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Studies of isopenicillin N synthase enzymatic properties using a continuous spectrophotometric assay

Alain Dubus[a,*], Malkit Sami[b], Toby J.N. Brown[b], Christopher J. Schofield[b], Jack E. Baldwin[b], Jean-Marie Frère[a]

[a] *University of Liège, Laboratoire d'Enzymologie, CIP, Institut de Chimie B6, B-4000 Sart-Tilman, Liège, Belgium*
[b] *The Oxford Centre for Molecular Sciences and The Dyson Perrins Laboratory, South Parks Road, Oxford OX1 3QY, UK*

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Abstract Isopenicillin N synthase (IPNS) from *Aspergillus nidulans* is a no-heme iron(II)-dependent oxygenase which catalyses, in a single reaction, the bicyclisation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine into isopenicillin N, the precursor of all other penicillins, cephalosporins and cephamycins. The IPNS reaction can be followed directly and continuously by a new assay which monitors the absorbance increase at 235 nm characteristic of penicillin nucleus formation. Using this assay, the effects of influential factors affecting the in vitro IPNS enzymatic reaction were investigated. Even under optimal conditions, enzyme inactivation occurred during catalysis. Iron(II) depletion and product inhibition were not the cause of this phenomenon, the addition of antioxidants or reducing agents failed to slow down inactivation or reactivate the enzyme. Therefore, this phenomenon appears to be irreversible and is attributed to oxidative damage caused to the enzyme by reactive oxygen species generated in solution during catalysis. Nevertheless, the steady-state kinetic parameters for the IPNS reaction were determined. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: β-Lactam biosynthesis; Isopenicillin N synthase; Oyxgenase; Enzyme assay; Oxidative damage

1. Introduction

β-Lactam antibiotics are widely used compounds for the treatment of bacterial infectious diseases. Various β-lactam-producing microorganisms are used in fermentation processes to directly produce penicillin antibiotics, or starting materials, such as benzylpenicillin or cephalosporin C, which are transformed by synthesis into other antibiotics.

Isopenicillin N synthase (IPNS) is a key enzyme in the β-lactam biosynthetic pathway. It catalyses the bicyclisation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) into isopenicillin N (IPN), the precursor of all other penicillins, cephalosporins and cephamycins [1].

One catalytic turnover proceeds through two oxidative ring closures in which four hydrogen atoms are removed from the tripeptide substrate and one molecule of dioxygen is transformed into two water molecules (Fig. 1). IPNS uses a single ferrous iron to capture and activate the oxidative molecular oxygen [2] and is a member of an extended family of mononuclear no-heme iron(II)-dependent oxygenases [3,4]. Significant homology is observed between the primary structures of IPNS and of an extended subgroup of this family, the 2-oxoglutarate-dependent oxygenases. This subgroup includes enzymes involved in the cephem biosynthesis pathway and another related enzyme, 1-aminocyclopropane-1-carboxylic acid oxidase (ACCO) [5], which, like IPNS, does not utilise a 2-oxoglutarate cosubstrate. The primary structure isology is reflected in the folding similarities observed between the tertiary structures of IPNS and deacetoxycephalosporin C synthase (DAOCS) [6,7].

Under certain conditions, the IPNS reaction can be studied in vitro using cell-free extracts or purified enzyme from various microorganisms. Among the discontinuous techniques employed to follow IPNS activity are the hole-plate bioassay [8,9], high performance liquid chromatography (HPLC) [10] and nuclear magnetic resonance [11,12]. However, these methods can be time consuming, labour intensive and tedious to perform when many samples are analysed. Two procedures, which allow a continuous monitoring of IPNS activity, an automatic pH titration-coupled enzyme assay using a β-lactamase [13] and an oxygen uptake assay [14] have been described. Whilst these methods are more appealing, they require special technical skills and careful setting and calibration of the experimental conditions. Moreover, the precision of the oxygen uptake assay is reduced owing to a background rate due to competing non-specific oxidation reactions.

We have developed a continuous spectrophotometric assay for IPNS based on the increased absorbance at 235 nm due to penicillin formation. It is readily performed and was used here to determine steady-state parameters and investigate the influence of the various cofactors on the in vitro IPNS enzymatic reaction.

2. Materials and methods

ACV was synthesised and the TEM-β-lactamase prepared as described [15,16]. All other chemicals were purchased from Sigma-Aldrich. A homogeneous preparation of recombinant wild-type *Aspergillus nidulans* IPNS [11] was used throughout this study.

