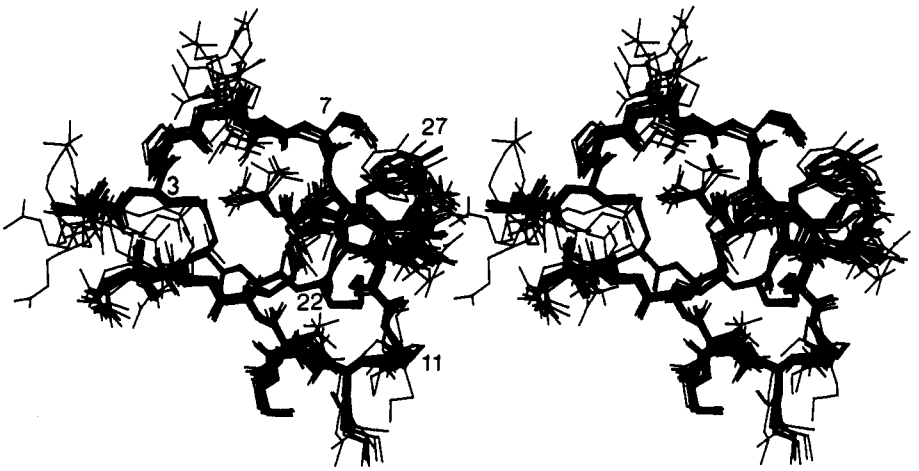

Figure 2. Best superposition of the backbone (N, C^{α}, C, O; residues 2-29) of the 12 structures. All heavy atoms are shown. The average of the root-mean-square differences among the 3D NOE-NOE structures is 0.51 ± 0.11 Å for the backbone atoms and 1.18 ± 0.13 Å for all heavy atoms.

distance constraint violations greater than 0.5 Å. The root-mean-square difference from the experimental constraints, which are calculated with respect to the upper and lower limits of the distance constraints,[6] is 0.060 ± 0.008 Å for all 541 distance constraints. When the new structures are checked for distance constraint violations against the 324 2D NOESY distance constraints (with the upper and lower bounds to distance constraints used in the present study), the root-mean-square difference is 0.081 ± 0.010 Å. The root-mean-square difference is lower than that for the 2D NOESY distance constraints while the number of NOEs is 65% greater for the new structures.

In one set of 12 structures, the disulfide bridges were defined neither as bonds nor as distance constraints. These structures were almost identical with those calculated with the disulfide bonds specified. The S-S pairing could be determined unambiguously from the NMR structures. Also, in contrast to the previous structures, the present structures exhibit unique conformations for all the disulfide bridges. The ability to obtain structures with the uniquely determined conformations of the disulfide bridges could be traced to the presence of new NOEs in the 3D spectrum involving cysteines.

In conclusion, we have shown that a single 3D NOE-NOE experiment of a protein in water can provide sufficient input data to calculate structures that could be deemed to correspond to high-resolution structures as defined in X-ray crystallography. A large number of NOEs can be extracted from the homonuclear 3D spectrum, together with NOEs not observed in 2D NOESY spectra. This is especially true for connectivities between the side-chain protons of different residues. Around 200 such long-range NOEs could be obtained from the 3D NOE-NOE spectrum. For the 2D spectrum, the aliphatic region of the NOESY spectrum is used to extract such connectivities; due to experimental limitations,[1] this requires a spectrum of protein dissolved in D_2O. In the 3D NOE-NOE spectrum in H_2O, the NOEs between the aliphatic protons can be observed at unique amide proton frequencies. It is also easier to assign such connectivities in the 3D than the 2D spectrum because of the possibility of multiple checking of the assignments due to the redundancy and mutual consistency of the 3D cross-peaks. Since 3D NOE-NOE spectroscopy does not rely on coherent transfers due to the scalar coupling, its sensitivity is not restricted by small J coupling or large line widths. The 3D NOE-NOE spectroscopy should therefore be useful in the structure determination of large biomolecules. Of course, the method is not limited to biomolecules; it should be useful for any organic compounds that give rise to the NOE effect. We expect that the homonuclear 3D NOE-NOE spectroscopy will replace or supplement the 2D NOESY for structure determination.

Acknowledgment. This work was supported by a research grant from the Bundesministerium für Forschung und Technologie (Grant No. 0318909A). J.O. is a recipient of a fellowship from the Humboldt Foundation.

Supplementary Material Available: Figure giving the F_3 NH cross section at amide of residues 22 and 8 and tables containing angle and distance constraints used in the calculations together with the 3D connectivities involved in these distance constraints (20 pages). Ordering information is given on any current masthead page. Tables containing constraints and coordinates of the five structures have been also deposited in the Brookhaven Protein Data Bank.

