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Factors affecting the isopenicillin N synthetase reaction

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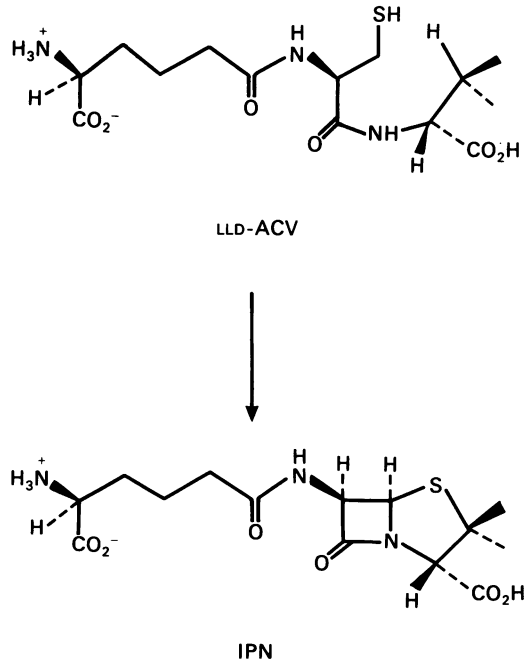
1. Isopenicillin N synthetase (IPNS) from *Cephalosporium acremonium*, which requires Fe^{2+} and O_2 for activity, was highly purified for studies of factors affecting its conversion of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (LLD-ACV) into isopenicillin N (IPN). EDTA was used to quench the reaction by removal of Fe^{2+}. 2. IPNS was inactivated during the course of the conversion of LLD-ACV into IPN, although it was relatively stable in the absence of LLD-ACV under otherwise similar conditions. In the presence of GSH and ascorbate each IPNS molecule carried out about 200 catalytic events before inactivation, but the turnover number was decreased 5-fold in the absence of ascorbate. 3. After trace metal ions had been removed from IPNS and other components of the reaction mixture by Chelex-100 resin, only about 10 μM-Fe^{2+} was required for maximum stimulation. Several other transition-metal ions were inhibitors of the enzyme. 4. Both dithiothreitol (DTT) and GSH stimulated IPNS activity, but GSH, unlike DTT, was not rapidly oxidized in the presence of O_2 and Fe^{2+}. 5. IPNS was rapidly inhibited by the thiol-blocking reagents *N*-ethylmaleimide and 2,2′- and 4,4′-dipyridyl disulphide, but not by 5,5′-dithiobis-(2-nitrobenzoic acid) in the same concentration. Inhibition by 2,2′-dipyridyl disulphide could be reversed by DTT.

INTRODUCTION

The soluble enzyme isopenicillin N synthetase (IPNS) catalyses the oxidative cyclization of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (LLD-ACV) to isopenicillin N (IPN) (Scheme 1) and has a key role in β-lactam antibiotic production by *Cephalosporium acremonium* (*Acremonium chrysogenum*), *Penicillium chrysogenum* and *Streptomyces* species (Abraham, 1986). It has been purified to homogeneity from cell-free extracts of *C. acremonium* (Pang *et al.*, 1984). The *N*-terminal amino acid sequence has been determined (Baldwin *et al.*, 1985*a*) and the complete sequence obtained from the corresponding gene cloned into *Escherichia coli* (Samson *et al.*, 1985). The enzyme from this source is a single polypeptide chain of 338 amino acid residues containing cysteine residues at positions 106 and 255 that can form a disulphide bridge (Baldwin *et al.*, 1985*a*). The *P. chrysogenum* IPNS gene has also been sequenced and the derived amino acid sequence shows substantial homology with that of the *C. acremonium* enzyme, including the presence of only two cysteine residues (Carr *et al.*, 1986). IPNS has been shown to exhibit substantial tolerance towards structural changes in the substrate LLD-ACV, enabling thereby the enzymic synthesis of a wide range of bicyclic β-lactams to be performed (Baldwin & Abraham, 1988).

Although detailed information has been obtained about the amino acid sequences and M_r values of IPNS from different sources, much still remains to be discovered about the factors affecting IPNS activity *in vitro*. Earlier experiments with crude cell-free extracts and partially purified preparations have shown that the enzyme activity is stimulated by Fe^{2+}, ascorbic acid and DTT (Abraham *et al.*, 1981; Kupka *et al.*, 1983). The thiol form of ACV and O_2 are required by the enzyme (Abraham *et al.*, 1981) and 1 mol of O_2 is consumed/mol of IPN formed (White *et al.*, 1982). A coupled enzyme assay for IPNS has been developed and used to determine the effect of pH, ionic strength and temperature on the rate of conversion of LLD-ACV into IPN (Baldwin *et al.*, 1985*b*).

