A Heuristic Approach to the Analysis of Enzymic Catalysis: Reaction of δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-α-aminobutyrate and δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-allylglycine Catalyzed by Isopenicillin N Synthase Isozymes†

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ABSTRACT: Isopenicillin N synthase (IPNS) catalyzes the oxidative cyclization of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine to isopenicillin N. It is proposed that the multiple products produced from certain substrate analogues result from pathway branching after formation of a ferryl oxene intermediate. We have been interested in ascertaining the reasons for multiple product formation. One possibility is that the products are *predisposed toward formation* once the β-lactam ring and the ferryl oxene are produced. Alternately, the products may be *persuaded into being* by the enzyme restricting conformations such that otherwise less favorable chemistry can take place. With the existing description of the IPNS catalytic cycle, this fundamental question has not been answerable. We describe here the application of a heuristic method to resolve this key issue. It was reasoned that by comparing the ratios of products formed by a set of perturbed IPNS variants it might be possible to generate qualitative information about the relative magnitude of certain activation parameters. If certain product ratios are affected but others are not, then it should be possible to say which steps in the reaction are dictated merely by chemical fundamentals and which steps are directly effected by the enzyme. In this paper we report the high-level expression, purification, and characterization of four IPNS isozymes. Comparison of the product ratios obtained on incubation of unnatural substrate analogues with four IPNS isozymes corresponding to perturbed active site variants shows substantial variation in some cases and little in others. Interpretation of the results obtained with δ-(L-α-aminoadipoyl)-L-cysteinyl-D-α-aminobutyrate (ACAB) allows conclusions to be drawn regarding the role of the enzyme in restricting available conformations of the natural substrate to disfavor certain otherwise chemically favorable pathways and hence products. The results obtained with δ-(L-α-aminoadipoyl)-L-cysteinyl-D-allylglycine, while rather more complex, substantiate the conclusions drawn from the ACAB data. A major conclusion is that, in the oxidation of ACV, IPNS is a negative catalyst of cepham formation but a positive catalyst of penam formation.

A key step in the biosynthesis of the penicillins and cephalosporins involves the double oxidative cyclization of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV)[1] (**1**) to isopenicillin N (IPN) (**2**) mediated by isopenicillin N synthase (IPNS) (Baldwin & Abraham, 1988). The gene sequences of six IPNS isozymes from both fungal and bacterial sources have been determined and show extensive homologies (Figure 1). The double cyclization of ACV (**1**) to IPN (**2**) is a chemically unusual process in which the full oxidizing power of molecular oxygen is used. This is unprecedented among enzymes of the desaturase/monooxygenase class and thus warranted an investigation of the possible mechanisms

	S. lipmanii	*S. clavuligerus*	*S. jumonjinensis*	*C. acremonium*	*A. nidulans*	*P. chrysogenum*
S. lipmanii		74	70	62	61	61
S. clavuligerus	81		81	56	57	56
S. jumonjinensis	81	85		60	59	58
C. acremonium	68	65	68		74	76
A. nidulans	64	61	61	70		81
P. chrysogenum	68	63	65	75	75	

amino acid identities/%

nucleotide identities/%

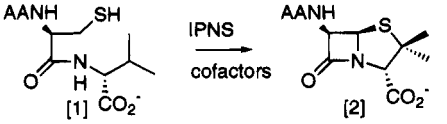

FIGURE 1: Pairwise sequence comparisons for six IPNS isozymes (upper) and the reaction catalyzed by IPNS (lower).

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[1] Abbreviations: AA, δ-(L-α-aminoadipoyl); ACAB, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-α-aminobutyrate; ACAG, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-allylglycine; ACV, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPN, isopenicillin N; IPNS, IPN synthase (aIPNS, cIPNS, pIPNS, and sIPNS refer to the isozymes from *Aspergillus nidulans, Cephalosporium acremonium, Penicillium chrysogenum* and *Streptomyces lipmanii,* respectively); IPTG, isopropyl thio-β-D-galactoside; IU, international unit; Tris, tris(hydroxymethyl)aminomethane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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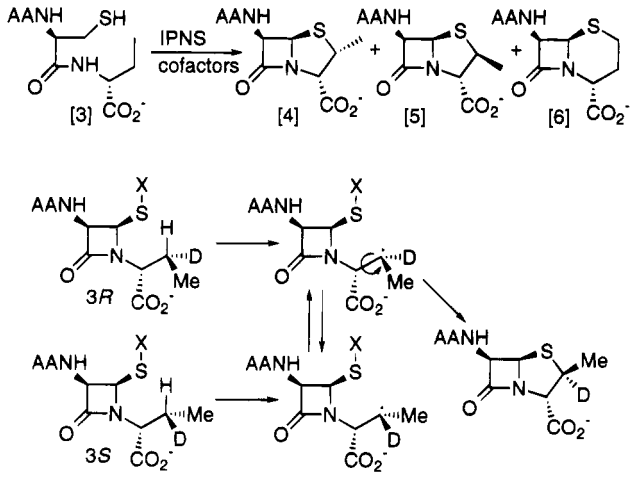


FIGURE 2: Conversion of AC-aminobutyrate tripeptide (**3**) by cIPNS.

involved. A wealth of substrate analogue studies designed to probe the mechanism have been carried out in this laboratory and form the subject of an extensive review (Baldwin & Bradley, 1990). While there has still been no direct observation of an intermediate, it is now generally accepted that the cyclization of ACV (**1**) to IPN (**2**) proceeds *via* an enzyme-bound monocyclic β-lactam. Incubation of AC-aminobutyrate (**3**), a sterically less demanding substrate than ACV (**1**), with IPNS gave rise to three products: a minor 2α-methyl penam (**4**), a major 2β-methyl penam (**5**), and a cepham (**6**) (Figure 2) (Baldwin et al., 1983a). The cepham product (**6**) was the first non-penam bicyclic product isolated and gave an indication of low selectivity in the second ring closure. Cepham formation from AC-aminobutyrate (**3**), but not from ACV (**1**), has previously been rationalized in terms of methyl (410 kJ mol^{-1}), methylene (398 kJ mol^{-1}), and methine (385 kJ mol^{-1}) C−H bond dissociation enthalpies. It was presumed that the enthalpy difference in the valinyl case is sufficient to dictate that only the penam product is formed, while in the aminobutyrate case, cepham formation becomes possible. When the aminobutyrate peptide was stereospecifically labeled with deuterium at the aminobutyrate C3 position and incubated with IPNS, the same penam products were isolated *irrespective* of the stereochemistry of the starting material. This result has been explained in terms of cleavage of the weaker C3−H bond of either epimeric substrate, providing a pair of rapidly equilibrating free secondary radicals. Closure of the radical form onto sulfur then results in the observed penam product (Figure 2) (Baldwin et al., 1983b). Thus the aminobutyrate tripeptide (**3**) appeared to provide the first firm evidence for a free radical pathway to a penam product. From this result and others it has been inferred that the second ring closure in the conversion of ACV (**1**) to IPN (**2**) proceeds *via* concerted insertion of an iron oxene into the C−H bond followed by a free radical pathway to form the C−S bond. That some β-lactam products appear to be formed stereospecifically (e.g., IPN (**2**)), while others are not (e.g., the methyl penams **4** and **5**), despite the proposed intermediacy of free radical species in all cases, has previously been explained in terms of the rates of inversion/rotation of primary, secondary, or tertiary free radicals relative to C−S bond formation rates.

Incubation of the substrate analogue AC-allylglycine (**7**) with IPNS gave six enzymatic products. Three of these (α- and β-vinyl penams **8** and **9** and homocephem **10**) arise *via*

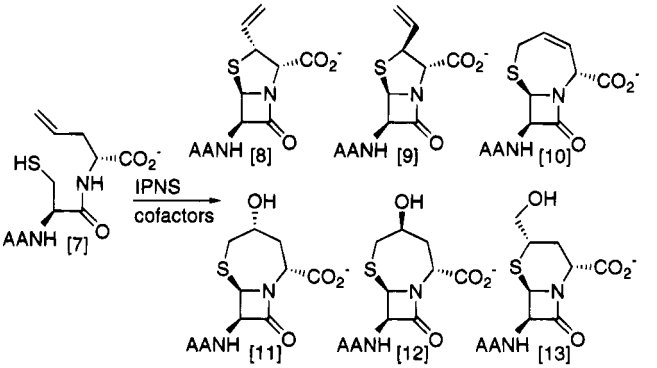


FIGURE 3: Conversion of AC-allylglycine tripeptide (**7**) by cIPNS.

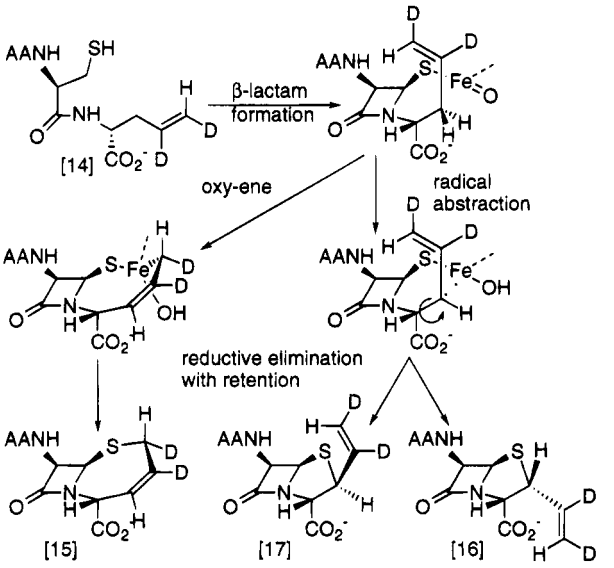


FIGURE 4: Conversion of AC-(*E*)-4,5-dideuterioallylglycine tripeptide (**14**) by cIPNS: Desaturase pathways.

a desaturase pathway, while the remaining three (α- and β-hydroxyhomocephams **11** and **12** and α-hydroxymethylcepham **13**) are derived by a monooxygenase pathway (Figure 3) (Baldwin et al., 1984b). In order to establish the stereochemistry of these products, AC-(*E*)-4,5-dideuterioallylglycine (**14**) was incubated with the enzyme. The desaturase products were found to be (5*R*)-4,5-dideuteriohomocephem (**15**), formed *via* an "oxy-ene" process, and α- and β-vinyl penams **16** and **17**, formed *via* a hydrogen atom abstraction pathway in which the olefinic stereochemistry has been retained (Figure 4) (Baldwin et al., 1991b). In addition, the synthesis and incubation of other stereospecifically deuterated AC-allylglycine tripeptides has shown that exclusive abstraction of the 3-*pro-S* hydrogen of the allylglycine residue occurs in both desaturase pathways (Baldwin et al., 1991b). Two of the monooxygenase products, the major (5*R*)-4,5-dideuterio-4α-hydroxyhomocepham (**18**) and the 4α-(deuteriomethylenehydroxy) cepham (**19**), can be rationalized by a [2+2] cycloaddition pathway from either of two possible conformations (the latter product is a single diastereomer but of unknown absolute stereochemistry in the hydroxymethylene group). The formation of the two other monooxygenase products observed, the minor (5*S*)-4,5-dideuterio-4α-hydroxyhomocepham (**20**) and the (5*R*)-4,5-dideuterio-4β-hydroxyhomocepham (**21**), has been explained in terms of the iron oxene effecting a *syn* epoxidation on either face of the olefin. Subsequent S_N2 attack by sulfur opens the two epoxides at the least