Isolation and Structure Elucidation of the 4-Amino-4-deoxychorismate Intermediate in the PABA# Enzymatic Pathway

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Received December 17, 1990

PABA (*p*-aminobenzoic acid), an important precursor in the bacterial biosynthetic pathway for folate coenzymes, is formed

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Abbreviations used: PABA, *p*-aminobenzoic acid; TRIS, tris(hydroxymethyl)aminomethane; PabA, PABA synthase subunit having glutaminase activity; PabB, PABA synthase subunit having aminodeoxychorismate synthase activity; PabC, PABA synthase subunit having aminodeoxychorismate lyase activity, formerly referred to as enzyme X.

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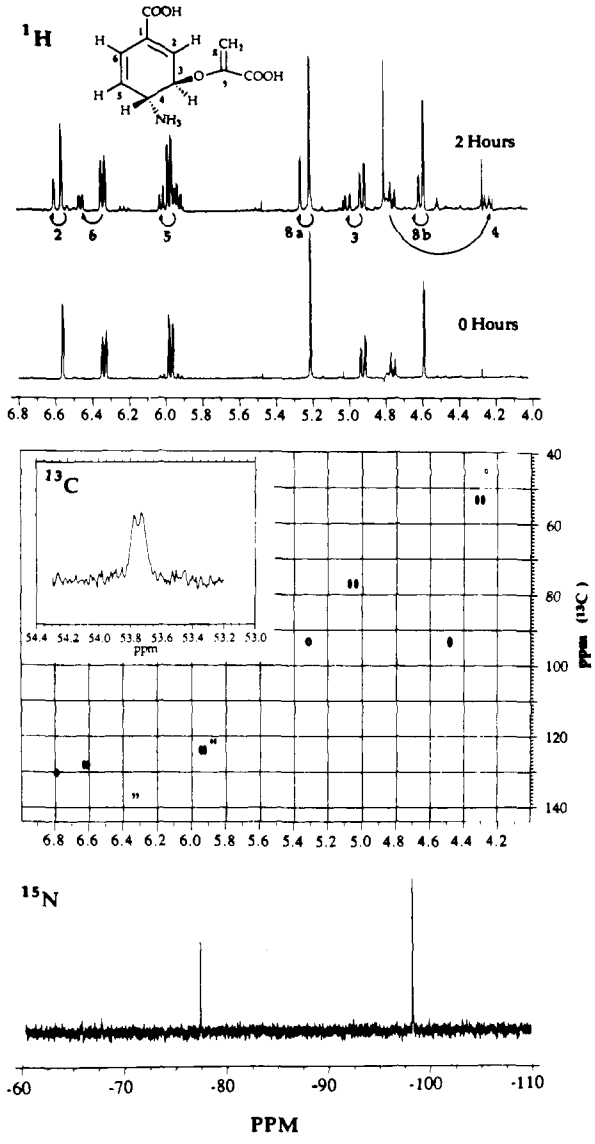