The present study was undertaken to examine further



Scheme 1. Cyclization of δ-L-(α-aminoadipoyl)-L-cysteinyl-D-valine (LLD-ACV) to isopenicillin N (IPN)

Abbreviations used: IPN, isopenicillin N; IPNS, isopenicillin N synthetase; LLD-ACV, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent); DTT, dithiothreitol.

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the effects of the various cofactors on the course of the IPNS-catalysed reaction with a highly purified preparation of enzyme from *C. acremonium* and to investigate the effects of thiol-blocking reagents on the enzyme's activity.

MATERIALS AND METHODS

Materials

Ferrozine, *N*-ethylmaleimide, 2,2′-dipyridyl disulphide and 4,4′-dipyridyl disulphide were from Aldrich Chemical Co., and Chelex-100 (200–400 mesh, Na^+ form) was from Bio-Rad Laboratories. With the exception of LLD-ACV all other reagents were from Sigma Chemical Co.

Preparation and assay of IPNS

IPNS was purified from extracts of *C. acremonium* C0728 as described by Pang *et al.* (1984), except that 100 g batches of damp-dry mycelium were used and the Dyno-Mill agitator discs were run at 2000 rev./min. The peak fractions containing IPNS were used for further purification at each chromatographic step. SDS/polyacrylamide-gel electrophoresis of the product revealed only traces of contaminating proteins after staining with Coomassie Blue. The protein content of solutions of IPNS was determined by the method of Bradford (1976), with bovine serum albumin as the standard.

The activity of IPNS was determined by bioassay of the IPN formed, with *Staphylococcus aureus* N.C.T.C. 6571 as the test organism, as described by Pang *et al.* (1984), except that the enzymic reaction (normally at pH 8.0) was quenched after 10 min by addition of EDTA (5 μl; 100 mM) and that in some experiments GSH (25 mM) was used in place of DTT (2 mM).

Removal of metal ions from IPNS reaction solutions

Solutions (2.5 ml) of IPNS, LLD-ACV, Mops and cofactors as described by Pang *et al.* (1984) were largely freed of iron and other metal ions by passage through columns (1 cm × 0.6 cm) of Chelex-100 (Na^+ form) in distilled water. The first few drops of the eluates were discarded to avoid dilution of the solutions.

O_2 uptake during the Fe^{2+}-catalysed oxidation of DTT

Warburg respirometers were used to determine O_2 uptake during oxidation of DTT in the presence of Fe^{2+} at 30 °C.

$FeSO_4$ (40 μl; 2 mM) was placed in each flask side arm and a solution of DTT (1.96 ml; either 2 or 4 mM) in Mops buffer (0.1 M, pH 8.0) in the main compartment. After equilibration for 10 min, the $FeSO_4$ and DTT solutions were mixed and readings were taken every 5 min for 30 min.

Loss of thiol groups during the Fe^{2+}-catalysed oxidation of DTT

Solutions of DTT (2 ml; 2 or 4 mM) in Mops buffer (0.1 M, pH 8.0) were shaken (100 rev./min) in 25 ml conical flasks at 30 °C. Samples (10 μl) were removed at 2 min intervals and transferred to DTNB (0.99 ml; 1 mM in 0.1 M-sodium phosphate buffer, pH 7.2), and the absorbance of the mixture at 412 nm was measured. After 10 min, $FeSO_4$ (100 μl; 2 mM) or distilled water (100 μl) was added and sampling into DTNB continued every 5 min for a further 30 min. Thiol-group concentrations were calculated by use of a molar absorption coefficient for the 3-carboxy-4-nitrothiophenolate anion of 13600 $M^{-1} \cdot cm^{-1}$ (Ellman, 1959).