*Corresponding author. Fax: (32)-4-366 3364.
E-mail: a.dubus@ulg.ac.be

Abbreviations: IPNS, isopenicillin N synthase; DAOCS, deacetoxycephalosporin C synthase; IPN, isopenicillin N; ACV, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine; DTT, dithiothreitol; TCEP, tris(carboxyethyl)phosphine; ROS, reactive oxygen species

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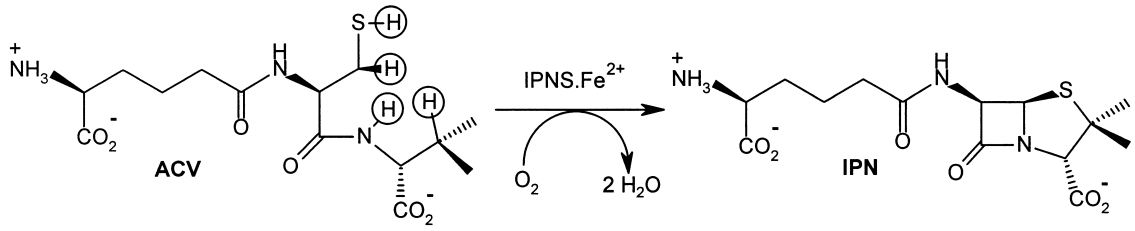

Fig. 1. Reaction catalysed by IPNS.

2.1. Preparation of ACV stock solution

Stock solutions of 10 to 20 mM ACV were made in distilled water and the pH adjusted to neutrality. ACV contains a thiol group and readily oxidises in solution into its disulfide form which is not an IPNS substrate. Thus, it is necessary to incubate the ACV stock solution with an equimolar amount of reducing agent before preparing the assay mixture. With tris(carboxyethyl)phosphine (TCEP), reaction for 10 min at 25°C was sufficient to reduce all the disulfides present. An independent determination of the ACV concentration in stock solution was made using the method described by Le and Means [17].

2.2. HPLC assay

IPNS activity was determined by monitoring IPN production. A standard assay mixture consisted of 200 μl of 50 mM HEPES (pH 7.0) containing up to 10 mM ACV, 1 mM TCEP, 100 μM ascorbate, 25 μM iron(II) and 0.5–4 μM IPNS enzyme. These values were modified in some of the experiments as indicated in the text or the figure legends. After 5–10 min at 30°C, the reaction was stopped by addition of an equal volume of 0.5 mM *o*-phenanthroline and kept on ice or stored at −20°C. The samples were centrifuged for 5 min at $10\,000\times g$ prior to injection (20–50 μl). Separation of the products was performed by reversed-phase HPLC on a 250×4 mm Merck Lichrospher RP18 100-5 μm column using a linear elution gradient of 2–25% acetonitrile in 25 mM sodium phosphate buffer pH 7.0 (1 ml/min). UV detection was at 215 nm.

2.3. Spectrophotometric assay of IPNS

UV spectrophotometric measurements were made in 1 cm light-path quartz cuvettes with a Beckman DU8 instrument interfaced with a computer for data collection or with a Hewlett-Packard HP8452A diode array spectrophotometer. Freshly prepared assay mixtures were prepared by dilution of stock solutions of ACV and other cofactors and contained 50 mM HEPES (pH 7.0), 1 mM TCEP and 25 μM ascorbate. The ACV concentration ranged from 0.2 to 10 mM. In practice, 2–3 ml mixes for each ACV concentration were made and kept on ice. Before running the assay, a small volume of concentrated ferrous ammonium sulphate solution was added (10 to 25 μM final concentration). The concentration of dioxygen in aqueous solution is dependent on the temperature and is about 0.23 mM at 30°C (21% in air at 0.1 MPa). Thus, the cofactors mixture was equilibrated for several minutes at the assay temperature before proceeding. A sample (450 μl) was withdrawn, added with concentrated IPNS enzyme solution (0.5–2 μM final concentration), and the absorbance at 235 nm recorded. During the measurement, the temperature was maintained at 30°C by a Peltier system. Under these conditions, initial rates are proportional to the enzyme concentration and can be calculated from the linear portion in the early part of the reaction time course. A blank assay (no enzyme) was similarly recorded and if a significant linear change in absorbance was observed, probably due to iron(II)-catalysed oxidation of ascorbate [18], the values obtained in the presence of enzyme were accordingly corrected. The rate values in the text and figures were obtained from at least four independent measurements and one blank assay for each condition or ACV concentration tested.

