Therefore the connectivities involving the Gly55 α-protons and the P56 γ-protons are obscured by the intense β-proton connectivities in Figure 1b,c.

The long-range connectivities in Figure 1, that link Pro residues to remote residues in the sequence, are consistent with reported[4] Nase assignments and the distances calculated from the crystal structure.[7] These results are further evidence that there is a close correspondence between the solution and crystal structure of the ternary complex throughout most of the protein, as concluded previously.[4,10,11]

The isotope editing approach described herein should be a generally useful method of assigning proline spin systems in moderate size proteins. Labeling with [3,5-$^{13}C_2$]proline should improve the efficiency of the method, because all relevant information could be obtained by using one double-labeled sample. The double-labeled proline could be synthesized by following the scheme used to obtain [4-^{13}C]proline.[12]

Acknowledgment. This work was supported by the Intramural AIDS Antiviral Program of the Office of the Director of the National Institutes of Health and by NIH Grant MBRS RR08153 (P.E.Y.). We thank Rolf Tschudin for technical support. We are grateful to Professor John Gerlt for transformed *E. coli*, to Professors Gerlt and Philp Bolton for discussions of their results on P117G, to Professor Eaton Lattman and Dr. Philip Loll for providing us with their X-ray coordinates prior to publication, and to Dr. Bernard Brooks for converting the coordinates into internuclear distances.

Registry No. Nase, 9013-53-0; Pro, 147-85-3.

Supplementary Material Available: Spectra (500 MHz $^1H/^{13}C$ HMQC) of Nase labeled with [2-^{13}C]Pro and [4-^{13}C]Pro and spectrum (500 MHz ^{13}C edited NOESY) of Nase labeled with [4-^{13}C]Pro (3 pages). Ordering information is given on any current masthead page.

(10) Cole, H. B. R.; Sparks, S. W.; Torchia, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6362–6365.
(11) Kay, L. E.; Brooks, B.; Sparks, S. W.; Torchia, D. A.; Bax, A. *J. Am. Chem. Soc.* **1989**, *111*, 5488–5490.
(12) Young, P. E.; Torchia, D. A. In *Peptides: Structure and Function*; Hruby, V. J., Rich, D. H., Eds.; Pierce, Rockford, IL, 1983; pp 155–158.
(13) Bax, A.; Weiss, M. A. *J. Magn. Reson.* **1987**, *71*, 571–575.

Observation and Sequence Assignment of a Cis Prolyl Peptide Bond in Unliganded Staphylococcal Nuclease

Susan M. Stanczyk and Philip H. Bolton*

Department of Chemistry, Wesleyan University Middletown, Connecticut 06457

Mark Dell'Acqua and John A. Gerlt*

Department of Chemistry and Biochemistry University of Maryland College Park, Maryland 20742

Received May 2, 1989

Cis–trans isomerization of X-prolyl peptide bonds is now considered as both a major determinant of the rate of protein folding and an explanation for the existence of multiple folded forms of proteins in solution.[1-3] Indirect evidence for these effects of prolyl peptide bonds has been recently obtained by examining the properties of mutant proteins in which specific prolyl residues have been replaced by site-directed mutagenesis techniques. For example, by investigating substitutions for specific proline residues, Richards and co-workers have deduced that isomerization of a single prolyl peptide bond is the rate-determining step in the folding of thioredoxin from *Escherichia coli*.[4] Also, Dobson and Fox have implicated cis and trans isomers of a single prolyl peptide bond in staphylococcal nuclease (SNase) as the explanation for the coexistence of multiple folded forms of the protein.[5-7] However, the conclusions reached in these and all other solution studies of isomerization of prolyl peptide bonds in proteins have not been based on the observation of cis prolyl peptide bonds but on the effects of this putative isomerization on biophysical properties such as the rates of folding and the ^{1}H NMR spectra of the resolved histidine H_ϵ resonances. In this communication we report observation and assignment of selected ^{1}H and ^{13}C NMR resonances of a single proline cis X-prolyl peptide bond in SNase.