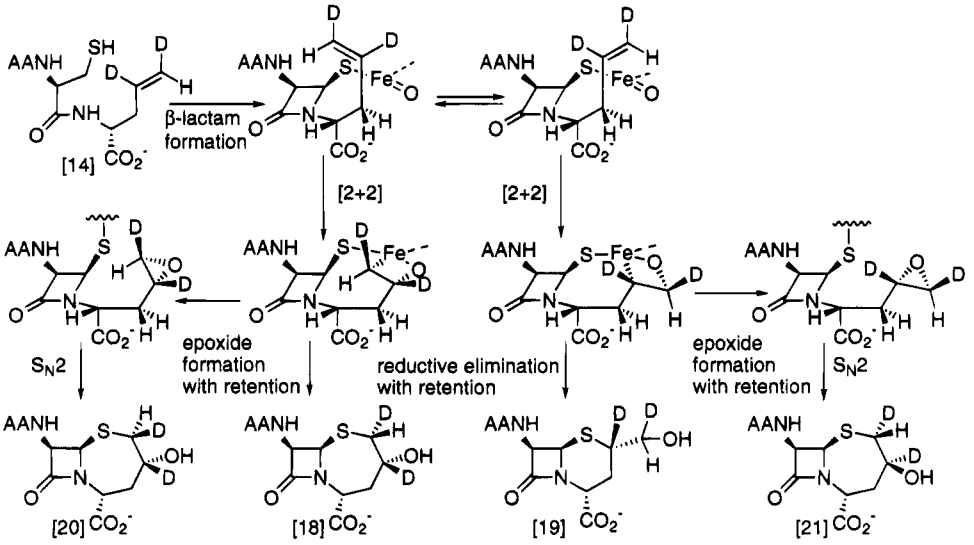


FIGURE 5: Conversion of AC-(*E*)-4,5-dideuterioallylglycine tripeptide (**14**) by cIPNS: Monooxygenase pathways.

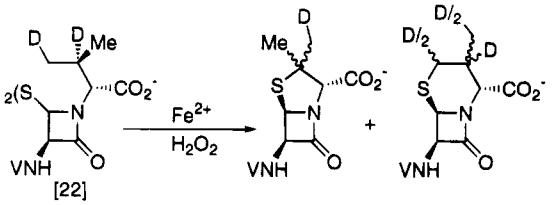


FIGURE 6: Biomimetic second-ring closure reaction.

hindered position to give the observed products (Figure 5) (Baldwin et al., 1991b).

Particularly pertinent in the context of this paper is a nonenzymatic reaction in which the second ring closure has been mimicked by treatment of a monocyclic β-lactam disulfide (**22**) with iron(II), hydrogen peroxide (or oxygen), and ascorbic acid (Figure 6) (Baldwin et al., 1985). However, the biomimetic route was found to lack the stereospecificity (retention of configuration in cyclization of ACV (**1**)) and regiospecificity (five-membered ring closure only from the valinyl substrate) of the enzyme-catalyzed reaction. This biomimetic reaction can be interpreted by suggesting either that the enzyme is imposing a modified set of "rules" on the chemical reactions occurring at the active site or that the chemistry carried out by the enzyme is *fundamentally different* from the uncatalyzed chemistry. The work described in this paper is aimed at resolving this issue.

A working model for the mechanism of the enzyme-catalyzed formation of isopenicillin N (**2**) from ACV (**1**) is shown in Figure 7.

The initial binding of the substrates, ACV (**1**) and dioxygen, results in a large decrease in entropy and thus free energy increases on formation of (**23**). At this stage, the iron can be formally considered to be either Fe^{2+} or Fe^{4+}, depending upon the extent of back donation from the iron *d*-orbitals into the dioxygen π-antibonding orbitals. This is difficult to predict since it will depend largely on the other ligands present, and these are not yet known with certainty.

After binding of the substrates, the next step involves the mildly exothermic reduction of dioxygen to a hydroperoxide ($\Delta G_o = -134$ kJ mol^{-1} in isolation) with concomitant oxidation of sulfur to give **24**. The cysteinyl 3-*pro-S* proton is then lost with electron density flowing from the iron-sulfur double bond onto iron. Simultaneously, the iron is oxidized to the ferryl-oxene species (**25**) with associated cleavage of the peroxide bond. Cleavage of the peroxide bond is expected to be highly exothermic ($\Delta G_o = -343$ kJ mol^{-1} in isolation). The net result of this step is the two-electron oxidation of C3 of the cysteinyl residue coupled with the two-electron reduction of hydrogen peroxide. In a reaction reminiscent of intramolecular Pummerer trapping, the valinyl nitrogen collapses in stereospecific fashion onto the *si* face of the sulfur-stabilized carbonium ion, generating the S-ferryl monocyclic β-lactam **26**. The intermediate (**26**) then undergoes concerted oxidative insertion of the iron-oxene moiety into the valinyl C3−H bond, giving the metallo heterocycle **27**. This species can then collapse to the bicyclic β-lactam (**2**) either directly by reductive elimination or *via* the intermediacy of the diradicaloid **28**. Either of these modes of collapse regenerates the enzyme in its original state and completes the reaction cycle.

We have been interested in investigating whether the production of IPN (**2**) from the monocyclic intermediate (**26**) is a consequence of active site control of chemistry or simply a manifestation of fundamental chemical reactivity principles, as alternate cepham products are conceivable (and have been observed in the biomimetic reaction described above) but have not been observed in the reaction catalyzed by cIPNS. A plethora of products have been produced from unnatural substrates, and one possibility is that these products are *predisposed toward formation* once analogous monocyclic intermediates have been produced. Alternately, they may be *persuaded into being* by the enzyme restricting conformations such that chemically more favorable reactions are inhibited, allowing otherwise less favorable chemistry to take place. With the existing description of the IPNS reaction cycle, these fundamental questions have been unanswerable, and thus a more detailed assessment of the enzyme's contribution to catalysis is required. In particular we wish to construct a Gibbs function profile for the enzyme mechanism, and the work described in this paper is directed toward this goal.

A Heuristic Approach. In the absence of a direct method for measuring internal activation barriers and Gibbs function changes, an alternate method must be used. We reasoned that an approach whereby the ratios of products from unnatural substrates were measured for various perturbed states of IPNS should give rise, at least in a *qualitative* sense, to the requisite information. Specifically, the actual mag-

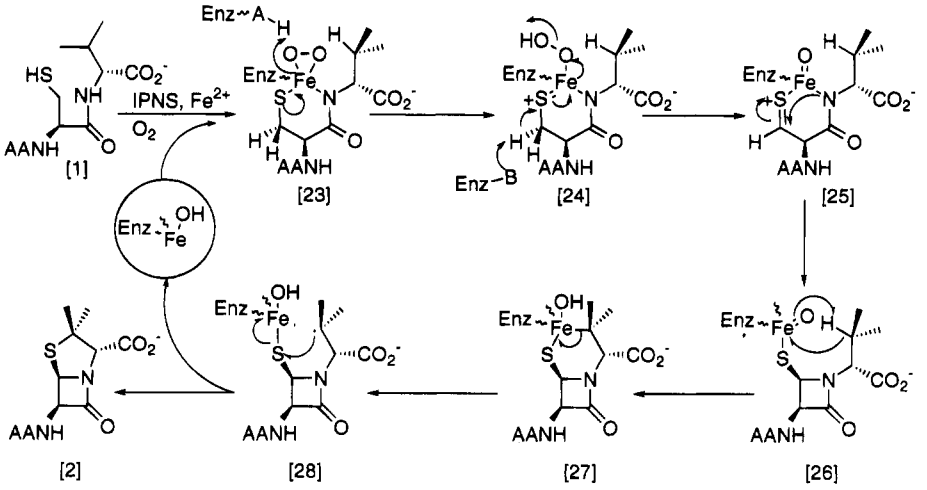


FIGURE 7: Proposed mechanism for the catalytic cycle of IPNS in the conversion of ACV (**1**) to IPN (**2**).

nitude of product ratios allows, if a valid conformation–reactivity picture is available, something to be said about the relative magnitudes of certain activation parameters. The Gibbs function difference between different internal states is accessible from an analysis of the way in which perturbation of the enzyme structure affects the variation in specific product ratios because of Hammond's postulate (Hammond, 1955). Thus, if it transpires that enzyme perturbation affects some product ratios but not others, and the states from which the products arise can be related, then it seems reasonable to infer that those products whose ratios are invariant upon perturbation are produced by exothermic reactions. For these exothermic (and the term is necessarily used in the *relative* fashion) processes, the transition states bear most structural similarity to the intermediates and thus are affected by the perturbation in a similar manner to the intermediates. On the other hand, products derived from common intermediate states and whose ratio changes upon perturbation are presumed to arise *via* reactions with *different* thermicities. The similarities between the transition states and the common intermediates will then be different, so the transition states will respond differentially to the active site perturbation.

The overall Gibbs function for the reaction is assumed to be strongly negative (*ca.* -150 kJ mol^{-1}). This follows from the known thermochemical data concerning dioxygen reduction to water ($\Delta G_o = -474$ kJ mol^{-1}) and necessarily crude calculations which suggest that the difference in Gibbs function values between ACV (**1**) and IPN (**2**) should be no more than 330 kJ mol^{-1}. Kinetic isotope effect data (Baldwin et al., 1984a) show that the first irreversible event is the cleavage of the cysteinyl C3–H bond and that the subsequent cleavage of the valinyl C3–H bond has a similar Gibbs function of activation. A consequence of this is that the initial binding event and the subsequent oxidation of sulfur (**23** to **24**) must have lower activation barriers. For both these events this seems reasonable; binding just involves ligand exchange, and oxidation of sulfur is chemically extremely facile. This leaves the intramolecular Pummerer-type trapping (**25** to **26**) and the overall collapse of **27** to **2** for consideration.

The unfavorable ring strain in the transition state leading from **25** to **26** would be expected to be offset by the associated charge neutralization. The same factors should also apply to the associated thermodynamics, and thus it is assumed that the conversion of **25** to **26** has a negative Gibbs function and a low activation barrier. It is difficult to

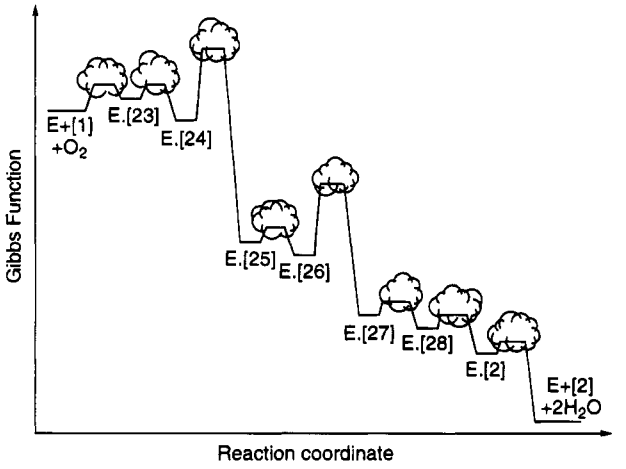


FIGURE 8: Prototype Gibbs function diagram for the oxidative cyclization of ACV (**1**) to IPN (**2**) catalyzed by IPNS.

speculate about the conversion of **27** to **2** (through **28** or not) because of a lack of chemical precedent. The release of the product (**2**) from the enzyme has also not been considered previously, and it was thought that this might be important, as the product does not have the iron-ligating capacity of the substrate. On this point, it is pertinent to stress that while all enzymes must bind substrates *and* products, in the case of IPNS the *gross* structural and chemical differences make comparable binding seem extremely unlikely. In the prototype Gibbs function diagram proposed here (Figure 8), the release of product is assumed to be accompanied by a large drop in Gibbs function.