2.4. Determination of Δε associated with ACV conversion to IPN

The total change in absorbance at 235 nm was recorded for six standard spectrophotometric assays of 450 μl samples containing 200 or 400 μM ACV. The reactions were stopped with *o*-phenanthroline and the amount of IPN accumulated in these samples was determined with the DD-peptidase assay [19]. This provided a value of 1130 ± 50 M^{-1} cm^{-1} for the variation of the molar absorption coefficient associated with the formation of IPN.

3. Results

3.1. Development of the spectrophotometric assay

The largest variation in the UV absorption spectrum observed during IPNS catalysis is found at 235 nm. A good agreement was found between the amount of IPN accumulated in the samples as determined by the HPLC and DD-peptidase assays and the recorded change in absorbance (Fig. 2). The absorbance progress curve indicates a rapid conversion of substrate followed by a decrease in rate leading to a final plateau. Excessive substrate utilisation can be ruled out as the cause of non-linearity since little IPN product is formed under the conditions used in the experiment. Thus, the observed non-linearity of the IPNS reaction rate is most likely the result of a gradual enzyme inactivation and several factors related to this phenomenon were investigated.

To examine product inhibition, two standard reaction samples were used, one of them containing a small amount of TEM-β-lactamase to hydrolyse IPN as it was formed. While no IPN peak was detected in the HPLC chromatogram of the β-lactamase-containing sample, a similar ACV peak was found in both samples. This result indicates that product inhibition is not the cause of the enzyme inactivation (data not shown).

It is reasonable to suggest that depletion of iron(II) could be responsible for this phenomenon. Fig. 3A depicts an experiment in which new aliquots of iron(II) or IPNS enzyme were added to reaction mixtures where almost complete en-

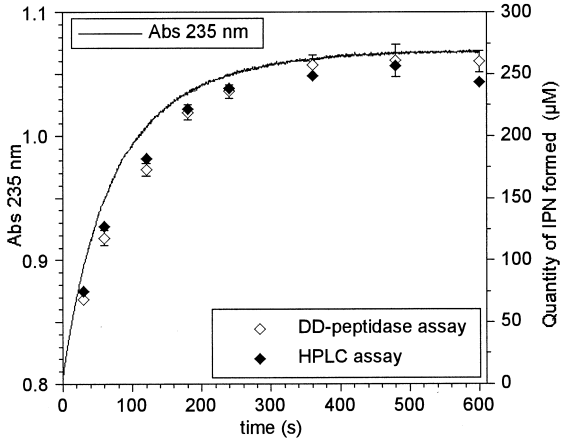

Fig. 2. IPNS activity determined with three different assay methods. Standard assay: 2 ml of 50 mM HEPES, pH 7.0 containing 800 μM ACV, 1.2 mM TCEP, 25 μM ascorbate, 10 μM iron(II) and 0.5 μM IPNS. After the enzyme was added, the change of absorbance at 235 nm was recorded on 450 μl of the mixture. The remaining solution was incubated at 30°C and 120 μl samples were removed after fixed time periods, the reaction rapidly stopped by addition of *o*-phenanthroline and the quantity of IPN determined by HPLC (♦) and DD-peptidase assays (◇).

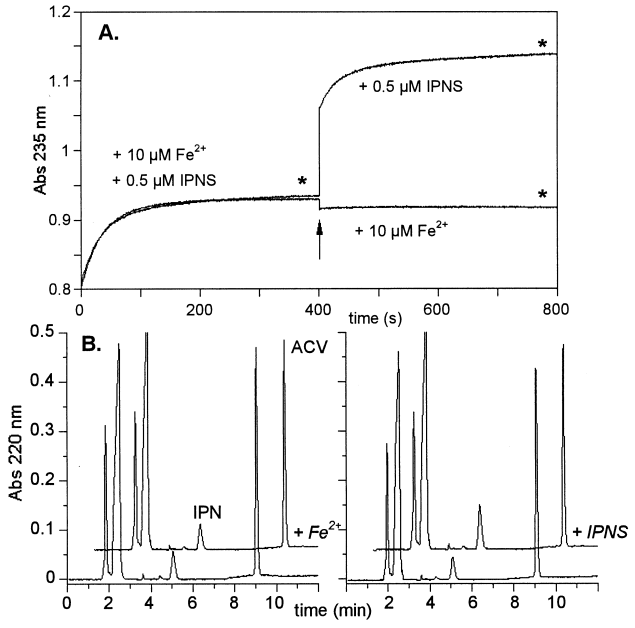

Fig. 3. Reactivation experiments. The assay mixtures contained 1 mM ACV, 2 mM TCEP, 25 μM ascorbate in 50 mM HEPES, pH 7.0 and additional compounds as indicated. A: Reaction followed at 235 nm. The arrow indicates the moment when new additions of either iron or IPNS were made and the asterisks when aliquots were withdrawn for HPLC analysis. B: HPLC chromatograms. The analyses performed at the end of the procedures (upper trace) are shown with a 1.25 min offset to allow comparison of the results.