Inhibition of IPNS activity by *N*-ethylmaleimide, 2,2′- and 4,4′-dipyridyl disulphides and DTNB

To solutions of IPNS (1.2 ml; 0.44 or 0.63 mg/ml) in 20 mM-Tris/HCl buffer, pH 8.0, was added dimethyl sulphoxide (40 μl) or dimethyl sulphoxide containing the thiol-blocking reagent (40 μl; 0.1 M) at room temperature. Samples (10 μl or 40 μl) were transferred immediately and then at intervals to an ice-cold IPNS reaction mixture to give a final volume of 200 μl. After incubation at 30 °C with shaking for 10 min, EDTA (5 μl; 50 mM) was added to quench the IPNS reaction and the IPN formed was determined by bioassay. IPNS activity was recorded as a percentage of that at zero time.

Time course of conversion of LLD-ACV into IPN

Samples (200 μl) were taken at intervals from an IPNS reaction solution (3.6 ml in a 25 ml conical flask), which was shaken at 30 °C and contained 34 or 17 μg of IPNS/ml, 1 mM-LLD-ACV, 1 mM-ascorbic acid, 25 mM-GSH, 0.1 mM-$FeSO_4$, 3000 Sigma units of catalase/ml and 0.1 M-Mops/NaOH buffer, pH 8.0. The samples were added to 5 μl of 100 mM-EDTA to quench the reaction and were kept at 0 °C until the determination of IPN by bioassay. After 60 min incubation, further LLD-ACV, or LLD-ACV plus $FeSO_4$ plus ascorbic acid (to double the initial concentrations), or IPNS (to give 17 μg/ml) was added to samples (200 μl) of the remainder of the initial solution that had contained 34 μg of IPNS/ml. After a further 10 min incubation with shaking at 30 °C, the reaction was quenched by the addition of EDTA, and IPN was determined by bioassay.

The stability of IPNS in the absence of substrate under these conditions was determined by incubation of IPNS in the presence of all cofactors but no LLD-ACV. Samples (186 μl, in duplicate) were transferred to solutions of ACV (14 μl; 14.3 mM) and the mixtures incubated with shaking for 10 min at 30 °C. The reaction was then quenched with EDTA, and IPN was determined by bioassay. Enzyme activity was recorded as a percentage of that at zero time.

RESULTS

Assay of IPNS and its inactivation during cyclization of LLD-ACV

Under the conditions used for assay of IPNS it was difficult to determine initial rates of reaction because the formation of IPN from LLD-ACV was not linear and decreased virtually to zero within 10 min. However, in a series of experiments, in which the concentration of IPN produced varied from 60 μM to 400 μM, it was found that the proportion of LLD-ACV converted into IPN after this time was approximately proportional to the concentration of IPNS. For example, in the presence of 34 and 17 μg of IPNS/ml the amount of IPN increased rapidly for a few minutes at 30 °C and then almost ceased when the conversion of 27% and 14% respectively of the substrate had occurred (Fig. 1). Thus assays of IPN after 10 min were used to obtain approximate values for the relative activities of enzyme preparations.

The addition of more LLD-ACV or cofactors after 60 min incubation did not change the final amount of conversion significantly, but a rapid and substantial

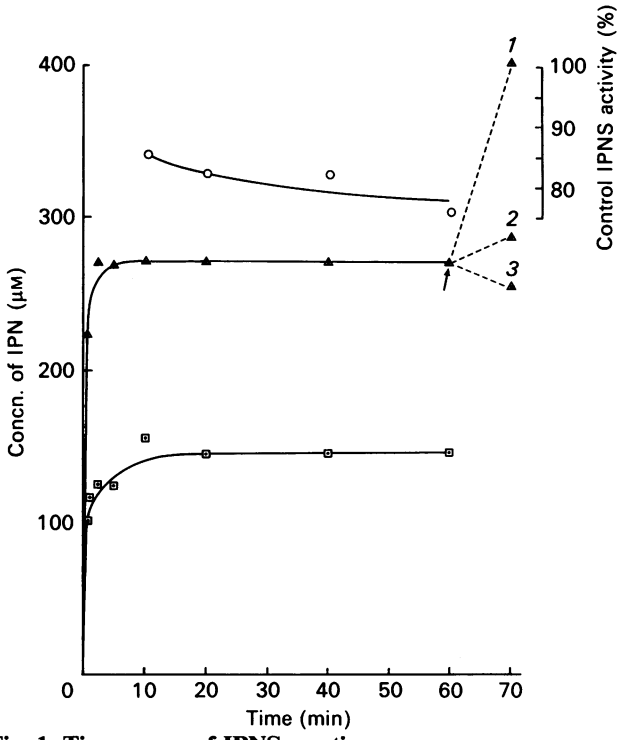

Fig. 1. Time course of IPNS reaction

The conversion of LLD-ACV (1 mM) into IPN in the presence of IPNS was monitored as described in the Materials and methods section. ○—○, Control enzyme activity in the presence of all cofactors but in the absence of substrate (percentage of that at zero time); △—△, IPN formed with 34 μg of IPNS/ml; ⊡—⊡, IPN formed with 17 μg of IPNS/ml; △---△, IPN formed on further addition (↑) of: *1*, IPNS; *2*, LLD-ACV and cofactors; *3*, LLD-ACV.