As is evident then, there are many uncertainties in the energetics of the IPNS-catalyzed conversion of ACV (**1**) to IPN (**2**). It was hoped that the novel approach outlined above might resolve some of these uncertainties and answer some of the fundamental questions posed in preceding paragraphs.

Crucial, then, to the comparative approach is a set of active but perturbed IPNS variants. The modern techniques of protein engineering allow perturbations to be made to an enzyme by site-directed mutagenesis (Leatherbarrow & Fersht, 1986). However, in the absence of a structural model for IPNS and with only limited data as to which residues constitute the active site (Baldwin et al., 1990), it is difficult to predict which site-directed mutations might alter the active site to *perturb activity significantly without destroying it altogether*. As has been discussed, IPNS has been isolated from many sources, and the isozymes have been shown to

have significantly *different* amino acid sequences (Figure 1). It is likely that some of the regions of strong homology are essential for catalytic activity and are presumably thus in the active site (or distant but crucial from a structural maintenance point of view). Other residues in the active site are of lesser importance, and change might be tolerated as regards processing of the natural substrate. The isozymes have evolved to process the natural substrate, and it is unlikely that evolutionary pressure has been brought to bear on the processing of unnatural substrates. Thus the isozymes comprise a class of IPNS variants which are all active toward the natural substrate but which have not been preselected on the basis of their ability to handle unnatural substrates (this is important to ensure that any comparison is not intrinsically predisposed to give one result or the other).

It is known that a single amino acid change, whether in the active site or not, can grossly affect both the structure and the activity of a protein (Loll & Lattman, 1990; Sowek et al., 1991). It therefore seems inconceivable that a 20−40% change in amino acid composition will not significantly perturb the active site of an enzyme, especially with respect to its interactions with an unnatural substrate. The minor active site changes assumed from the sequence heterology of the isozymes are therefore exactly the sort of perturbations required to test our assumptions concerning the chemistry of this enzyme.

We have chosen, for initial comparison, the enzymes from the fungi *Cephalosporium acremonium* (cIPNS) (Samson et al., 1985), *Aspergillus nidulans* (aIPNS) (Weigel et al., 1988), and *Penicillium chrysogenum* (pIPNS) (Carr et al., 1986) and from the bacterium *Streptomyces lipmanii* (sIPNS) (Weigel et al., 1988). For the purposes of comparing product ratios from unnatural substrate conversions, it was envisaged that large quantities of each of these enzymes would be required. We have recently reported the high-level overexpression of the isozymes from *C. acremonium*, *A. nidulans*, and *P. chrysogenum* in *E. coli* (Baldwin et al., 1991a). In this paper we describe the high-level over-expression of the *S. lipmanii* IPNS (sIPNS) in *Escherichia coli* and the purification and characterization of the four different isozymes from recombinant sources. In addition we describe the results obtained upon incubation of ACV (**1**), δ-(L-α-aminoadipoyl)-L-cysteinyl-D-α-aminobutyrate (ACAB, **3**), and δ-(L-α-aminoadipoyl)-L-cysteinyl-D-allylglycine (ACAG, **7**) with these isozymes.

MATERIALS AND METHODS

Materials. δ-L-α-Aminoadipoyl-L-cysteinyl-D-valine (ACV) (**1**) and cephalosporin C were the generous gift of Eli Lilly and Co., Indianapolis, IN. δ-L-α-Aminoadipoyl-L-cysteinyl-D-aminobutyrate (**3**) and δ-L-α-aminoadipoyl-L-cysteinyl-D-allylglycine (**7**) were prepared according to published procedures (Baldwin et al., 1983a, 1984b, 1991b). Nitrocefin was from Glaxo Group Research Ltd., and lithium clavulanate was the gift of SmithKline Beecham Pharmaceuticals. Bacto penicillinase was from Difco. Ampicillin, chloramphenicol, kanamycin, and tetracycline were purchased from Sigma. Plasmid pOGO249 was the generous gift of Eli Lilly and Co., and plasmid pRH1090 was supplied by Mr. R. J. Heath of this Laboratory.

Restriction endonucleases, DNA-modifying enzymes, and appropriate 10× buffer concentrates were purchased from Gibco-BRL, Amersham International, Pharmacia, New England Biolabs, or Northumbria Biologicals Ltd. Molecular weight markers for protein and DNA gels were purchased from Gibco-BRL. Deoxynucleotide triphosphates, dideoxynucleotide triphosphates, and Sequenase 2.0 for DNA sequencing were purchased in kit form from USB through Cambridge Bioscience. Deoxynucleotide triphosphates and dideoxynucleotide triphosphates for purposes other than sequencing were purchased from Pharmacia (Ultrapure grade). Deoxynucleotide α-^{35}S-thiotriphosphates were from Amersham International. Sequagel, buffer, and diluent for polyacrylamide DNA sequencing gels and Protogel for SDS−PAGE protein gels were obtained from Flowgen.

Anhydrous chemicals and biochemicals used to prepare buffers and reagents were of the highest purity available and were purchased from BDH, Gibco-BRL, May & Baker, or Sigma.

Bacterial Strains. The following strains of *E. coli* were used: TG1 (*supE*, *hsd*Δ5, *thi*, Δ(*lac-proAB*), {F′, *traD*36, *proAB*$^+$, *lac I*^{q}, *lacZ*ΔM15}) (Sambrook et al., 1989), JM109 (*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, Δ(*lac-proAB*), {F′, *traD*36, *proAB*$^+$, *lac I*^{q}, *lacZ*ΔM15}) (Yanisch-Perron et al., 1985), and NM554 (*recA*1, *araD*139, Δ(*araABC-leu*) 7697, Δ*lacX*74, *galU*$^-$, *galK*$^-$, *hsdR*, *hsdM*$^+$, *rspL*, *strA*, *thi*, *mcrA*(−), *mcrB*(−)) (Raleigh et al., 1988).

Plasmid Construction. pRH1090 was digested to completion with *Nde*I and *Bam*HI, and the ~4.6-kb fragment comprising the vector backbone was purified by preparative agarose gel electrophoresis. pOGO249 was digested to completion with *Nde*I and partially with *Bam*HI, and the ~1.3-kb fragment containing the sIPNS gene was purified by preparative agarose gel electrophoresis. Ligation of the pRH1090- and pOGO249-derived fragments followed by transformation into *E. coli* JM109 and selection for chloramphenicol resistance gave pJB800.

Expression of IPNS Isozymes in E. coli. E. coli strains NM554 and JM109 were transformed with the appropriate plasmid according to the IPNS isozyme to be expressed. Transformation mixtures were plated at 27 °C onto LB agar containing either chloramphenicol (20 μg mL^{-1}) or kanamycin (50 μg mL^{-1}) as required. Single colonies were used to inoculate 3 mL of LB containing the appropriate antibiotic, and the cultures were grown overnight at 27 °C and 250 rpm. The overnight cultures were used as 1% inocula into 100 mL of 2× TY or M9 minimal medium containing the appropriate antibiotic. Cultures were incubated at temperatures between 27 and 42 °C and at 250 rpm. When required, IPTG was added to the cultures at a final concentration of 1 mM. Cell growth was monitored by measuring the OD_{550}. Cells were harvested by centrifugation at 3500 rpm and lysed by sonication. Total soluble cell protein was quantified by the method of Bradford (Bradford, 1976), and IPNS specific activity was measured by plate bioassay (Pang et al., 1984). Levels of IPNS activity too low to be detected by bioassay were assessed by a β-lactamase induction assay (Sykes & Wells, 1985). SDS-PAGE was carried out using the discontinuous buffer system (Laemmli, 1970).

When optimal conditions for growth and expression had been established on a small scale, large-scale fermentations were carried out using the same conditions in either 5- or 40-L vessels using a 1% inoculum grown at 27 °C.

Purification of C. acremonium IPNS. E. coli NM554 transformed with pJB508 (Baldwin et al., 1991a) was fermented at 37 °C in 10 L 2× TY*kan* and harvested,

yielding 110 g of cells (wet weight). One hundred grams of cells was lysed, and the soluble protein (~5.2 g) was applied at 4 °C to a Q-Sepharose HP 60/100 anion-exchange column previously equilibrated with 50 mM Tris·HCl, pH 7.4, on a Biopilot FPLC system. The column was washed with 600 mL of 50 mM Tris·HCl, pH 7.4, and 50 mM NaCl, and proteins were eluted by application of a 50−250 mM NaCl linear gradient over 2.25 L at 20 mL min^{-1}. Fractions containing IPNS activity were assessed for purity by SDS−PAGE and were pooled and concentrated by ultrafiltration through YM10 membranes (Amicon, Beverly, MA) as appropriate, yielding 595 mg of purified protein.

Purification of A. nidulans IPNS. E. coli NM554 transformed with pJB703 (Baldwin et al., 1991a) was grown at 37 °C in 5 L of 2× TY*cam* and harvested yielding 25 g of cells (wet weight). These cells were lysed, and the soluble protein (~2.3 g) was applied in two equal batches at 4 °C to a Q-Sepharose HP 60/100 anion-exchange column previously equilibrated with 50 mM Tris·HCl, pH 7.4. The column was washed with 600 mL of 50 mM Tris·HCl, pH 7.4, and 50 mM NaCl, and proteins were eluted by application of a 50−200 mM NaCl linear gradient over 2.25 L at 20 mL min^{-1}. Fractions were assayed for IPNS activity and by SDS−PAGE and were pooled and concentrated by ultrafiltration through YM10 membranes as appropriate, yielding 900 mg of purified material.

Purification of S. lipmanii IPNS. E. coli JM109 transformed with pJB800 was grown at 30 °C in 10 L of 2× TY*cam* containing 1 mM IPTG and harvested, yielding 97 g of cells (wet weight). Forty-seven grams of cells was lysed, and the soluble protein (3.55 g) was applied to a Q-Sepharose HP 60/100 anion-exchange column previously equilibrated at 4 °C with 50 mM Tris·HCl, pH 7.8. The column was washed with 600 mL of 50 mM Tris·HCl, pH 7.8, and 75 mM NaCl, and proteins were eluted by application of a 75−300 mM NaCl linear gradient over 2.25 L at 20 mL min^{-1}. Fractions were assayed for IPNS activity and by SDS−PAGE and were pooled and concentrated by ultrafiltration through YM10 membranes as appropriate, yielding 285 mg of protein. A portion of this material (17.5 mg) was loaded in 2.1 mL onto a Superdex S-200 35/600 gel filtration column equilibrated at 4 °C with 50 mM Tris·HCl, pH 7.4, and eluted in the same buffer. Fractions containing IPNS activity were assessed for purity by SDS−PAGE and were pooled and concentrated by ultrafiltration through YM10 membranes as appropriate. The protein solution (4.8 mg, 21 mL) was applied to a Dynochrom Neobar AQ column preequilibrated at 4 °C with 50 mM Tris·HCl, pH 7.5, 10 mM KCl, and 10 mM $MgCl_2$. Proteins were eluted with a linear gradient of 50−300 mM NaCl over 80 mL at 4 mL min^{-1}. The fractions containing IPNS activity were pooled and concentrated as above, yielding 1.7 mg of purified protein.