zyme inactivation had occurred. Fresh addition of iron did not restore enzyme activity while addition of enzyme resulted in a burst of product formation. Similarly, HPLC analysis of samples removed before and 6 min after the respective additions (Fig. 3B) showed that iron addition did not result in an IPN peak increase while enzyme addition did. These results demonstrate that inactivation caused by iron(II) depletion can be excluded but also that all the other assay components (i.e. ACV, ascorbate and TCEP) are still functional and present in sufficient quantity to promote IPNS activity.

In subsequent experiments, no recovery of enzyme activity upon addition of two reducing agents, 1 mM TCEP or 5 mM sodium dithionite, could be obtained in either case. Likewise, addition of 200 μM *t*-butyl-4-hydroxyanisole or 100 μM ethyl gallate, two substances known for their antioxidant properties, also did not result in any significant improvement of the enzyme lifetime.

Therefore, if the enzyme inactivation results from an oxidative process, the modification appears to be irreversible under the conditions used.

3.2. Influence of ascorbate on the IPNS reaction

The effect of ascorbate on the in vitro IPNS reaction was examined both by the HPLC and spectrophotometric assays (Fig. 4). The HPLC assay was used initially, because of the large UV absorbance associated with high ascorbate concentrations. In this experiment, the largest amount of IPN formed after 20 min reached a plateau above 1 mM ascorbate, corresponding to about three times the quantity obtained in the absence of ascorbate. In the light of our previous results (see Fig. 2) we can assume that the enzyme was completely inactivated at the end of the 20 min incubation period.

In the spectrophotometric assays, lower ascorbate concentrations were used and initial reaction rates were directly measured on a much shorter time period (less than 1 min). The maximum rate was observed at 0.2 mM ascorbate and represented less than twice the value obtained in the absence of ascorbate. The discrepancy between these results is explained by the different techniques and conditions and would probably be less marked if a shorter incubation period had been used for the HPLC assay. But this would have rendered the method less precise since less product would have been detected. Nonetheless, both experiments indicate that ascorbate has a positive effect on the in vitro enzyme activity with different influences on the magnitude of the turnover (initial rates)

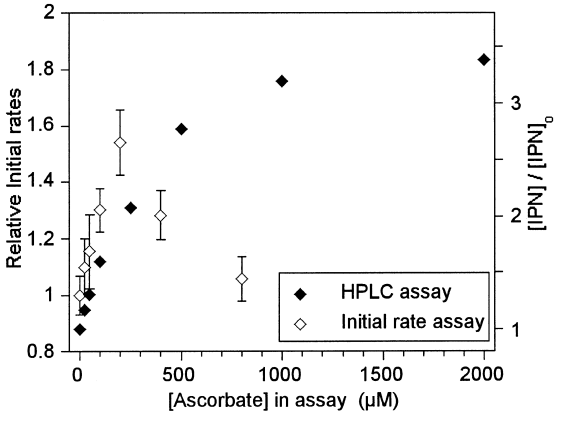

Fig. 4. Effect of ascorbate on IPNS activity. ◆: HPLC assay. 100 μl reaction mixtures contained 2 mM ACV, 4 mM TCEP, 20 μM iron(II), 1 μM IPNS in 50 mM HEPES (pH 7.0) and were incubated for 20 min at 30°C. ◇: Initial rates calculated from absorbance change. 450 μl assay mixtures contained 250 μM ACV, 2 mM TCEP, 25 μM iron(II), 0.5 μM IPNS in 50 mM HEPES, pH 7.0. Relative values are the ratios of values measured at different ascorbate concentrations to those obtained in the absence of ascorbate (i.e. $v_0 = 2.6$ μmol s^{-1} μmol^{-1}, $[IPN]_0 = 0.23$ mM).