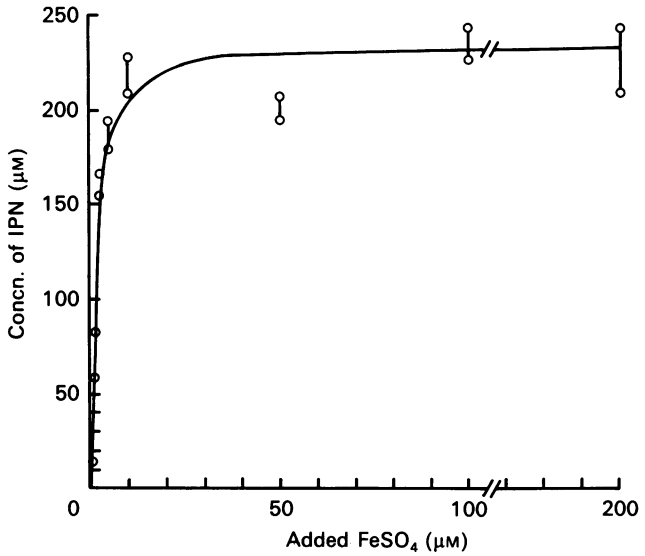

Fig. 2. Effect on IPNS activity of the addition of Fe^{2+} to a metal-ion-depleted reaction mixture

Components of the reaction mixture were depleted of metal ions by passage through Chelex 100. IPNS activity in the presence of added Fe^{2+} was determined by bioassay after a 10 min incubation at 30 °C (for details see the Materials and methods section). The IPNS concentration was 90 μg/ml, except that the low activity when no $FeSO_4$ was added was determined with a higher enzyme concentration. The bars refer to IPN assays from duplicate samples.

increase in IPN occurred after addition of further IPNS. When IPNS was incubated under similar conditions in the presence of cofactors but in the absence of LLD-ACV it lost less than 25% of its activity.

In the absence of ascorbate the progress curves of the IPNS-catalysed reaction were similar in shape to those in Fig. 1, but there was about a 5-fold decrease in the amount of LLD-ACV converted into IPN by a given amount of enzyme before the reaction ceased. Measurements of absorbance at 265 nm indicated that most of the ascorbate remained unchanged at the end of the reaction.

Reversible changes in activity with pH

It was reported previously (Kupka *et al.*, 1983) that the activity of IPNS fell sharply when the pH was lowered below 7.6. In the present study the purified enzyme showed less than 10% of its maximum activity when assayed at pH 6.0 instead of at pH 8.0. But on assay at pH 8.0 after preincubation at pH 6.0 (30 °C for 10 min) the activity rose to 90% of its original value. The readily reversible loss of activity at pH 6.0 could thus be attributed to changes in the ionization of one or more amino acid residues.

Effect of EDTA and metal ions on IPNS activity

In previous studies with IPNS the enzymic reaction was terminated before the bioassay of IPN by the addition of either acetone (Abraham *et al.*, 1981) or methanol (Kupka *et al.*, 1983) as a protein denaturant. Since IPNS is stimulated by Fe^{2+} ions, it seemed that the addition of EDTA might be more convenient for this purpose. Concentrations of EDTA up to 5 mM had virtually no effect on the growth of *Staph. aureus* N.C.T.C. 6571 when used in the hole–plate bioassay procedure. Under the conditions used for the standard determination of IPNS activity, the inclusion in the reaction mixture of 0.2 mM-EDTA resulted in 75% inhibition and 1 mM-EDTA was usually sufficient to prevent the formation of detectable IPN. In subsequent work 2.5 mM-EDTA was used routinely to quench the enzymic reaction.