Partial Purification of P. chrysogenum IPNS. E. coli JM109 transformed with pJB603 (Baldwin et al., 1991a) was fermented at 37 °C in 25 L of 2× TY*cam* containing 1 mM IPTG and harvested, yielding 155 g of cells (wet weight). Ninety grams of cells was lysed, and the crude soluble protein (~6 g) was applied in three batches (3 × 60 mL) to a Superdex S-200 60/600 column equilibrated with 50 mM Tris·HCl, pH7.4, 50 mM KCl, 1 mM DTT, and 1 mM ascorbate at 4 °C. Proteins were eluted in the same buffer, and fractions containing IPNS activity were pooled and concentrated as above, yielding 375 mg of this partially purified material.

Characterization of the IPNS Isozymes. The N-terminal amino acid sequences of the isozymes were obtained by Mr. A. C. Willis of the MRC Immunochemistry Unit of the Department of Biochemistry, Oxford University, using an Applied Biosystems 470 sequenator.

Mass spectra of the purified proteins were determined by Dr. R. T. Aplin of this Laboratory using a VG BIO-Q electrospray mass spectrometer. Proteins were desalted and exchanged into 20 mM Tris·HCl, pH 7.5, using a NAP 5 Sephadex G-25 column. The samples were diluted with methanol and acetic acid to give a final concentration of ~13 pmol μL^{-1} (0.5 mg mL^{-1}) enzyme, 50% methanol, and 1% acetic acid, and the solutions were then introduced into the electrospray system. The mass scale was calibrated using multiply charged peaks from a separate introduction of myoglobin.

Isoelectric focusing gel electrophoresis using Ampholine-PAG plates (Pharmacia) was used to estimate the p*I*s of the purified proteins.

The purity of the proteins was assessed, where appropriate, by analytical gel filtration on a Gilson HPLC and otherwise by Coomassie Blue stained SDS−PAGE gels.

Determination of pH Dependence of Specific Activity. A 50 mM solution of each buffer, Tris, phosphate, and NH_4HCO_3, was prepared and adjusted to a variety of pHs with concentrated HCl or 5 M NaOH. Only pHs that were compatible with the buffering range of each compound were used. A volume of a solution containing 7.8 mM ACV-disulfide (**1**), 25 mM DTT, 12.5 mM ascorbate, 1.25 mM $FeSO_4$, and 50 mM Tris·HCl, pH 7.7, sufficient to add 30 μL to each reaction mixture was prepared immediately before use. In this way, the tube to tube variation in concentration of each of the substances was minimized. Similarly, a sufficient volume of each enzyme preparation was diluted immediately prior to use in Milli-Q water. Two hundred forty microliters of buffer and 30 μL of substrate concentrate were mixed in a glass vial, and the reaction was started by addition of 60 μL of freshly diluted enzyme. The final dilutions of each enzyme were as follows: cIPNS, 1:300; aIPNS, 1:300; sIPNS, 1:50; and pIPNS, 1:10. The reaction mixtures were incubated in a bench-top orbital shaker at 27 °C and 250 rpm for 10 min, and the reactions were quenched by addition of 330 μL of cold methanol. Assays were carried out in duplicate. Precipitated protein was removed by centrifugation, and 100 μL of each assay mixture was then plated into 10-mm wells in *S. aureus* NCTC 6571 bioassay plates. Plates were incubated overnight at 37 °C, and the specific activities were plotted against pH.

β-Lactamase Activity Assay. The IPNS isozyme preparations to be used for ^1H NMR experiments were assessed for β-lactamase activity using the following assay adapted from the β-lactamase induction assay (Sykes & Wells, 1985): Nitrocefin (0.4 mg mL^{-1} in 5% DMSO) was diluted to a final concentration of 80 μM in 50 mM sodium phosphate, pH 7.5. Enzyme preparations were diluted in 50 mM sodium phosphate, pH 7.5, to the following concentrations: cIPNS, 3 mg mL^{-1}; aIPNS, 4 mg mL^{-1}; sIPNS, 8.5 mg mL^{-1}; and pIPNS, 9 mg mL^{-1}. BSA was made up at 10 mg mL^{-1}. Nine hundred microliters of the nitrocefin solution was mixed with 100 μL of the diluted enzyme solution. The absorbance at 485 nm corresponding to the λ_{max} of the hydrolysis product [λ_{max}(nitrocefin) = 385 nm] was monitored continuously over

30 min. Controls were carried out with BSA and Bacto penicillinase and with the equivalent concentration of Tris·HCl in the absence of protein.

Inhibition of β-Lactamase Activity. Enzyme preparations showing β-lactamase activity were incubated with 1 mg mL^{-1} lithium clavulanate for 10 min at room temperature. Protein was then buffer exchanged into 50 mM sodium phosphate, pH 7.5, using a Sephadex G-25 NICK column (Pharmacia) to remove excess clavulanate and then reassayed for β-lactamase activity.

Incubation of ACV Analogues with IPNS Isozymes. Incubations were carried out using cIPNS, aIPNS, and sIPNS purified by Q-Sepharose ion-exchange chromatography and pIPNS purified by Superdex S-200 gel filtration. Before use, the pIPNS preparations were preincubated for 10 min at room temperature with 1 mg mL^{-1} lithium clavulanate. Approximately 5 IU of each enzyme was used per milligram of substrate. Protein samples were thawed as required and exchanged into 50 mM NH_4HCO_3, pH 7.7 on a Sephadex G-25 NAP 5 column immediately prior to use. One milligram of ACV (**1**), or an analogue thereof, was dissolved in 50 μL of buffer, and 50 μL each of 100 mM DTT, 50 mM ascorbate, and 5 mM $FeSO_4$ (all made up in 50 mM Tris·HCl, pH 7.5) was added. This substrate and cofactor mix was added to the enzyme preparation in a glass vial, and the reaction mixture was incubated for 15 min at 27 °C and 250 rpm in a bench-top orbital shaker. Cold acetone (~4 °C) was added to 70% by volume, and the protein precipitated for 5 min at room temperature. Precipitated protein was removed by centrifugation at 13 500 rpm for 5 min, and the acetone then removed on a vacuum centrifuge. The aqueous remainder was frozen and lyophilized. The lyophilysate was dissolved in 400 μL of D_2O, passed through a sterile 0.22-μm filter, and the 500-MHz ^1H NMR spectrum was examined for the presence of the characteristic β-lactam resonances in the region of 4.8−6.0 ppm. The β-lactam signals were integrated and used to determine the ratios of the various products formed. One spectrum for each of the three different substrates was re-recorded ~2 h after the original acquisition had finished. Each reaction was repeated using double the incubation time, and the spectra were recorded.

The β-lactam resonances observed were assigned on the basis of ^1H NMR spectra of previously purified metabolites, as detailed below:

*Incubation of δ-L-α-Aminoadipoyl-L-cysteinyl-D-valine (**1**) (Baldwin et al., 1986a) (2S,5R,6R)-6-[(5S)-5-Amino-5-carboxypentanamido]-3,3-dimethyl-7-oxo-1-aza-4-thiabicyclo-[3.2.0]heptane-2-carboxylic Acid (IPN (**2**)).* δ_H(500 MHz, D_2O), 5.35 and 5.45 (2H, ABq, *J* 4Hz, 5-*H* and 6-*H*).

*Incubation of δ-L-α-Aminoadipoyl-L-cysteinyl-D-aminobutyrate (**3**) (Baldwin et al., 1983a) (2S,3R,5R,6R)-6-[(5S)-5-Amino-5-carboxypentanamido]-3-methyl-7-oxo-1-aza-4-thiabicyclo[3.2.0]heptane-2-carboxylic Acid (α-Methyl Penam (**4**)).* δ_H(500 MHz, D_2O), 5.36 and 5.45 (2H, ABq, *J* 4Hz, 5-*H* and 6-*H*).

*(2S,3S,5R,6R)-6-[(5S)-5-Amino-5-carboxypentanamido]-3-methyl-7-oxo-1-aza-4-thiabicyclo[3.2.0]heptane-2-carboxylic Acid (β-*Methyl Penam (**5**)). δ_H(500 MHz, D_2O), 5.31 and 5.37 (2H, ABq, *J* 4Hz, 5-*H* and 6-*H*).

*(2R,6R,7R)-7-[(5S)-5-Amino-5-carboxypentanamido]-8-oxo-1-aza-5-thiabicyclo[4.2.0]octane-2-carboxylic Acid (Cepham (**6**)).* δ_H(500 MHz, D_2O), 5.15 and 5.28 (2H, ABq, *J* 4Hz, 6-*H* and 7-*H*).

*Incubation of δ-L-α-Aminoadipoyl-L-cysteinyl-D-allylglycine (**7**) (Baldwin et al., 1984b; Baldwin et al., 1991b). (2S,3R,5R,6R)-6-[(5S)-5-Amino-5-carboxypentanamido]-3-vinyl-7-oxo-1-aza-4-thiabicyclo[3.2.0]heptane-2-carboxylic Acid (α-Vinyl Penam (**8**)).* δ_H(500 MHz, D_2O), 5.47 and 5.58 (2H, ABq, *J* 4Hz, 5-*H* and 6-*H*).

*(2S,3S,5R,6R)-6-[(5S)-5-Amino-5-carboxypentanamido]-3-vinyl-7-oxo-1-aza-4-thiabicyclo[3.2.0]heptane-2-carboxylic Acid (β-Vinyl Penam (**9**)).* δ_H(500 MHz, D_2O), 5.45 and 5.47 (2H, ABq, *J* 4Hz, 5-*H* and 6-*H*).

*(2R,7R,8R)-8-[(5S)-5-Amino-5-carboxypentanamido]-9-oxo-1-aza-6-thiabicyclo[5.2.0]non-3-ene-2-carboxylic Acid (Homocephem (**10**)).* δ_H(500 MHz, D_2O), 5.30 and 5.48 (2H, ABq, *J* 4Hz, 7-*H* and 8-*H*).

*(2R,4R,7R,8R)-8-[(5S)-5-Amino-5-carboxypentanamido]-4-hydroxy-9-oxo-1-aza-6-thiabicyclo[5.2.0]nonane-2-carboxylic Acid (α-Hydroxyhomocepham (**11**)).* δ_H(500 MHz, D_2O), 5.26 and 5.38 (2H, ABq, *J* 4Hz, 7-*H* and 8-*H*).