Figure 1. NMR spectra of the 4-amino-4-deoxychorismate. Top panel: The time course of enzymatic conversion of chorismate to aminochorismate was monitored at 25 °C with a Bruker AM-500 spectrometer resonating at 500.13 MHz for ^1H. Chemical shifts are reported in parts per million relative to tetramethylsilane. Internal acetone (δ = 2.22 ppm) was used as a secondary reference. The resonances for the ring protons are designated by the conventional numbering of the ring (2–6). The reaction mixture contained 20 mM diammonium chorismate, 15 μM PabB, 10 mM $MgCl_2$, and 200 mM NH_4HCO_3 pD 8.6 in D_2O. The HDO resonance at about 4.8 ppm was partially suppressed by presaturation. Inset: Structure of the aminated intermediate, 4-deoxy-4-aminochorismate. Middle panel: The inverse ^1H–^{13}C heteronuclear 2D correlated spectrum of the purified aminated intermediate was obtained at 0 °C in CD_3OD with a 5-mm inverse BB probe on a Bruker AM-500 spectrometer at 500.13 MHz. The ^1H and ^{13}C chemical shifts were referenced relative to the methyl group of methanol. the inverse ^1H–^{13}C 2D correlation was performed with the Bruker pulse sequence BIRDDP9.A88, which includes a Bird delay of 1.4 s, a relaxation delay of 1 s, and an acquisition time of 0.512 s. The ^1H chemical shifts differ slightly from those reported in the top panel because of differences in solvent, pH, and temperature. Inset: Proton-decoupled natural abundance ^{13}C spectrum in the 53 ppm region showing splitting of the ^{13}C resonance at C-4 by the ^{15}N of the amino group. Bottom panel: The proton-decoupled ^{15}N NMR spectrum of the purified aminated intermediate was obtained at 0 °C in CD_3OD with a VSP 10-mm BB probe on a Bruker AM-500 spectrometer. The ^{15}N chemical shifts were referenced to a sample of 50% formamide in CD_3OD (δ = −267 ppm). The resonances at −98.3 and −77.2 ppm were from NH_4^+ and the nitrogen attached to C-4 of the intermediate, respectively. Methods: A 200 mM $^{15}NH_4HCO_3$ pH 8.0 solution was prepared by ion exchange of 10 mL of a solution of 100 mM $(^{15}NH_4)_2SO_4$ through a Bio Rad AG1-X8 column (5 mL) in the HCO_3 form. Chorismic acid (25 mg) was neutralized with 2 mL of 200 mM $^{15}NH_4HCO_3$, lyophilized overnight, and reconstituted with 2.2 mL of the $^{15}NH_4HCO_3$ buffer containing 20 mM $MgCl_2$ and 16.8 μM PabB enzyme. The reaction mixture was incubated at 37 °C for 2.5 h and then quenched with 2.4 mL of chloroform. The aqueous layer was removed, acidified with 2 N trifluoroacetic acid, extracted with another 2.4-mL portion of chloroform, and then extracted three times with 2.4 mL of ethyl acetate. The aqueous layer was then lyophilized, and the residue was redissolved and lyophilized three times with CD_3OD prior to NMR analysis. The progress of the reaction was monitored by using RP-HPLC with radioactivity and/or UV detection at 280 nm. The following gradient was employed for HPLC analysis: 0–4 min 100% A, 4–20 min 40% B, where solvent A is 50 mM ammonium acetate, pH 4.0, and solvent B is methanol. Analysis of the aqueous and ethyl acetate layers by HPLC showed that the organic extractions were a very efficient method for separating intermediate from chorismate with less than 2% chorismic acid remaining in the aqueous phase.

from chorismic acid and glutamine in the reaction catalyzed by PABA synthase. As shown in Scheme I, 4-amino-4-deoxychorismate (**2**) has been suggested as an intermediate in the enzymatic reaction on the basis of two lines of indirect evidence. Earlier it was shown that a racemic mixture of **2** can be converted to PABA (**3**) upon incubation with crude bacterial extracts.[1] Recently it was shown that the conversion of chorismate to PABA requires three separate proteins, PabA, PabB, and "enzyme X",[2] the first two of which have been cloned and overexpressed. PabA is a glutaminase which provides nascent ammonia in vivo, but is not required if ammonia is provided in vitro. PabB catalyzes the conversion of chorismate to PABA in the presence of ammonia and enzyme X, which has been shown to have an aminodeoxychorismate lyase activity[3] and will hereafter be termed as PabC. In our preliminary report,[3] we described an intermediate formed by the action of PabB and subsequently converted to PABA by PabC. In this report, we describe the isolation and structure proof for **2**.

Rapid chemical quench studies[4,5] provided the first direct evidence for **2**. PabB was incubated with [^{14}C]chorismate and ammonia, the reaction was quenched with chloroform, and the products were analyzed by HPLC. A new peak was observed only in the presence of ammonia, suggesting an aminated intermediate. If PabC was included in the reaction mixture, only the final product, PABA, was observed and the intermediate did not accumulate.

The reaction catalyzed by PabB requires magnesium while that catalyzed by PabC does not. By preincubating PabB with [^{14}C]chorismate and ammonium sulfate in the presence of magnesium, we could observe the formation of **2** followed by its subsequent conversion to PABA after stopping the PabB-catalyzed reaction with EDTA and then adding PabC. Analysis by HPLC revealed that only chorismate and PABA were observed at the end of the second reaction. Upon addition of PabC and EDTA, all of the intermediate **2** formed in the first reaction was converted to the product, PABA, while additional **2** could not be produced from the remaining chorismate due to the chelation of magnesium.

The reaction catalyzed by PabB reaches an equilibrium between **1** and **2** in the absence of PabC. The equilibrium constant was measured by mixing [^{14}C]chorismate with a low concentration of enzyme and then monitoring the ratio of chorismate to **2**. The overall solution equilibrium constant was determined to be 6.1 M^{-1}, including the concentration of ammonium ions in the expression.[6] The K_m for chorismate with PabB is 67 μM, and the k_{cat} is 0.098 s^{-1}.