A quantitative study of the requirement of IPNS for Fe^{2+} was complicated by the ubiquity of iron as a contaminant in biochemical reagents. We therefore first removed iron and other metal ions from the buffer, substrate, cofactor and enzyme solutions by use of the metal-ion-chelating resin Chelex 100. Fig. 2 shows that after passage of all reaction components (other than $FeSO_4$) through the resin, maximum enzyme activity was achieved by the addition of Fe^{2+} to give a concentration as low as 10 μM. In contrast, 100–200 μM-$FeSO_4$ was required before Chelex treatment.

In the absence of added Fe^{2+} the Chelex-treated IPNS showed only 5% of its maximum activity. Since this residual activity was completely inhibited by EDTA it was presumably due to traces of Fe^{2+} (< 1 μM) remaining after Chelex treatment. The data shown in Fig. 2 can be fitted approximately to a rectangular hyperbola and suggest an apparent K_m for Fe^{2+} of 1–2 μM under the

Table 1. Inhibition of IPNS by transition-metal ions and ferrozine

Metal ions were added to a reaction mixture from which metal ions had previously been removed by passage through Chelex 100. The assay conditions were as described in the Materials and methods section, except that 10 μM-Fe^{2+} was used.

Inhibitor	Concentration (μM)	Inhibition (%)
$CoCl_2$	10	100
$CuSO_4$	10	83
$ZnSO_4$	10	58
$MnCl_2$	10	27
$NiSO_4$	10	7
$MgSO_4$	10	0
Ferrozine	500	82

conditions used. No attempt was made to assess the effect of any binding of Fe^{2+} to components of the assay system other than IPNS.

Several transition-metal ions were inhibitory when added to the metal-depleted reaction system to give the same concentration (10 μM) as Fe^{2+} (Table 1). Atomic absorption spectrophotometry of an untreated system revealed the presence of low concentrations of cobalt (3 μM), copper (0.3 μM), zinc (0.45 μM), magnesium (5 μM) and iron (4 μM). The Fe^{2+}-specific chelator ferrozine (Stookey, 1970) also inhibits IPNS (Table 1).

Roles of DTT and GSH

With LLD-ACV as the substrate IPNS activity is stimulated by DTT (Abraham *et al.*, 1981; Kupka *et al.*, 1983). In most subsequent studies the disulphide form of LLD-ACV was used because it could be obtained more readily by chemical synthesis or could be isolated from the culture supernatants of a mutant of *C. acremonium* blocked in IPNS biosynthesis (Shirafuji *et al.*, 1979). However, the disulphide form of LLD-ACV is not a substrate and must be converted *in situ* into the thiol form. Hence the thiol form of LLD-ACV was used in the following experiments to study the effect of cofactors on IPNS activity.

During the preparation of mixtures containing DTT and Fe^{2+} for the IPNS reaction a pink coloration was observed that faded upon aeration at 30 °C and suggested the formation of a complex. The combination of DTT and Fe^{2+} was therefore investigated further. The addition of Fe^{2+} (to give 0.1 mM) to a solution of DTT (2 or 4 mM) at 30 °C resulted in the uptake of 0.5 mol of O_2/mol of DTT (Fig. 3). The O_2 uptake was virtually complete in 15 min. Ascorbic acid (1 mM) had no effect on the amount of O_2 consumed. In a separate experiment it was shown with DTNB that during the same time both thiol groups of DTT had become oxidized, presumably to the disulphide form (Fig. 3). Such a rapid Fe^{2+}-catalysed oxidation did not occur with GSH.

Under the conditions normally used, with assay of IPN after 10 min, the maximum stimulation of IPNS occurred with 2 mM-DTT in the presence of ascorbic acid (1 mM) and Fe^{2+} (0.1 mM). In the absence of DTT the activity was only about 15% of the maximum value. GSH could produce a similar stimulation, but for this it was necessary to use a concentration of 10 mM; increasing concentrations up to 50 mM had no further effect. In long-term experiments (up to 70 min) 20 mM-GSH was used so that the concentration of the thiol form would not fall below 10 mM as a result of oxidation in air.