*(2R,4S,6R,7R)-7-[(5S)-5-Amino-5-carboxypentanamido]-4-(hydroxymethyl)-8-oxo-1-aza-5-thiabicyclo[4.2.0]octane-2-carboxylic Acid (α-Hydroxymethylcepham (**13**)).* δ_H(500 MHz, D_2O), 5.24 and 5.34 (2H, ABq, *J* 4Hz, 6-*H* and 7-*H*).

RESULTS

Expression of Streptomyces lipmanii IPNS. The gene encoding isopenicillin N synthase in *Streptomyces lipmanii* (sIPNS) has been cloned and sequenced by workers at Eli Lilly. Plasmid pOGO249 was constructed in which the sIPNS gene was under the control of the λP_L promoter, but transformation of this plasmid into *E. coli* and temperature shift induction resulted in production of sIPNS almost exclusively in insoluble, inactive inclusion bodies (Weigel et al., 1988). Since attempts at refolding cIPNS from inclusion bodies had met with only limited success (J. E. Baldwin, J. M. Blackburn, and J. D. Sutherland, unpublished results), it was therefore thought that this expression system would be unsuitable for the purposes of this work, and so it was necessary to construct an alternative. The gene was available as an *Nde*I/*Bam*HI fragment which was directly suitable for transfer into a high copy number vector analogous to those used previously for high-level, soluble expression of other IPNS isozymes (Baldwin et al., 1991a). Plasmid pRH1090 (Baldwin et al., 1992) was constructed from pJS62 specifically for expression of genes containing an *Nde*I site rather than an *Nco*I site at the start codon as described previously.

The sIPNS gene was subcloned into the pRH1090 *Bam*HI/*Nde*I backbone as a 1.3-kb *Bam*HI partial/*Nde*I fragment, giving pJB800 (Figure 9). The fine structure restriction map of pJB800 was confirmed by double-strand DNA sequencing across the tandem promoter region, which clearly showed an intact *trc* promoter (data not shown) and correctly positioned 5′ coding region of the sIPNS gene.

Plasmid pJB800 was transformed into *E. coli* strains NM554 and JM109 for expression studies. Crude lysates from both transformed strains grown at 37 °C were tested by bioassay, but only very low levels of IPNS activity were found. SDS−PAGE of the crude lysates was also carried out, but no discernible band could be seen on the gel at the expected size of ~35 kDa (data not shown). The cell debris was then resolubilized and analyzed by SDS−PAGE and bioassay. The bioassay indicated only very low levels of activity, but SDS−PAGE showed the presence of a very intense band at ~35 kDa in both samples which was absent

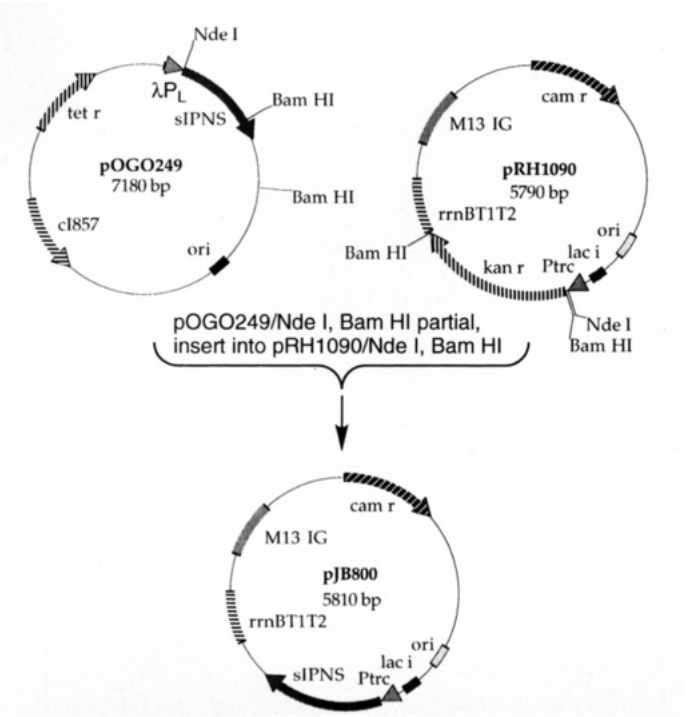
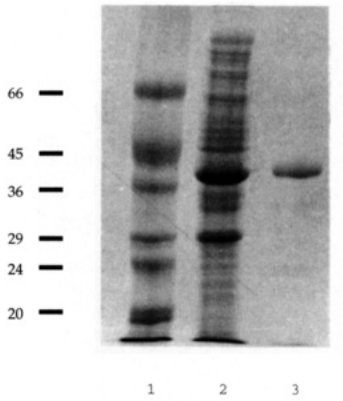


FIGURE 9: Construction of high-level sIPNS expression vector pJB800.

in control samples of pJS62/JM109 and pJS62/NM554 (data not shown). Thus under these initial conditions, expression of sIPNS was occurring in the form of insoluble, inactive inclusion bodies, and it was considered unlikely that the sIPNS protein would prove any more amenable to refolding than had the cIPNS protein extracted from inclusion bodies. In an attempt to produce soluble enzyme, the transformed strains were grown at 30 °C without IPTG induction. Analysis of the crude lysate by bioassay showed that at this lower temperature there was an increase in the level of *soluble* IPNS activity compared to cultures grown at 37 °C (data not shown). SDS−PAGE showed the presence of an ~35-kDa protein in the soluble lysates from the 30 °C cultures which was absent in the pJS62 controls and also absent in the soluble lysates from the 37 °C cultures (data not shown). Thus it was concluded that while growth of cultures of pJB800/NM554 or pJB800/JM109 at 37 °C resulted in the formation of inclusion bodies, growth at a lower temperature, 30 °C, could switch expression to the soluble, active form.

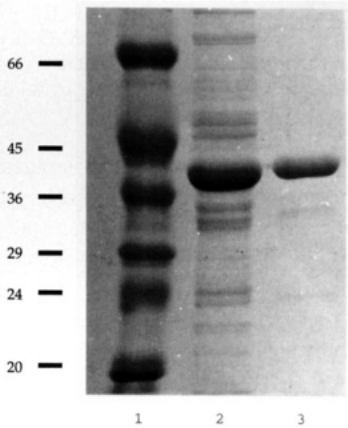
Further optimization experiments suggested that 30 °C was the growth temperature of choice and that, with NM554 as the host strain, IPTG induction had little effect, the crude lysates from both cultures having specific activities of ~0.15 IU mg^{-1}. However, not surprisingly, with JM109 as the host strain, IPTG induction did have a significant effect, the crude lysate from the induced culture having a specific activity of ~0.3 IU mg^{-1} compared to ~0.1 IU mg^{-1} for that from the uninduced culture. This result was confirmed by SDS−PAGE analysis of the crude lysates (data not shown) which showed that in the induced culture of pJB800/JM109, the ~35 kDa protein assumed to be sIPNS constituted ~5% of the total soluble cell protein.

Purification of IPNS Isozymes. The very high levels of expression observed for cIPNS and aIPNS (Baldwin et al., 1991a) suggested that material of sufficient purity for unnatural substrate conversion experiments might be obtained by single-column purification. We have found this to be



Purification Step	Volume (ml)	Total Protein (mg)	Total Activity (I.U.)	Specific Activity (I.U.mg^{-1})	Yield (%)	Purification (-fold)
Crude Extract	400	5 200	3 900	0.75	100	-
Q-Sepharose	45	595	1 490	2.5	38	3.3

FIGURE 10: Purification of cIPNS from NM554/pJB508: (1) molecular weight markers, (2) crude extract, and (3) Q-Sepharose.



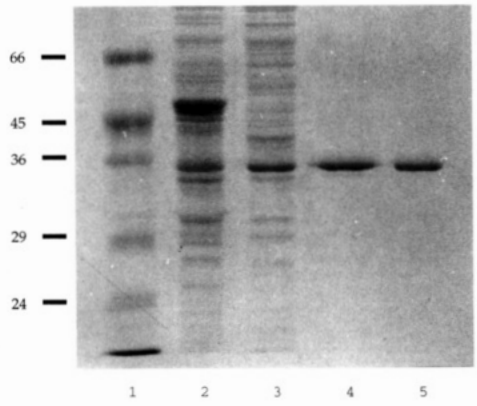
Purification Step	Volume (ml)	Total Protein (mg)	Total Activity (I.U.)	Specific Activity (I.U.mg^{-1})	Yield (%)	Purification (-fold)
Crude Extract	100	2 300	4 025	1.75	100	-
Q-Sepharose	90	900	3 870	4.3	96	2.5

FIGURE 11: Purification of aIPNS from NM554/pJB703: (1) molecular weight markers, (2) crude extract, and (3) Q-Sepharose.

the case, and > 100-mg quantities of both enzymes (at purities in excess of 95%) can be obtained from medium-scale cultures after chromatography on Q-Sepharose (Figures 10 and 11).

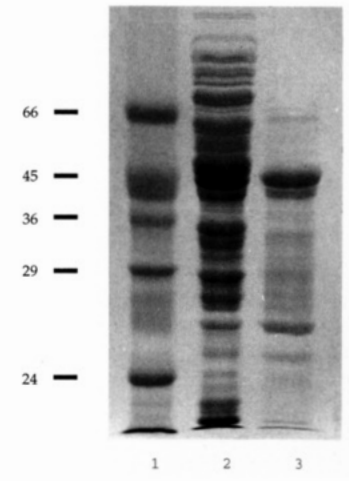
The lower level of expression of sIPNS necessitated the development of a three-column purification to obtain material of similar purity (Figure 12); nevertheless, sIPNS after a single Q-Sepharose step was found to be of sufficient purity (>30%) and high enough specific activity (0.45 IU mg^{-1}) for unnatural substrate conversion. A 10-L fermentation yielded 285 mg of sIPNS of this grade.

Although pIPNS is expressed to a moderate level in *E. coli*, we have found that purification of this enzyme is difficult because of extreme instability. The instability of a highly homologous IPNS partially purified from a strain of *P. chrysogenum* has previously been noted by other workers



Purification Step	Volume (ml)	Total Protein (mg)	Total Activity (I.U.)	Specific Activity (I.U.mg^{-1})	Yield (%)	Purification (-fold)
Crude Extract[a]	15	218	15.3	0.07	100	-
Q-Sepharose[a]	2	17.5	7.9	0.45	52	6.4
Superdex S-200	21	4.8	4.2	0.87	27	12.4
Neobar AQ	2	1.7	2.4	1.43	16	20.4

FIGURE 12: Purification of sIPNS from JM109/pJB800: (1) molecular weight markers, (2) crude extract, (3) Q-Sepharose, (4) Superdex S-200, and (5) Neobar AQ. [a]Adjusted to allow for the fact that only a small amount of material was carried through after the first purification step.



Purification Step	Volume (ml)	Total Protein (mg)	Total Activity (I.U.)	Specific Activity (I.U.mg^{-1})	Yield (%)	Purification (-fold)
Crude Extract	180	6000	90	0.015	100	-
Superdex S-200	21	375	75	0.2	83	13

FIGURE 13: Partial purification of pIPNS from JM109/pJB603: (1) molecular weight markers, (2) crude extract, and (3) Superdex S-200.