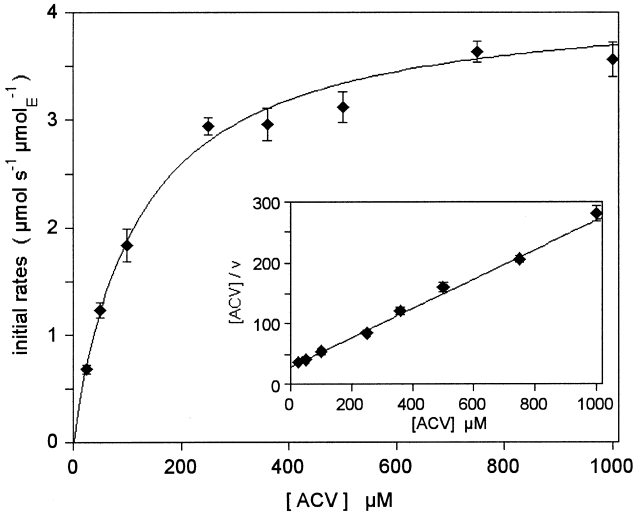

Fig. 5. Initial rate measurements as a function of ACV concentration. Initial rate values were determined with 400 μl reaction mixtures containing ACV in 50 mM HEPES (pH 7.0), 1 mM TCEP, 25 μM ascorbate, 10 μM iron(II) and 1.22 μM IPNS. The curve results from non-linear regression fitting of the data to the Henri–Michaelis equation with $k_{cat} = 4.14$ (±0.11) s^{-1} and $K_m = 120$ (±10) μM. Inset: linear regression according to the Hanes–Woolf equation.

and inactivation rates. The increase of the turnover rate reaches an optimum value at 0.2 mM ascorbate and then decreases back to its initial value as the concentration of ascorbate increases. On the contrary, the amount of IPN formed after 20 min, which also reflects the enzyme stability, continues to increase up to 1 mM ascorbate.

3.3. Determination of steady-state kinetic parameters from initial rate measurements

The plot of initial rates against ACV concentrations shows that the enzyme reaction obeys simple saturation kinetics (Fig. 5). The steady-state kinetic parameters are $k_{cat} = 4.1$ (± 0.11) s^{-1}, $K_m = 120$ (± 10) μM, specific activity = 6.64 U per mg_E (1 U equals 1 μmol/min). These values are significantly different from those obtained previously with the same *A. nidulans* enzyme using the HPLC assay [11]: $k_{cat} = 0.2$ s^{-1}, $K_m = 1220$ μM, specific activity = 0.32 U per mg_E (assay conditions: 50 mM Tris pH 8.0, 4 mM dithiothreitol (DTT), 1 mM ascorbate, 100 μM iron(II) and up to 100 μM IPNS; 5 min incubation at 28°C). The difference in methodology and experimental conditions must be the cause for the difference observed in the measured parameters. It is remarkable that the present spectrophotometric assay yields a much larger specific activity meaning that either the assay conditions are more favourable for enzyme activity or that this new method supplies better estimates of the initial rates.

4. Discussion

Ferrous iron is an absolute requirement for IPNS catalytic activity and its concentration in the assay buffer is inversely proportional to the enzyme degree of purity. Spectrophotometric initial rate determinations with purified enzyme (1 μM) and 0.5 mM ACV in 50 mM HEPES, 2 mM TCEP, pH 7.0 were performed at iron to enzyme molar ratios ranging from 30 to 240 and no significant variation was observed. Ascorbate was omitted in these assays because its interaction with iron would have unnecessarily complicated the interpretation of the results. With purified enzyme a 25- to 50-fold iron molar excess is sufficient for optimum activity and the minimum amount necessary to activate the enzyme should be used.

The utilisation of Tris as buffer generally rests on historical reasons rather than on its intrinsic qualities. In fact, Tris presents some problems such as a reactive primary amine and metal chelating properties [20] which can have undesirable effects. Consequently, we used HEPES buffer, but a related compound such as Mops is probably also well suited [21]. However, as pointed out by others [22,23] there is no ideal buffer when studying biochemical reactions involving a transition metal.

At pH 6.0, the enzymatic activity of the *Cephalosporium acremonium* IPNS is 10% of that observed at pH 8 [21]. Using the spectrophotometric assay, with the recombinant *A. nidulans* enzyme, there was no marked difference between initial rates measured at pH 7.0 and 7.5. However, the choice of a pH value below 7.5 was chosen on the basis of the following considerations. Above neutral pH ferric iron rapidly forms insoluble polymeric hydroxyl complexes and this process has two adverse effects on the assay: it rapidly decreases the amount of soluble iron(II) available to bind to IPNS, and interferes with the spectrophotometric detection. For this reason, phosphate buffer which dramatically increases the ferrous iron oxidation rate should be avoided [22,24]. Moreover, in solution the generally slow 'auto-oxidation' of thiols to their disulfide form by molecular oxygen is increased by trace amounts of transition metals and is generally faster at alkaline pH [22].