Effect of thiol-blocking reagents on IPNS activity

IPNS was allowed to react at room temperature with *N*-ethylmaleimide. For the removal of unchanged reagent and the determination of IPNS activity samples of the solution were diluted 20-fold into a mixture containing an excess of GSH and LLD-ACV. After the usual 10 min

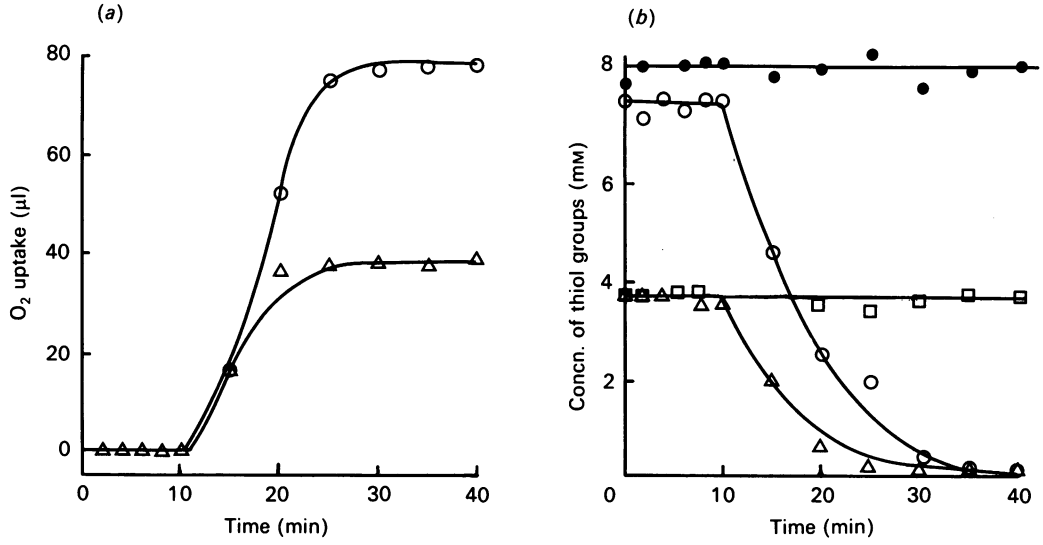

Fig. 3. Uptake of O_2 and loss of thiol groups during the iron-catalysed oxidation of DTT

O_2 uptake (*a*) and loss of thiol groups (*b*) were measured as described in the Materials and methods section. ●, 4 mM-DTT without Fe^{2+}; ○, 4 mM-DTT plus Fe^{2+}; □, 2 mM-DTT without Fe^{2+}; △, 2 mM-DTT plus Fe^{2+}. DTT and Fe^{2+} were mixed after 10 min.

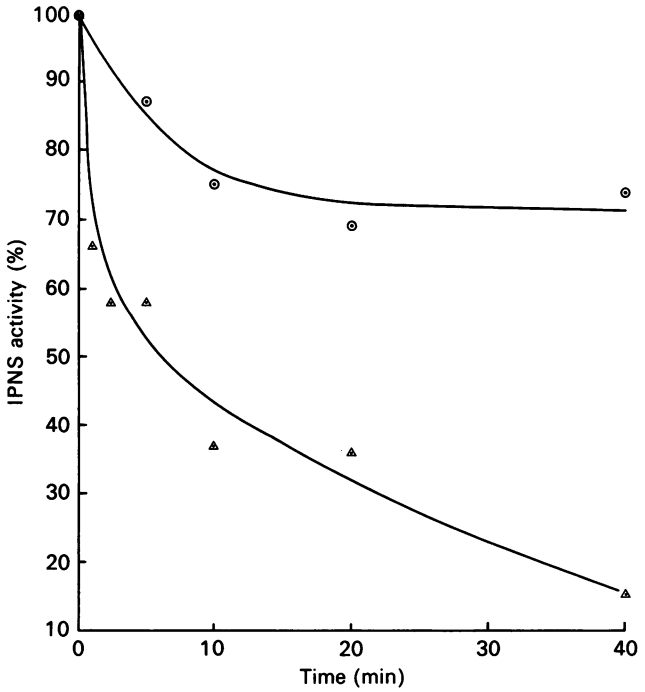

Fig. 4. Inhibition of IPNS by *N*-ethylmaleimide

IPNS was allowed to react with *N*-ethylmaleimide (2.5 mM) under the conditions given in the Materials and methods section. The control sample contained dimethyl sulphoxide but no *N*-ethylmaleimide. ⊙, control; △, 5 mM-*N*-ethylmaleimide.