(Barredo et al., 1989). This tends to suggest that the instability is an intrinsic property of the protein rather than being due to any external factors. Attempts to obtain substantial enrichment by a variety of ion-exchange chromatography techniques resulted in very low recovery of activity although it was found that the presence of DTT, KCl, and ascorbate increased stability. Gel filtration on Superdex S-200 was found to give moderate enrichment and good recovery of activity. A 15-L fermentation yielded 375 mg of pIPNS with a specific activity of 0.2 IU mg^{-1} and a purity in excess of 20% (Figure 13). This material was found to be suitable for unnatural substrate conversion if further treated with lithium clavulanate to reduce β-lactamase activity (*vide infra*).

Characterization of IPNS Isozymes. Prior to an assessment of their substrate specificity, the IPNS isozymes were characterized by a variety of techniques (Table 1). The p*I*s of cIPNS, aIPNS, and sIPNS were all found, by isoelectric focusing, to be *ca.* 4.9, although minor bands were observed for cIPNS and aIPNS. The same three isozymes were shown by N-terminal amino acid sequencing to have undergone correct posttranslational cleavage of *N*-formylmethionine (such processing is rarely complete when the isozymes are expressed insolubly), but only the aIPNS had a molecular weight consistent with that predicted by the nucleotide sequence. For cIPNS the mass discrepancy of 85 Da has been attributed previously to the binding of one molecule of Fe^{2+} and one molecule of H_2O (combined mass of 74 Da) to the protein (Aplin et al., 1990). However, we have recently resequenced the gene (J. E. Baldwin, J. M. Blackburn, J. D. Sutherland, C. J. Rowe, K. Mofford, and E. J. Wilson, unpublished results) and have found two errors in the previously determined sequence (Samson et al., 1985). One of these errors is silent (codon 232, AAT, Asn, should be AAC, Asn), but the other results in an amino acid change (codon 177, TCC, Ser, should be TTC, Phe) that accounts for the previously reported mass discrepancy within experimental error.

The mass difference of 342 Da between the observed and predicted masses for sIPNS could be accounted for by loss of C-terminal threonine, glutamine, and valine (combined mass loss of 328 Da) or could also be due to errors made during the original determination of the GC-rich open reading frame sequence. The close agreement between calculated and observed masses for cIPNS and aIPNS tends to support the view that the mass discrepancy observed with sIPNS is due to sequence errors rather than any binding of cofactors or additional posttranslational processing.

All of the IPNS isozymes were more active in Tris·HCl buffer relative to sodium phosphate or ammonium bicarbonate, but the activities in these latter ^{1}H NMR compatible buffers were still sufficiently high for NMR analysis of unnatural substrate turnover experiments.

β-Lactamase Assay. For the purposes of this work, it was of the utmost importance to demonstrate that the protein preparations to be used in the substrate conversion experiments were not contaminated with β-lactamase activity. At

Table 1: Characterization of IPNS Isozymes

isozyme	p*I*	calcd mass (Da)	obsd mass (Da)	N-terminal fMet	purity (%)	pH optimum		
						Tris·HCl	Na_2HPO_4	NH_4HCO_3
cIPNS	4.90	38 300	38 385 ± 12	yes	97	7.8	7.9	8.0
aIPNS	4.95	37 391	37 403 ± 7	yes	99.7	7.8	7.3	7.5
sIPNS	4.85	37 948	37 606 ± 8	yes	92	7.8	7.8	7.1
pIPNS	N/D	N/A	N/D	N/D	>20	7.4	7.3	7.5

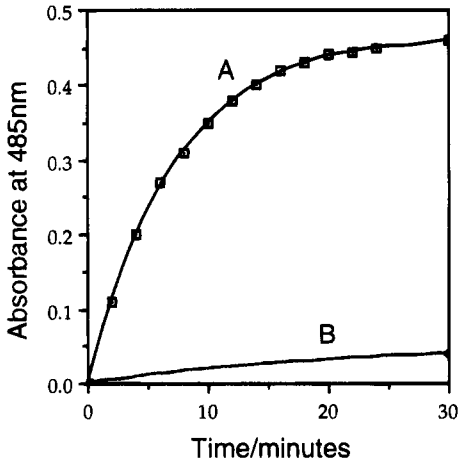


FIGURE 14: Hydrolysis of nitrocefin by partially purified pIPNS: (A) before incubation with clavulanate; (B) after incubation with clavulanate.

the outset of this work, this was not considered probable because the plasmid-encoded ampicillin resistance genes which encode the R_{TEM} β-lactamase had been insertionally inactivated during the construction of the expression vectors, and the chromosomally encoded β-lactamase (*ampC* gene product) is only produced in small amounts and is not thought to contribute to the penicillin resistance of wild-type *E. coli* K-12 strains (Normark & Burman, 1977). The *ampC* β-lactamase is a more potent cephalosporinase than penicillinase (the reverse being true of the R_{TEM} β-lactamase), and so nitrocefin was an appropriate choice of substrate for a β-lactamase assay (Lindstrom et al., 1970). The assay was based on the color change from yellow to red on hydrolysis of nitrocefin, which could be followed by monitoring the absorbance at 485 nm, corresponding to the absorption maximum of the hydrolysis product. At this wavelength, nitrocefin has no absorbance, and so the increase in this absorption with time could be correlated directly with a rate of hydrolysis of nitrocefin. BSA, a penicillinase, and Tris·HCl were used as controls in the assay.

Samples of purified cIPNS, aIPNS, and sIPNS showed no hydrolysis of nitrocefin over the course of a 30-min assay (data not shown), but surprisingly, partially purified pIPNS did demonstrate substantial β-lactamase activity, estimated to be ~10 nmol of nitrocefin per minute per mg of protein in the region where the reaction rate was approximately linear (Figure 14). At this stage, it was decided that further purification of pIPNS to try to remove the contaminating β-lactamase activity was unlikely to be successful given the instability of the pIPNS activity. However, it was known that clavulanic acid inhibited the *ampC* gene product with an I_{50} of 56 μg mL^{-1}, which is relatively poor compared to the inhibition of the R_{TEM} β-lactamase (I_{50} = 0.08 μg mL^{-1}) but still more than adequate for *in vitro* inhibition (Reading & Cole, 1977). Thus an aliquot of the pIPNS preparation was incubated for 10 min at room temperature in the presence of lithium clavulanate (1 mg mL^{-1}), and the excess clavulanate was removed by gel filtration over Sephadex G-25. This preparation was then reassayed for β-lactamase activity alongside a control sample, and this clearly showed that the β-lactamase activity had been irreversibly inactivated by the preincubation (Fisher et al., 1978), with minimal hydrolysis of nitrocefin being observed over a 30-min assay (Figure 14). That very slow hydrolysis occurred at all was not surprising since an equivalent concentration of BSA hydrolyzed nitrocefin at the same rate (data not shown), and so this could be attributed to nonspecific protein hydrolysis of the labile nitrocefin nucleus rather than any specific β-lactamase activity.

These results showed that the IPNS preparations (after preincubation with clavulanate if necessary) were suitable for subsequent use in NMR studies since it was essential that the product ratios observed with the unnatural analogues were indicative of the true ratios of formation rather than of selective β-lactam hydrolysis.

Substrate Conversions with IPNS Isozymes. The isozyme preparations used had been through only a single round of purification since such material was found in each case to be of sufficient activity to achieve substantial cyclization of the starting materials. It had also been demonstrated previously that these preparations had insignificant hydrolytic activity toward nitrocefin. All incubations were carried out in ammonium bicarbonate to provide a ^{1}H NMR-transparent, semivolatile buffer for the reactions. The incubations were carried out at pH values close to but not identical with the optimum for each isozyme. This was not considered to be important since, for our purposes, IPNS variants with altered structure/activity are required, and any additional perturbation caused by operating away from the pH optimum would not interfere with interpretation of the results. The 500-MHz ^{1}H NMR spectra of the samples were recorded in D_2O, and integration of the characteristic resonances between 4.8 and 6.0 ppm allowed determination of the ratios of the various products formed. Controls were carried out in which the length of incubation and the processing times were varied for each isozyme and each substrate. The first control was designed to show that the products were not degraded by protein(s) in the incubation mixture, and this was found to be true for each isozyme preparation with the exception of pIPNS, which still had trace residual hydrolytic activity toward β-lactams even after preincubation with clavulanate. The second control was designed to show that the products were stable under the NMR conditions, confirming that the product distributions were not affected by small differences in processing times, and this was found to be true in each case. Since all the compounds detected by NMR had been previously purified and fully characterized, the products of these incubations were not purified and the resonances observed were assigned on the basis of previously reported data (Baldwin et al., 1983a, 1984b).

It is important to stress at this point that while the data obtained from the incubations with cIPNS, aIPNS, and sIPNS appear to be authentic within the limits of the controls employed, the data from the incubations with pIPNS must be viewed with caution because the residual β-lactamase activity was shown to be affecting the product ratios in the longer incubations. This caveat will be discussed in more detail at the appropriate juncture.

Incubation of ACV. The conversion of ACV (**1**) to IPN (**2**) is the principal reaction catalyzed in nature by each of the IPNS isozymes. Although it might be considered possible to cyclize this tripeptide to give a 4,6-bicyclic cepham product via the proposed mechanism, no evidence of this was observed by ^{1}H NMR. The spectra for each isozyme showed quantitative conversion of the substrate to a single product having the characteristic proton resonances of isopenicillin N (**2**).

Table 2: Distribution of Products from Incubations of ACAB with the IPNS Isozymes

IPNS isozyme source	β-lactam product		
	α-methyl penam (**5**)	β-methyl penam (**6**)	cepham(**7**)
C. acremonium	1	15	6
A. nidulans	1	9	4
S. lipmanii	1	9	4
P. chrysogenum	1	19	12

Table 3: Distribution of Products from Incubations of ACAG with the IPNS Isozymes

IPNS isozyme source	β-lactam product				
	α-vinyl penam (**8**)	β-vinyl penam (**9**)	homo-cephem (**10**)	α-hydroxy-homocepham (**11**)	hydroxy-methyl cepham (**12**)
C. acremonium	1	4.5	12	9	N/D
A. nidulans	1	2	6	4	1
S. lipmanii	1	5	4.5	3.5	1.5
P. chrysogenum	N/D	N/D	3.5	2	1

Incubation of L-α-*Aminoadipoyl-*L*-cysteinyl-*D*-aminobutyrate.* The products of the incubation of ACAB (**3**) with cIPNS have previously been characterized as the α-methyl penam (**4**), the β-methyl penam (**5**) and the cepham (**6**) (Figure 2) (Baldwin et al., 1983a). Incubation of this substrate with each isozyme resulted in the same three products in all cases, but the product distribution observed appeared to be affected by the catalyst used to effect the transformation. Table 2 shows the product ratios resulting from each incubation as adjudged by integration of the resonances, normalized with respect to the α-methyl penam (**4**). The ratio of methyl penams to cepham with pIPNS was anomalously low because, as the controls indicated, the penam products were being hydrolyzed faster than the cepham product (i.e. this ratio decreased with time) by the residual hydrolytic activity associated with this enzyme preparation. However, the ratio of α- to β-methyl penams remained constant irrespective of the length of incubation, strongly suggesting that the two penams were hydrolyzed at the same rate and therefore that the ratio found is indicative of the true ratio.