The first structural proof of **2** was provided by examining the changes in the proton NMR spectrum, comparing the spectrum before enzyme addition (0 h) and 2 h after the addition of enzyme

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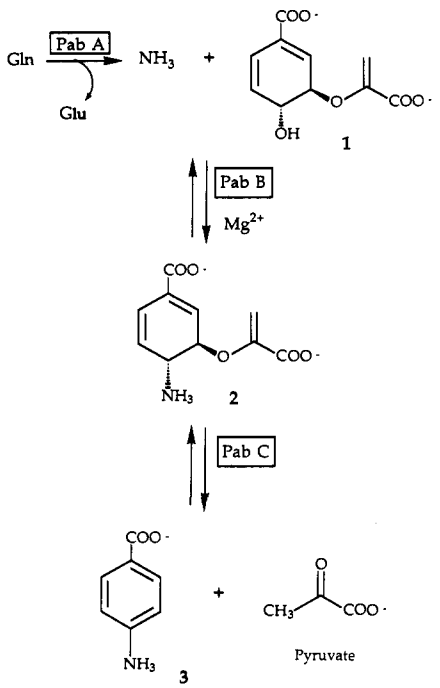
Scheme I



to chorismate. As shown in the spectra in the top panel of Figure 1, a duplicate set of resonances appeared after addition of enzyme. The ratio of peak heights of the original and new resonances reached a constant value which reflected the equilibrium constant for the reaction measured by chemical-quench methods.

The proton chemical shifts are in agreement with those previously reported by Teng et al. for the chemically synthesized 4-amino-4-deoxychorismate.[1] The resonances were shifted downfield (relative to chorismate) for all of the protons except those at the 4-position. The 4-H proton was shifted *upfield* by approximately 0.5 ppm to 4.25 ppm, as anticipated in replacing the C-4 oxygen with nitrogen. The coupling constant measured between H-3 and H-4 is −14 Hz, establishing a trans stereochemical relationship analogous to chorismic acid.

Further structural information was provided by enzymatically synthesizing **2** using ^{15}N-labeled ammonium bicarbonate (see legend to Figure 1). A 2D proton–carbon correlated spectrum is shown in the middle panel of Figure 1. The spectrum shows the anticipated chemical shifts for carbons and protons, with C-4, the allylic carbon, bearing the amino group and H-4 located at 53.7 ppm. A proton-decoupled natural abundance ^{13}C NMR spectrum (53 ppm region) is shown in the inset to the middle panel. The resonance having a chemical shift of 53.7 ppm was split into a doublet with a coupling constant, $^1J_{CN} = -8$ Hz, demonstrating that the [^{15}N]amino group is attached to C-4. The final confirmation of the structure for **2** was provided by the ^{15}N NMR spectrum shown in the bottom panel. The resonance observed upfield at −98.3 ppm is attributable to residual NH_4^+ while the resonance downfield at −77.2 ppm is due to the intermediate.

The enzymatic and structural data in this report provide definitive identification of **2** as a true intermediate which is formed by the action of PabB on chorismate. As discussed previously,[7-9] isomeric hydroxy- or aminochorismate compounds have been isolated or proposed also in the biosynthesis of isochorismate and anthranilate, respectively. It is interesting to note that the absolute stereochemistry of chorismate is retained in the corresponding isomeric intermediates, implying that a double inversion of configuration must have taken place. In each of these cases, the enzymatic transformation requires magnesium. It is reasonable to suggest that all three pathways utilize a common intermediate which can be attacked by the appropriate nucleophile to give the desired product. We are currently exploring this possibility in studies underway to establish the complete kinetic pathway for the conversion of chorismate to PABA.

Acknowledgment. We thank J. Lecomte for helpful discussions.

(6) The value of the equilibrium constant was measured from reaction mixtures containing 50 μM [^{14}C]chorismate, 1 μM PabB, 50 mM $(NH_4)_2SO_4$, and 10 mM $MgCl_2$ in 50 mM TRIS pH 8.6 buffer at 37 °C. The reactions were quenched at 35 and 75 min with $CHCl_3$ and analyzed by RP-HPLC. The results showed that the equilibrium was established within 35 min and that the value for the equilibrium constant was 6.1 M^{-1}.

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Design of a Novel Type of Zinc-Containing Protease Inhibitor

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Received November 13, 1990

Carboxypeptidase A (CPA, EC 3.4.17.1)[1,2] serves as the prototypic enzyme for zinc-containing proteases.[3] The key catalytic groups of CPA are Zn^{2+}, Glu-270, and Arg-145. The carbonyl group of the scissile peptide bond is polarized by the Zn^{2+}, and the activated carbon is attacked by the carboxylate of Glu-270 (anhydride pathway[4]) or by a water molecule with the assistance

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