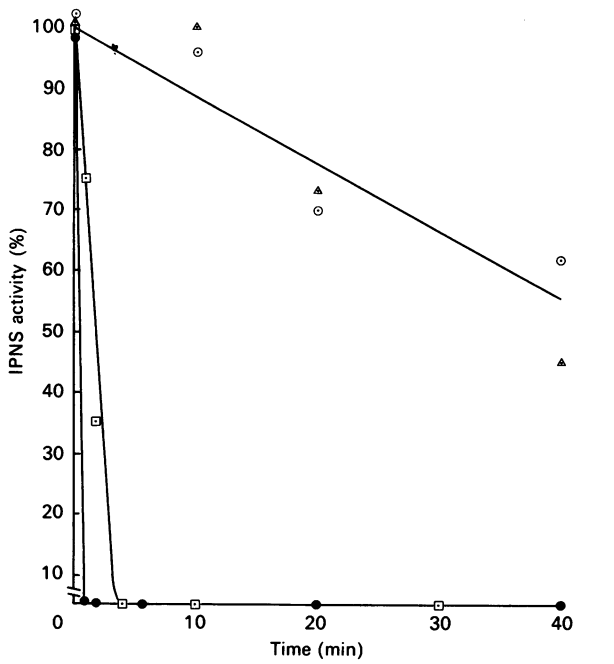

Fig. 5. Inhibition of IPNS by DTNB, 2,2′- and 4,4′-dipyridyl disulphides

The inhibition of IPNS (440 μg/ml) by the three reagents was determined as described for Fig. 4 except that from 6 min onwards 40 μl samples were removed for assay from the IPNS solutions containing the two dipyridyl disulphides. No Fe^{2+} was added to the reaction mixture. Points are the average of duplicate assays: ⊙, control; △, 2.5 mM-DTNB; ⊡, 2.5 mM-2,2′-dipyridyl disulphide; ●, 2.5 mM-4,4′-dipyridyl disulphide.

incubation period at 30 °C, the IPN formed was measured by bioassay. Fig. 4 shows that IPNS was largely inactivated by treatment with *N*-ethylmaleimide and that a small loss in activity also occurred in its absence. Jensen *et al.* (1986) have found that IPNS from *Streptomyces clavuligerus* is also inhibited by *N*-ethylmaleimide, but have reported that much lower concentrations than those used here with the *C. acremonium* enzyme produce complete inhibition.

The results of experiments with the reversible thiol-group inhibitors DTNB, 2,2′-dipyridyl disulphide and 4,4′-dipyridyl disulphide are shown in Fig. 5. The last-mentioned reagent inhibited IPNS very rapidly at the concentration used (2.5 mM) and 2,2′-dipyridyl disulphide inhibited the enzyme somewhat more slowly. In contrast, the loss of activity in the presence of DTNB did not differ significantly from that in the control. In the presence of Fe^{2+} (125 or 250 μM) the time required for half-inactivation of the enzyme by 2,2′-dipyridyl disulphide (1.0 mM) was about half of that required when no Fe^{2+} was added.

The inhibition by the two dipyridyl disulphides was at least 90% within 5 min, but any residual activity would not have been detected by the bioassay procedure. Incubation of these reagents in the reaction mixture for 10 min before the addition of IPNS did not diminish the subsequent conversion of LLD-ACV into IPN in the presence of the enzyme. There was thus no indication that formation of mixed disulphides of the thiol-blocking reagents with the LLD-ACV substrate contributed to the decline in IPN synthesis.

IPNS inactivated by treatment with 2,2′-dipyridyl disulphide (Fig. 5) could be re-activated by the addition of DTT. After 45 min incubation at room temperature with 2.5 mM-DTT, in excess of that required to react with the reagent present, the IPNS activity was 40% of that obtained from a control solution of enzyme to which the reagent had not been added. After similar treatment with 16 mM-DTT the IPNS activity regained was 80% of the control sample.

Reaction of IPNS with thiol-blocking reagents

The reactions of thiol groups in IPNS with 2,2′-dipyridyl disulphide and DTNB were monitored by measurement of the increase in absorbance at 343 nm and 412 nm respectively under conditions similar to those used to determine the effect of these reagents on IPNS activity. With 2,2′-dipyridyl disulphide a rapid reaction was about half complete within 3 min, comparable with the time in which the enzyme was half-inactivated. The final value obtained for thiol-group content was about 48% of that found when the enzyme used was denatured in 4 M-urea. The latter value corresponded to about 1.3 mol of SH groups/mol of enzyme.

Reaction with DTNB in 4 M-urea gave a value for thiol-group content close to that obtained under these conditions with 2,2′-dipyridyl disulphide. However, with the native enzyme the thiol-group content found was only 28% of that obtained with DTNB and the denatured enzyme.