Incubation of L-α-*Aminoadipoyl-*L*-cysteinyl-*D*-allylglycine.* Incubation of ACAG (**7**) with cIPNS has previously been shown to give rise to five major enzymatic products characterized as the α- and β-vinyl penams (**8** and **9**), the homocephem (**10**), the α-hydroxyhomocepham (**11**), and the α-hydroxymethylcepham (**13**) (Figure 3) (Baldwin et al., 1984b). Incubation of ACAG (**7**) with the four IPNS isozymes gave rise to the same products as previously reported, but in different proportions. Where they could be determined with sufficient accuracy, the normalized product ratios are given in Table 3.

The α- and β-vinyl penam integrals found with pIPNS were too low to be determined with any accuracy and were also found to decrease with increasing incubation time, and so this data is not presented here. The integrals of the other three products observed, however, did appear to be stable in the presence of protein, suggesting that the residual β-lactamase activity was selective for the penam products rather than the cepham, homocepham, or homocephem products, in accord with the results found with the ACAB incubations with pIPNS.

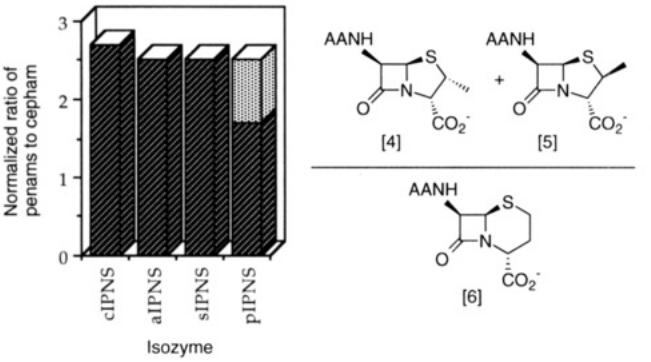


FIGURE 15: Normalized ratios of penam to cepham products in the conversion of ACAB (**3**) by IPNS isozymes. For pIPNS, estimated experimental error is indicated by the lighter shading.

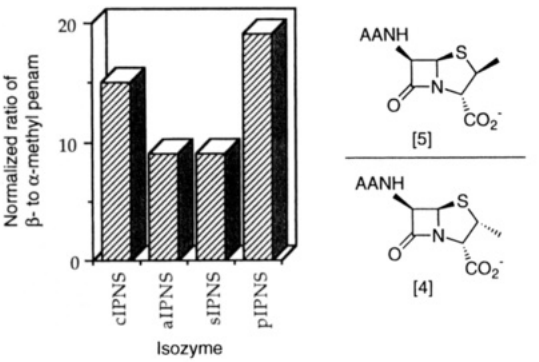


FIGURE 16: Normalized ratios of β- to α-methyl penam products in the conversion of ACAB (**3**) by IPNS isozymes.

DISCUSSION

L-α-Aminoadipoyl-L-cysteinyl-D-aminobutyrate. For the purposes of comparative analysis as discussed in the introduction, the ACAB product ratios shown in Table 2 are best represented histographically (Figures 15 and 16). As can be seen, the ratio of the combined penam products (**4** and **5**) to the cepham product (**6**) (Figure 15) is essentially invariant across the range of IPNS isozymes (and approximately 2.5:1), whereas the β- to α-penam ratio (**5**:**4**) (Figure 16) varies significantly (between approximately 10:1 and 20:1). These data can be interpreted according to the rationale presented in the introduction with reference to Figure 17.

The monocyclic intermediate for this conversion is assumed to exist in three staggered conformational states rotameric about the valinyl C2−C3 bond. For the sake of simplicity, these conformations will be described as "methyl-up" (**29**), "methyl-front" (**30**), and "methyl-back" (**31**). Throughout the ensuing discussion it is assumed that the *S*-ferryl oxene moiety of the intermediates is held fixed by the enzyme. Since the absolute orientation is unknown, that depicted is chosen to allow conformations to be described in a relative fashion. In the methyl-up and methyl-front conformers, the methyl group is in either of the positions occupied by the two valinyl methyl groups of ACV (**1**), and these are assumed to be the favored states. The penam products can arise from either of these two conformations because of the equilibration of the subsequent diradicaloid species. This interconversion is suggested by the observation that replacement of either the 3-*pro-S* or the 3-*pro-R* hydrogens of the aminobutyrate moiety by deuterium results in the same 3-deuterio-3-methyl penam stereochemistry via exclusive insertion into the remaining C3−H bond (Figure

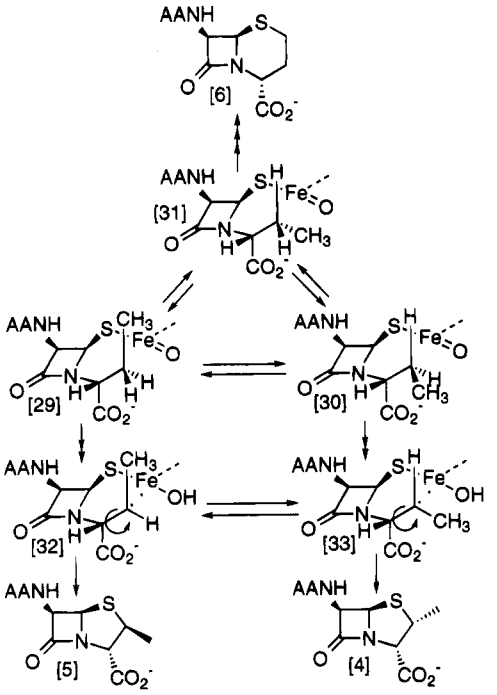


FIGURE 17: Mechanistic rationale for conversion of ACAB (**3**) by IPNS isozymes.

2) (Baldwin et al., 1983b). All three conformations are assumed to be interconvertible with low barriers, as indicated by these isotope labeling studies and the fact that the methyl-back conformation must be available to allow for the formation of the cepham product. The invariance of the combined penam to cepham ratio (Figure 15) suggests that the activation barriers for formation of either ring sized product are equally affected by active site perturbations and that these committed steps are strongly exothermic. In addition, the magnitude of the combined penam to cepham ratio (ca. 2.5:1) indicates that the barriers are of similar height, consistent with similar oxidative insertion chemistry being involved in each case. According to the Hammond postulate (Hammond, 1955), the transition-state structures should thus be very similar to the structures of the intermediate states and so any perturbation in the enzyme will affect the related intermediate states and transition states equally. Thus, for example, any change to the enzyme that stabilizes the methyl-back conformer (**31**) over the other two conformers (**29** and **30**) will be expected to stabilize the subsequent transition state to a similar extent and hence will not change the activation barrier for the committed step in the conversion through to cepham (**6**). The penam−cepham ratio will therefore remain unchanged.

As mentioned previously, the results with ACAB stereospecifically deuterated at C3 of the aminobutyrate moiety indicate that the diradicaloid intermediates **32** and **33** can equilibrate easily by rotation about the C2−C3 bond. The observation of large variation in the β- to α-methyl penam ratio (Figure 16), coupled with the invariance of the combined penams to cepham ratio, requires that steps after formation of the diradicaloid intermediates determine the β- to α-methyl penam ratio. As the diradicaloid equilibration must have a low barrier, then according to the Curtin−Hammett principle the stereochemical discrimination must be in the transition states of the subsequent steps. Using the Hammond postulate argument detailed earlier, the implication is that the collapse of the diradicaloid to give the enzyme-bound products is less exothermic and that the transition states display significant product character. Thus, factors which favor either of the intermediate diradicaloids will lower the transition states for the subsequent collapses differentially. The relative activation barriers are therefore affected discriminately by enzyme perturbation.

The observation of a cepham product has previously been interpreted as further evidence for C−H bond homolysis in the IPNS reaction, but the preceding discussion suggests that the situation is more subtle. Indeed, if the 12 kJ mol^{-1} difference in bond dissociation energy between the primary and secondary C−H bond homolyses was fully manifested in the committed transition state, then no cepham would be observable. Thus the very observation of the cepham (taken in conjunction with the above argument) suggests that the committed step for second ring closure is oxidative insertion of the *S*-ferryl oxene into a C−H bond. The magnitude of the ratio of penams to cepham (neglecting statistical effects) suggests that insertion into a primary C−H bond is, chemically, an intrinsically *favorable* process. The selectivity of oxidation of primary, secondary, and tertiary C−H bonds by the Gif system [in the more decisive but chemically simpler work of Barton (Barton, 1991)] is also different from that predicted on the basis of bond dissociation energies and varies substantially with reaction conditions. Although the order of selectivity is not the same as that suggested here, the results taken together argue for the lack of involvement of radicals in the committed steps. The data suggest that one of the roles of IPNS in the conversion of ACV (**1**) to IPN (**2**) is to stabilize a conformation of the four-membered ring intermediate, and hence also the transition state for insertion into the tertiary C−H bond. Conversely, the effect may be viewed as destabilization of conformers (and hence transition states) leading to cepham products; this would constitute an example of negative catalysis (Retey, 1990).

The observation of a substantial V_{max} kinetic isotope effect at the valinyl C3 position of ACV (**1**) (Baldwin et al., 1984a) suggests that even when the conformationally favored activation barrier is isotopically raised, the activation barrier for cepham formation is still unsurmountably high and so a shunt metabolite (Baldwin et al., 1988) is formed preferentially. The inaccessibility of the methyl-back conformation in the case of the ACV (**1**) to IPN (**2**) conversion explains why the sulfur−carbon bond is formed with retention of configuration (Baldwin et al., 1986a) since the tertiary diradicaloid intermediate would also thus be expected not to rotate. All of the isozymes tested showed the same discrimination with ACV (**1**), and no cepham products were observed. It is however possible that isotopic substrate perturbation at the valinyl C3 position will allow cepham formation with IPNS from an organism other than *C. acremonium*. This possibility is currently being evaluated. In addition, the above analysis suggests that cavity-creating point mutations in the putative valine binding site will allow cepham formation from the natural, non-isotopically labeled substrate by making the methyl-back conformation accessible. Investigation of this prediction awaits the determination of a crystal structure of an IPNS isozyme.

L-α-Aminoadipoyl-L-cysteinyl-D-allylglycine. For the ACAG product ratios shown in Table 3, the percentage variabilities are shown in Figure 18. The ratios of these five products are sufficient for an analysis to be carried out with reference to Figure 19. Again, the monocyclic *S*-ferryl monocyclic intermediate is taken as the starting point for the discussion.