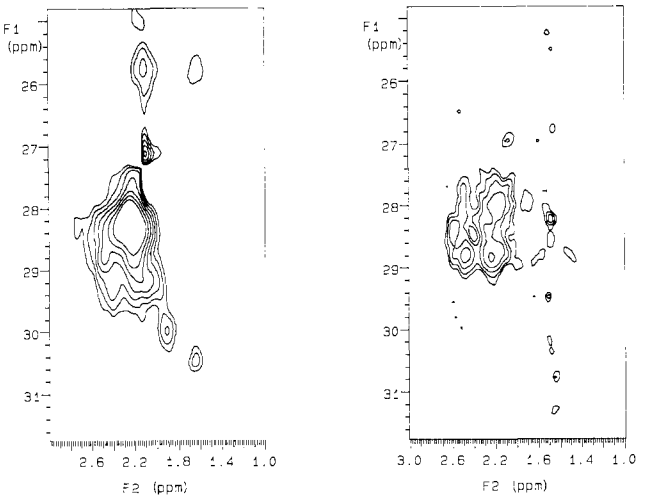

Figure 1. ^{1}H–^{13}C heteronuclear chemical shift correlation spectra of wild-type SNase (left panel) and its P117G mutant (right panel) labeled with [4-^{13}C]proline. The data were obtained in proton detection experiments of ^{1}H–^{13}C chemical shift correlations at pH 5.5 and 40 °C.[17] Assignments are made in the text.

A number of studies of the resonances of the resolved H_ϵ protons of the four histidine residues in SNase have suggested the presence of an equilibrium mixture of two monomeric folded forms since two resonances are detected for each of these protons.[5-10] Investigation of the temperature dependence of these resonances reveals that the multiplicity is preserved in the unfolded form. In the presence of the active site ligands Ca^{2+} and thymidine 3′,5′-bisphosphate (pdTp), the multiplicity of resonances in the folded state is eliminated. Crystallographic studies of SNase complexed with the active-site ligands have revealed that the peptide bond between Lys 116 and Pro 117 is cis whereas the remaining six X-prolyl peptide bonds are trans.[11] The ^{1}H NMR spectrum of the aromatic region of the site-directed mutant in which Pro 117 is replaced with a glycine residue (P117G) reveals only a single resonance for each H_ϵ proton. The simplest explanation for these observations is that the two folded forms of unliganded SNase in solution as detected by ^{1}H NMR spectroscopy can be associated with a major cis and a minor trans isomer of the Lys_{116}-Pro_{117} peptide bond. However, the ^{1}H NMR spectrum of P117G differs from that of the wild-type enzyme,

(1) Brandts, J. F.; Halvorson, H. R.; Brennan, M. *Biochemistry* **1975**, *14*, 4953.
(2) Lin, L. N.; Brandts, J. F. *Biochemistry* **1984**, *23*, 5713.
(3) Creighton, T. E. *Proteins: Structure, Function, and Molecular Properties*; Freeman: San Francisco, 1984.
(4) Kelley, R. F.; Richards, F. M. *Biochemistry* **1987**, *26*, 6765–6774.
(5) Fox, R. O.; Evans, P. A.; Dobson, C. M. *Nature (London)* **1986**, *320*, 192–194.
(6) Evans, P. A.; Dobson, C. M.; Kautz, R. A.; Hatfull, G.; Fox, R. O. *Nature (London)* **1987**, *329*, 266–268.
(7) Evans, P. A.; Kautz, R. A.; Fox, R. O.; Dobson, C. M. *Biochemistry* **1989**, *28*, 362–370.
(8) Markley, J. L.; Jardetzky, O. *J. Mol. Biol.* **1970**, *50*, 223.
(9) Alexandrescu, A. T.; Ulrich, E. L.; Markley, J. L. *Biochemistry* **1989**, *28*, 204–211.
(10) Stanczyk, S. M.; Bolton, P. H.; Dell'Acqua, M.; Pourmotabbed, T.; Gerlt, J. A. *J. Am. Chem. Soc.* **1988**, *110*, 7908–7910.
(11) Cotton, F. A.; Hazen, E. E.; Legg, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *79*, 2551–2555.

0002-7863/89/1511-8317$01.50/0

suggesting that the solution structure of the mutant protein may differ from that of the wild type.[12] In addition, a number of amino acid replacements remote from Pro 117, both within the active site[12] and elsewhere,[9] alter the equilibrium between the conformational forms revealed by the H_ϵ protons. These last two observations suggest the desirability of additional information about the presence of a cis prolyl peptide bond in SNase in solution.