DISCUSSION

The finding that the removal of trace metal ions from the IPNS reaction system greatly diminished the concentration of added Fe^{2+} needed for maximum activity may be explained by the ability of a number of transition-metal ions to compete for the Fe^{2+}-binding site of the enzyme.

The simplest explanation of the rapid and major loss of activity when IPNS reacted with *N*-ethylmaleimide and pyridine disulphides is that at least one of the two thiol groups in IPNS is required for a maximum rate of catalysis, although the introduction of these relatively large blocking groups could also have a less direct effect on the enzyme. The lack of a comparable inhibition by DTNB, which carries two negative charges, may be due to steric effects or to the location of the cysteine residues in a hydrophobic environment. The observation that the reversible inactivation by 2,2′-dipyridyl disulphide was significantly faster in the presence of Fe^{2+} than in its absence could be due to the blocking of a thiol group by other transition-metal ions or to a conformational change on the addition of Fe^{2+} that increased the reactivity of a thiol group. But the presence of a thiol group in the enzyme is not essential for it to show any activity at all: Samson *et al.* (1987) have modified IPNS by mutagenesis *in vitro* and found that 5% of the activity is retained and that the K_m for LLD-ACV is increased approx. 5-fold when cysteine-106 is changed to serine. When cysteine-255 is changed to serine approx. 50% of the activity remains. We therefore suggest that the thiol group of cysteine-106, at least, reacts readily with 2,2′-dipyridyl disulphide. The fact that some reaction of DTNB with the enzyme was observed, although DTNB caused no significant loss in IPNS activity, might be due to the presence of some partially denatured IPNS in the purified preparation. But the precise stoichiometry of the reactions of the enzyme with thiol-blocking reagents has not yet been clarified.

The precise roles of the cysteine (or serine) residues of IPNS in the binding of Fe^{2+} or the binding and cyclization of LLD-ACV have still to be determined, and the loss of the ability of the enzyme to function when the pH is lowered from 8.0 to 6.0 raises the question whether a histidine or dicarboxylic amino acid residue is also involved in one of these processes.

In earlier experiments with relatively low concentrations of IPNS it had been found that the amount of IPN produced from LLD-ACV in 30 min was substantially increased in the presence of DTT, but that an initial rapid reaction virtually ceased within 15 min although most of the substrate was still unchanged. Under the conditions used in the present work DTT was completely oxidized by O_2 in the presence of Fe^{2+} within 30 min. Since it is known that this Fe^{2+}-catalysed reaction generates reactive oxygen species, such as $O_2^{\cdot -}$, H_2O_2 and $HO^{\cdot}$, and that these species can inactivate certain enzymes (Kim *et al.*, 1985), it seemed possible that the much less readily oxidizable GSH would prolong the reaction by maintaining an optimum thiol-group concentration for IPNS activity. However, in the presence of Fe^{2+}, O_2, ascorbate and GSH IPNS catalysed a rapid conversion of LLD-ACV into IPN for only about 5 min, when virtually all the added GSH was still in the thiol form. This phenomenon was not due to a lack of substrate or cofactors, but to inactivation of the enzyme, since a rapid conversion was only renewed by the addition of further IPNS. The amount of IPN formed during the rapid phase of the reaction was approximately proportional to the concentration of enzyme used, and the results obtained with a reaction mixture containing 20 mM-GSH indicated that inactivation was nearly complete after about 200 catalytic events. Hence in the presence of enough enzyme virtually all the LLD-ACV substrate can be rapidly converted into IPN.

It thus appears likely that inactivation of IPNS during the conversion of LLD-ACV into IPN can be brought about by a mechanism that depends on the nature of the catalytic reaction. The present results could be accounted for if a derivative of the substrate underwent non-productive binding to the enzyme in competition with the oxidative cyclization of the substrate to IPN, but no evidence for this has been obtained. An alternative hypothesis is that inactivation is brought about by certain highly reactive species formed during the catalytic cycle, such as those formulated as containing iron–carbon bonds that have been proposed to account for the cyclizations observed with this enzyme (Baldwin, 1985). The large decrease in the number of catalytic events that was observed in the absence of ascorbate could be due to the ability of ascorbate to remove such species or to convert enzyme-bound Fe^{3+} into Fe^{2+}, as has been suggested for prolyl 4-hydroxylase by De Jong *et al.* (1982). Further studies of the nature of the inactivated enzyme should throw light on these questions.

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