Early studies with cell-free extracts or partially purified IPNS preparations showed that in vitro enzyme activity is stimulated by the addition of DTT and ascorbate in the assay buffer [9,21,25]. These reducing agents do not directly participate in the catalytic mechanism and their role seems to be restricted to maintaining both ACV and iron (either active site bound or in solution) in their reduced states. It has been suggested in the case of the related prolyl hydroxylase that ascorbate could reduce in situ inactive Fe^{3+}–enzyme complexes [26]. Another potential role for these substances rests on their ability to scavenge fortuitous reactive oxygen species (ROS, i.e. superoxide, hydrogen peroxide and hydroxyl radical) formed during the reaction and to prevent oxidative damage to the enzyme [27].

The simultaneous presence of a transition metal, a reducing agent and dioxygen can have very detrimental effects on biological systems studied in vitro. In fact DTT, the most commonly used reducing agent, is known to ligate iron in a bidentate manner and this complex favours the rapid metal-catalysed oxidation of the dithiol in solution [23,24]. This auto-oxidation reaction has two adverse effects on the IPNS assay: it utilises oxygen, a co-reactant in IPNS catalysis, and generates ROS and sulfur radicals [23]. It has been shown that reduced glutathione undergoes the same metal-catalysed auto-oxidation phenomenon [28] and presumably ACV would behave similarly. TCEP is a soluble non-volatile phosphine, which does not interact with iron in solution, is more stable than DTT to oxidation and is a better reducing agent at pH values below 7.5 [29,30]. The replacement of DTT by TCEP in the IPNS assay buffer enhanced the enzyme activity probably by removing some of the problems associated with DTT.

Ascorbate has been reported to stimulate most 2-oxoglutarate related and dependent oxygenases, but under certain conditions to inhibit others, e.g. proline 4-hydroxylase [31]. It is also known to exhibit prooxidant activities in the presence of iron(II) [32]. When included in the IPNS assay mixture, ascorbate appears to have a rather direct activating effect on the enzyme catalytic process which becomes inhibitory at higher concentrations but it also exhibits a more indirect action probably linked to its antioxidant protective properties which increase the enzyme lifetime. Interestingly, with the *C. acremonium* enzyme it was observed that the shape of the progress curves were similar with or without ascorbate but a five-fold decrease in the amount of converted ACV was obtained in the absence of ascorbate [21].

Nevertheless, even under optimal assay conditions, a progressive enzyme inactivation phenomenon still occurred. Instability under catalytic conditions had already been observed with IPNS [21] and with related oxygenases such as DAOCS [33], ACCO [34] or prolyl hydroxylase [26]. The mechanism leading to inactivation remains to be elucidated but our results have ruled out certain causes such as iron depletion or product inhibition. The formation of a dead-end complex during catalysis seems to be unlikely as it is inconsistent with the high efficiency of the biosynthetic process. This inactivation also could not be reversed by addition of reducing

agents. A likely explanation involves some irreversible oxidative damage caused by ROS reacting with the backbone or the side-chain of residues close to the active site. These ROS could be generated either by the enzyme catalytic chemistry itself or by redox side-reactions occurring in the assay mixture whose products would diffuse to the active site. It is noteworthy that the addition of catalase (0.5 mg/ml) has been reported to substantially reduce enzyme inactivation (M. Sami, unpublished work), and suggests the generation of hydrogen peroxide outside the catalytic site. However since the catalase protection is incomplete it does not exclude active-site-specific formation of ROS.

The situation is very different in vivo where sophisticated mechanisms may have evolved to sense and tightly regulate any oxidative stress and where the concentration of free transition metals inside the cell is very low.

In conclusion, reliable enzymatic kinetic data rest on a precise and easy assay which preferentially allows a continuous monitoring of the enzyme activity. We have developed such an assay for IPNS which can be used to routinely monitor enzymatic activity during purification or for precise analysis of kinetic properties. The present assay, which is amenable to automation with minor modifications, has yielded kinetic parameters which are probably the most accurate yet reported.

Moreover, there is a broad interest in the economic, technical and social aspects of the production of β-lactam antibiotics and it is certainly valuable to gain a better access to the in vitro kinetic properties of a key enzyme in their biosynthetic pathway.

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