We have obtained additional evidence for the presence of a cis prolyl peptide bond involving Pro 117 in wild-type SNase by using samples of wild type and the P117G mutant biosynthetically labeled with [4-^{13}C]proline.[13,14] Previous studies have shown that the ^{13}C NMR chemical shift of the labeled 4-carbon in a cis X-prolyl peptide bond is about 3 ppm upfield of the labeled carbon in a trans peptide bond.[15] We have used proton detection methodology[16] to observe the ^{13}C resonances in the labeled proteins. ^{1}H-^{13}C heteronuclear chemical shift spectra of the labeled samples of wild-type SNase and its P117G mutant obtained at 40 °C in the absence of the active-site ligands Ca^{2+} and pdTp are reproduced in Figure 1. The intense correlations at ^{13}C chemical shifts of 28 ppm in both spectra are characteristic of proline residues in trans peptide bond geometries. Prolines in cis peptide bond geometries are expected to be approximately 3 ppm upfield of these correlations.[15] Although several correlations associated with natural abundance ^{13}C nuclei are present in these spectra (as evidenced by spectra obtained on unlabeled samples), the correlation having a ^{13}C shift of 25.7 ppm and a ^{1}H shift of 2.1 ppm in the spectrum of wild-type SNase is associated with the labeled proline and, therefore, can be assigned to proline in a cis peptide bond geometry. The correlations associated with prolines in trans peptide bond geometries in P117G show small changes in dispersion in both the ^{13}C and ^{1}H dimensions, but it is clear that the correlation associated with proline in a cis peptide bond geometry is missing. Our experiments do not allow a quantitative measure of the position of the cis/trans equilibrium but reveal that the cis isomer predominates. Thus, the proline residue participating in a cis peptide bond is Pro 117.

These experiments provide persuasive experimental evidence that a cis X-prolyl peptide bond predominates in solution. Independent confirmation of our assignment of the cis prolyl residue to Pro 117 is detailed in the accompanying communication, in which sequence-specific assignments are used to detect and assign the cis geometry to the Lys_{116}-Pro_{117} peptide bond in the presence of Ca^{2+} and pdTp.[18] Thus, we conclude that, in solution both in the presence and absence of ligands and in the crystalline state[11] in the presence of ligands, this X-prolyl peptide bond exists predominantly in the cis geometry.

Our observation does not identify which structural feature(s) are responsible for the multiple conformations which are apparent via the resonances of the resolved histidine H_ϵ protons. For example, the X-prolyl peptide isomerization may serve to slow the rate of interconversion between two conformations that differ in hydrogen-bonding networks or steric interactions rather than the isomerization itself being the driving force for two conformations.[7,10]

Acknowledgment. We thank Dr. Dennis A. Torchia and Professor Paul E. Young for a generous sample of [4-^{13}C]proline and Dr. Torchia for sharing his experimental results during the course of this work. The P117G mutant was generously provided by Professor Robert O. Fox. This research was supported by NIH GM-34573 to J.A.G. and P.H.B. and NSF DMB 88-06744 to P.H.B.

Supplementary Material Available: One-dimensional ^{13}C NMR spectra of [4-^{13}C]proline-labeled SNase and P117G in the absence of Ca^{2+} and pdTp (2 pages). Ordering information is given on any current masthead page.

(12) Stanczyk, S. M.; Bolton, P. H.; Dell'Acqua, M.; Pourmotabbed, T.; Gerlt, J. A., unpublished observations.
(13) Hibler, D. W.; Stolowich, N. J.; Reynolds, M. A.; Gerlt, J. A.; Wilde, J. A.; Bolton, P. H. *Biochemistry* **1987**, *26*, 6278-6286.
(14) Wilde, J. A.; Bolton, P. H.; Dell'Acqua, M.; Hibler, D. W.; Pourmotabbed, T.; Gerlt, J. A. *Biochemistry* **1988**, *27*, 4127-4132.
(15) Torchia, D. A. *Annu. Rev. Biophys. Bioeng.* **1984**, *13*, 125-144.
(16) The pulse sequence used was

