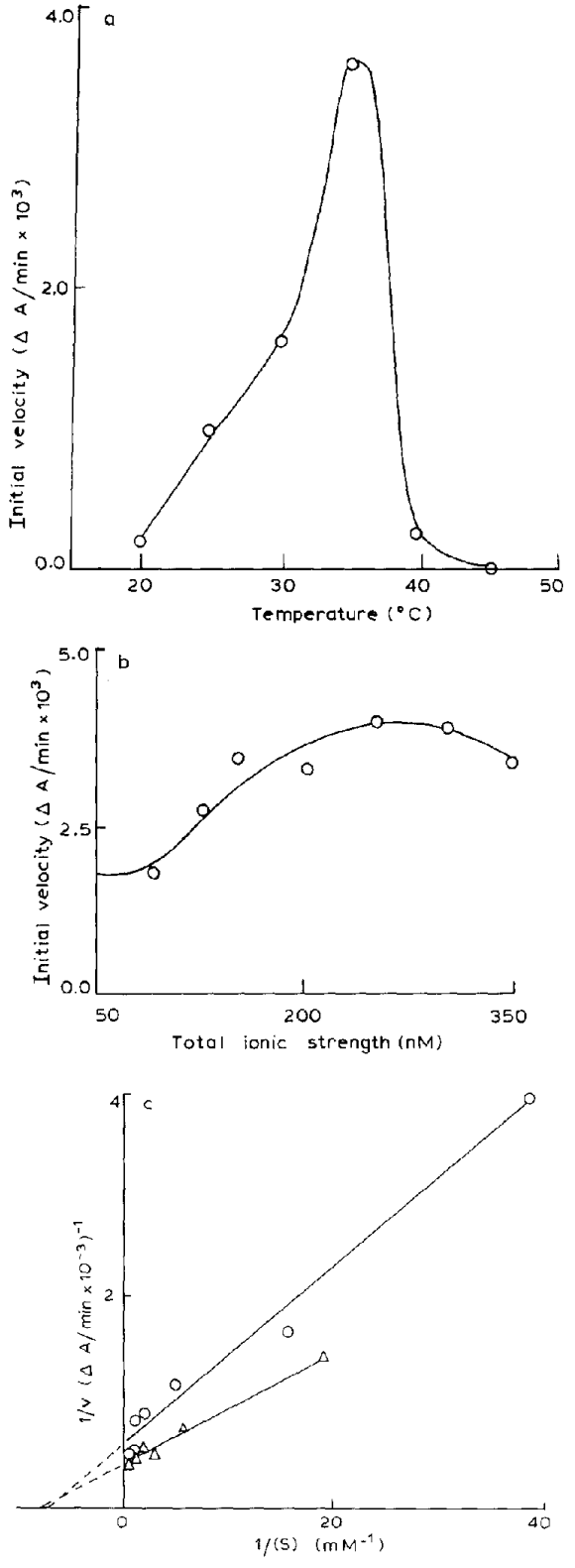

Fig.2. Characterisation of the direct spectrophotometric assay. (a) The effect of temperature; (b) the effect of $(NH_4)_2SO_4$; (c) double reciprocal plots for varied concentrations of Pen N (○) and α-ketoglutarate (△). For details see the text.

Table 1

Comparison of assays of deacetoxycephalosporin C expandase/hydroxylase by bioassay, direct and coupled spectrophotometric assays

Method	μmol·min^{-1}·mg^{-1} of enzyme ($\times 10^5$)
1. Hole plate bioassay (expandase)	1.30
2. Direct assay of cephem at 260 nm (expandase)	1.60
3. Coupled assay for succinate production from Pen N and α-ketoglutarate (expandase)	1.70
4. Coupled assay for succinate production from DAOC and α-ketoglutarate (hydroxylase)	1.04

Aliquots of a single enzyme preparation were used in all incubations. For details see the text

ylase reactions unless Pen N or DAOC were present.

These results are in accord with the finding that $^{14}CO_2$ was produced by oxidative decarboxylation of α-[1-^{14}C]ketoglutarate by a partially purified preparation of expandase in the presence of Pen N [12]. The enzyme shows some analogy to prolyl hydroxylase, an enzyme requiring Fe^{2+}, α-ketoglutarate, O_2 and ascorbate, where succinate release is dependent upon binding and subsequent hydroxylation of the peptide substrate [13].

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