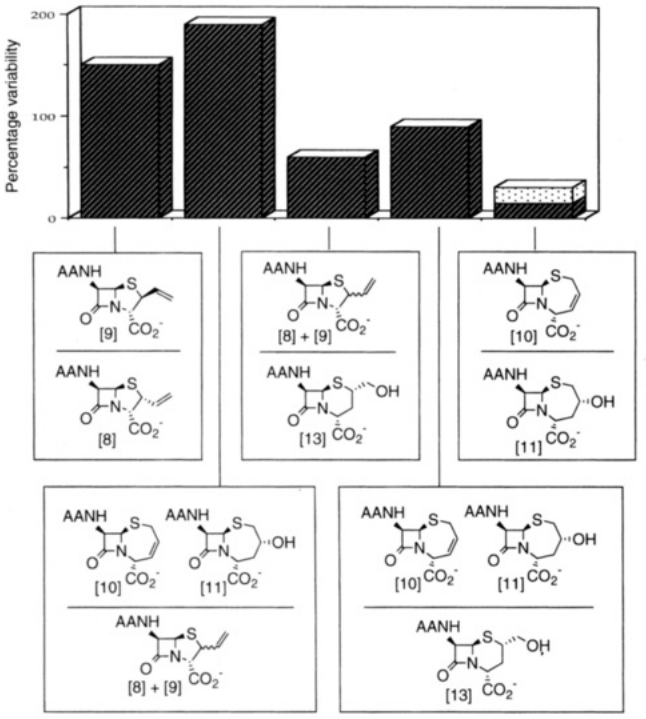


FIGURE 18: Percentage variabilities for various product ratios in the conversion of ACAG (**7**) by IPNS isozymes.

Of the three potential conformers about the C2−C3 valinyl bond, only the "vinyl-up" conformer is considered accessible. This consideration follows from two arguments. First, the greater steric bulk of the vinyl group relative to a methyl group (at least insofar as fitting into a pocket evolved to bind a methyl group) suggests, in conjunction with the aforementioned arguments about the unfavorability of the methyl-back conformation in the ACAB case, that the "vinyl-back" conformation with this larger substrate analogue will be even more unfavorable. Second, the lack of formation of any penam products from the allylglycinyl 3-*pro-S* deuterated tripeptide (Baldwin et al., 1991b) suggests that the "vinyl-front" conformation, from which the 3-*pro-R* hydrogen would be removed, is also inaccessible. Thus, the C2−C3 bond rotation is frozen for this substrate. The C3−C4 bond, however, must be free to rotate, as certain of the products must result from altered (relative to the presumed fixed ferryl oxene moiety, again with the caveat that the arbitrary depiction of the ferryl oxene position does not affect a relative conformational argument) conformations about this bond. The two rotational states about the C3−C4 bond that must be considered are the "vinyl-left" (**34**) and the "vinyl-right" (**35**) conformers. Of the five products observed, the homocephem (**10**) and the hydroxyhomocepham (**11**) must come from the vinyl-left conformer (**34**), while the α-hydroxymethylcepham (**13**) must come from the vinyl-right conformer (**35**). The α- and *β*-vinyl penams, **8** and **9**, could arise from either the vinyl-left or the vinyl-right conformers. The observation of very low variability in the ratio of homocephem (**10**) to α-hydroxyhomocepham (**11**) (and there is essentially no variability after allowing for experimental error) suggests that the transition states in the committed steps leading to these compounds (via oxy-ene and [2+2] pathways, respectively) are affected by enzyme perturbation in a very similar fashion. This is most consistent with both of the transition states being very similar in structure to the intermediate vinyl-left conformer (**34**). This suggests that, in accord with chemical intuition, both processes are relatively exothermic. The magnitude of the ratio (ca. 1:1) suggests that the activation barriers are very similar, consistent with this line of reasoning. Since the two seven-membered ring compounds are the major products in the ACAG conversion, the activation barrier in the committed step to either one must be lower than the corresponding activation barriers leading to any of the other products. The ratio of the seven-membered ring products (**10** and **11**) to the α-hydroxymethylcepham (**13**) (obligate vinyl-left products to obligate vinyl-right product) is substantially variable. Since the formation of either of the seven-membered ring products is nonselective (their relative amounts are invariant), the activation barrier in the committed step leading to α-hydroxymethylcepham (**13**) therefore obviously varies with isozyme change. The implication of this is that since the perturbation differentially affects the stability of the vinyl-right conformer and the subsequent committed transition state leading to (**13**), then the transition state must be more product-like than the transition states leading to the obligate vinyl-left products. According to Hammond's postulate, this suggests that the committed step leading to the α-hydroxymethylcepham (**13**) is less exothermic than those leading to **10** and **11**.

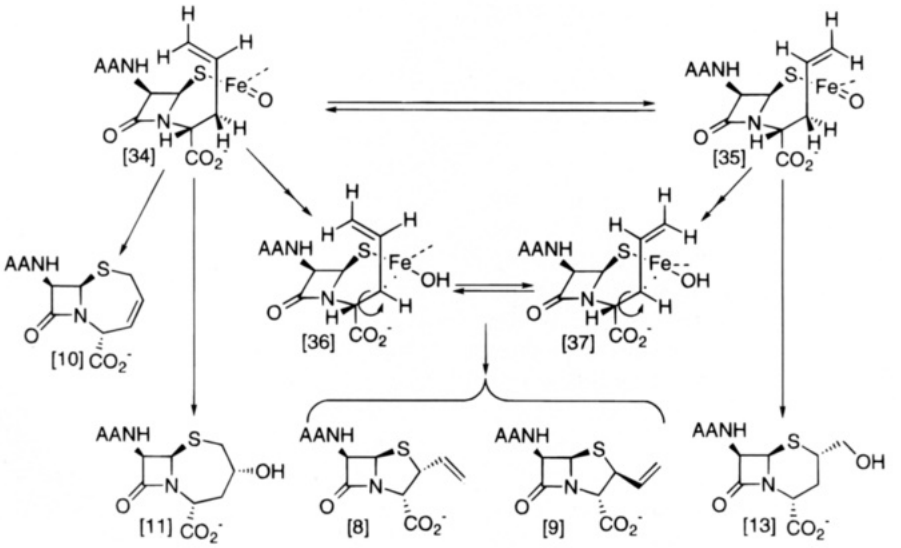


FIGURE 19: Mechanistic rationale for conversion of ACAG (**7**) by IPNS isozymes.

The observation of both α- and β-vinyl penams (**8** and **9**) suggests that a preceding state must have interconverted by rotation about the allylglycinyl C2−C3 bond. This rotation could, in theory, have occurred in the four membered ring intermediate **34/35**, but as discussed previously, the vinyl-front conformer (which would lead to the α-vinyl penam) is presumed to be inaccessible at this stage. Since the allylglycinyl C2−C3 bond is prevented from rotating in the metalloheterocyclic oxidative insertion products (not shown), the interconversion must take place at the diradicaloid intermediate stage **36/37**. The large variation in the β- to α-vinyl penam ratios (Figure 18) requires that the activation barriers leading to either penam product are differentially affected by enzyme perturbation. This in turn suggests that the relative semblance of the two transition states to the two conformationally distinct diradicaloid intermediates is different and therefore that the thermicities of the two processes are different. The decreased preference for the β-stereochemistry in the ACAG penam products (ca. 2−4:1) relative to the ACAB penam products (ca. 10−20:1) suggests that the activation barrier for closure to the β-substituted penam product is relatively increased in the ACAG case. The reasons for this are not clear but might reflect increased product-like nature of the transition state, with the β-vinyl penam (**9**) being less stable for steric reasons. More product-like transition states in the ACAG case suggests lower thermicity for closure of the diradicaloid states than in the ACAB case. This would be consistent with the expected allylic stabilization of the diradicaloid intermediates in the ACAG case.

The large variability of the ratios of the combined penams to either obligate vinyl-left or obligate vinyl-right products suggests that the activation barrier for the committed step (the oxidative insertion) leading to the penams is differentially affected by perturbation relative to the corresponding activation barriers for processes leading to the other products. Interpretation of this in terms of Hammond's postulate is difficult because in this case the chemistries being compared are substantially different. Consequently, the degree to which structural similarity between intermediate and transition state correlates with the relative activation barriers leading to the transition state would be expected to be changed for penam formation relative to the other products. Similarity of this degree of correlation is a necessary requirement for the validity of the Hammond postulate. It is thus difficult to say which of the two conformations, **34** or **35**, gives rise to the penam products.

The presumed rotation about the C2−C3 bond in the AC-allylglycinyl diradicaloid intermediates is similar to the situation deduced for the rotation about the C2−C3 bond in the AC-aminobutyryl diradicaloid but contrasts to the case with the natural substrate where no rotation takes place. This is an intriguing demonstration of the extent to which conformational change in a substrate can be controlled by an enzyme. The above analysis shows therefore that IPNS, which has previously been considered to be a highly nonselective, "promiscuous" enzyme, has in fact evolved to the extent that it can fine-tune the available conformational states differing by carbon−carbon bond rotation in a substrate. Put differently, the IPNS isozymes appear to be positively controlling the reactions of the high-energy intermediates formed with the natural substrate such that only a single product is produced. With unnatural substrates, however, this control is relaxed to varying degrees, allowing

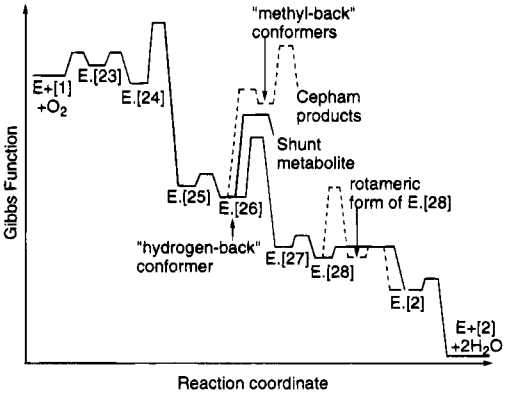


FIGURE 20: Refined Gibbs function profile for conversion of ACV (**1**) by IPNS isozymes.

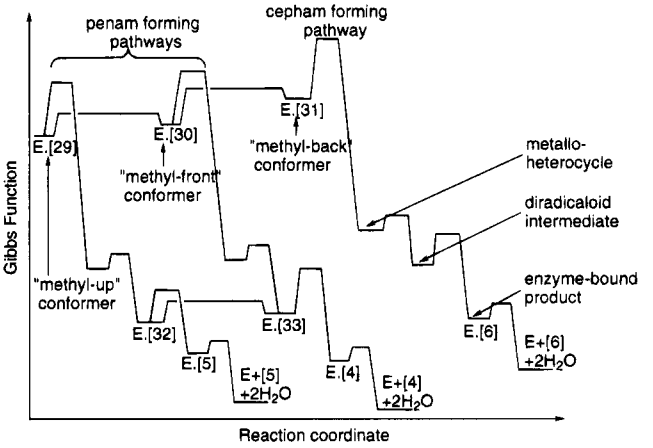


FIGURE 21: Gibbs Function profile for conversion of ACAB (**3**) by IPNS isozymes.

fundamental chemical reactivity to take over, typically resulting in formation of multiple products. Whether or not this is exemplary of induced fit will have to await the three-dimensional structure determination of an IPNS isozyme.

The results obtained and discussed above allow substantial improvements to be made to the prototype Gibbs function profile for the IPNS-catalyzed conversion of ACV (**1**) to IPN (**2**) (Figure 8). A refined profile (Figure 20) for the reaction with the natural substrate reveals that the changes made to the previous profile are essentially pathways that are not followed. These alternate pathways are, however, followed with certain unnatural substrates, and to demonstrate this for the simpler case of ACAB, a Gibbs function profile is presented in Figure 21.

The data and arguments presented in this paper have revealed the extent to which IPNS contributes to the catalysis of product formation from natural and unnatural substrates. That an analysis of the variability of product ratios with isozyme change can reveal so many subtle features of catalysis is surely testimony to the power of this novel, heuristic approach.

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