^{1}H: 90°-τ- -180°-$t_{1/2}$- -τ-acquisition

^{13}C: -90°-$t_{1/2}$- -90°- -decoupling

with the appropriate phase cycling.[17] The data was obtained by using a Varian XL-400 NMR spectrometer in conjunction with a Nalorac proton detection probe. ^{13}C NMR chemical shifts were referenced with respect to DSS. ^{1}H NMR chemical shifts were referenced with respect to H^2HO, which in turn was referenced to DSS.
(17) Griffey, R. H.; Redfield, A. G. *Q. Rev. Biophys.* **1987**, *19*, 51-82.
(18) Torchia, D. A.; Sparks, S. W.; Young, P. E.; Bax, A. *J. Am. Chem. Soc.*, preceding paper in this issue.

Lack of Inhibition in the Cleavage of *p*-Nitrophenyl Acetate by β-Cyclodextrin: Evidence for the Absence of Aryl Group Inclusion in the Transition State for Esterolysis

Oswald S. Tee* and Jorgen J. Hoeven

Department of Chemistry
Sir George Williams Campus
Concordia University
Montrêal, Québec, Canada H3G 1M8

Received June 19, 1989

The cleavage of phenyl acetates by cyclodextrins (CDs)[1] in aqueous base is generally believed to take place from an ester·CD complex in which the aryl group of the ester is included in the cavity of the CD.[1-4] Thus, other species that bind to the CD should inhibit the reaction by competition.[2a,5] Such is the case for the cleavage of *m*-nitrophenyl acetate (mNPA) by α-CD[2a] and, as described below, by β-CD. In contrast, the cleavage of *p*-nitrophenyl acetate (pNPA) by β-CD *is not strongly retarded* by various potential inhibitors (PIs), and in some cases the reaction is actually *faster*!

Studies of the inhibition of mNPA cleavage[6] gave dissociation constants (K_i) in good agreement with values determined by other methods (Table I), but comparable studies with pNPA did not. Experiments with fixed [PI] and varying [β-CD] showed that the cleavage of pNPA is faster with added 1-butanol; it is not inhibited (Figure 1). Also, addition of 1-hexanol or cyclohexanol brings about rate increases, suggesting that there is a reaction between alcohols and the pNPA·CD complex. To obtain rate constants for this reaction, we carried out experiments with a fixed, high [CD] and varying [PI].[7] As shown by the example in Table II, values of k_{obsd} are very different from those expected for simple inhibition (k_{inh}). Analysis of such data was based on the following approach.

(1) (a) Bender, M.; Komiyama, M. *Cyclodextrin Chemistry*; Springer Verlag: New York, 1978. (b) Szejtli, J. *Cyclodextrins and their Inclusion Complexes*; Akademiai Kiado: Budapest, 1982. (c) Saenger, W. *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 344.
(2) (a) Van Etten, R. L.; Sebastian, J. F.; Clowes, G. A.; Bender, M. L. *J. Am. Chem. Soc.* **1967**, *89*, 3242. (b) Van Etten, R. L.; Clowes, G. A.; Sebastian, J. F.; Bender, M. L. *J. Am. Chem. Soc.* **1967**, *89*, 3253. (c) Komiyama, M.; Bender, M. L. *J. Am. Chem. Soc.* **1978**, *100*, 4576.
(3) Tee, O. S.; Takasaki, B. K. *Can. J. Chem.* **1985**, *63*, 3540.
(4) Matsui, Y.; Nishioka, T.; Fujita, T. *Top. Curr. Chem.* **1985**, *128*, 61.
(5) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; Freeman: New York, 1985.
(6) Inhibition studies followed the method of Van Etten et al.[2a] Cleavage of mNPA (0.2 mM) in a 0.2 M phosphate buffer (pH 11.6) containing β-CD (1 mM) and various concentrations of PI was monitored by UV-visible spectrophotometry, with stopped-flow mixing.[3]
(7) Cleavage of pNPA (0.05 mM) in a 0.2 M phosphate buffer (pH 11.6) containing β-CD (10 mM) and various concentrations of PI was monitored by UV-visible spectrophotometry, with stopped-flow mixing.[3]

0002-7863/89/1511-8318$01.50/0