and C(7)′ and the other by C(8) and C(8)′. One of the two axial positions was nearly shared by C(7) and C(8)′ while the other was shared by C(7)′ and C(8). The hydrogens were thus assigned occupancies of 1.0. Since the electron density was dominated by those hydrogens attached to C(7) and C(8), the refined hydrogen positions are biased: the bond distances and angles are better for contacts with C(7) and C(8) than with C(7)′ and C(8)′.

Molecular Orbital Calculations. Calculations were performed by using the TRIBBLE package,[32] an extended Hückel method incorporating two-body repulsion corrections as introduced by Anderson.[33] Orbital exponents and H_{ii} values for H, C, and Zr were taken from ref 34, those for P and Pt from ref 35 (see Table VI). Double-ζ wave functions were used for the d-orbitals of Pt and Zr, with coefficients given in parentheses.

Supplementary Material Available: Final positional parameters (as fractional coordinates) and tables of thermal parameters (10 pages); tables of structure factor amplitudes (observed and calculated) (56 pages). Ordering information is given on any current masthead page.

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Proline Signals in Ultraviolet Resonance Raman Spectra of Proteins: Cis-Trans Isomerism in Polyproline and Ribonuclease A

Debra S. Caswell and Thomas G. Spiro*

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Abstract: The Raman spectrum of aqueous polyproline excited at 200 or 218 nm is dominated by a single band at ~1465 cm^{-1}, assigned to the imide C-N stretching vibration. It is analogous to the amide II′ band at ~1465 cm^{-1} of polypeptides in D_2O, which is also enhanced strongly at 200 nm. The amide II′ band loses much more intensity between 200- and 218-nm excitation (×5.6) than does the polyproline band (×1.2). This difference is attributable to a red shift of the imide vs. amide $\pi-\pi^*$ electronic transition: the first UV absorption band maximizes at 192 nm for poly-L-lysine but at 203 nm for polyproline. Because of this effect, proline residues are readily detected in protein ultraviolet Raman spectra excited at 218 nm. For example, histidine-rich glycoprotein (HRG) shows a major band at 1457 cm^{-1} due to the high proline content, 16.6% of the residues; this is much stronger than the 1547-cm^{-1} band arising from the amide II mode of the remaining residues. When polyproline is dissolved in 1:9 H_2O-propanol containing ~0.15 M $HClO_4$, a 1435-cm^{-1} band grows in at the expense of the original 1465-cm^{-1} band. This spectral change is associated with the transition from polyproline II to polyproline I, which contain trans and cis imide bonds, respectively. The frequency shift is in the same direction as that seen for the amide II mode of cis peptides. Ribonuclease A shows a band at 1458 cm^{-1} in its 218 nm excited Raman spectrum, attributable to its four proline residues, two of which are in the cis conformation. When the protein is incubated at pH 1.5 this band shifts to 1466 cm^{-1}; the upshift is consistent with the expected conversion to a higher percentage of prolines in the trans conformation in the unfolded protein. UVRR spectroscopy offers a direct probe of proline conformation in protein structural studies.

Because its side chain ties back on itself to form a secondary linkage (Figure 1) proline is an important structural determinant in proteins. The absence of a hydrogen atom on the imide nitrogen atom eliminates H-bonded interactions, while the presence of the ring constrains the rotation angle of the N-C^α bond.[1] Proline residues are frequently found at bends in the polypeptide chain.[2] On the other hand cis and trans isomers about the N-C(O) bond are much closer in energy for proline than for other amino acids. Steric constraints normally destabilize the cis peptide bond by a factor of ~10^3 over the normal trans conformation, but for proline the steric inhibition is relieved by the ring structure, and the trans:cis equilibrium ratio is reduced to ~4.[3] There are several examples of cis proline residues in protein structures,[4] but there are only four instances of cis peptides not involving proline (three in carboxypeptidase A and one in dihydrofolate reductase).[5] This conformational flexibility of the proline imide bond may be an important element in protein dynamics. It has been argued, for example, that the proline-rich region of immunoglobins might regulate antigen binding by mediating a structural transition in the hinge region of the protein.[6] Cis/trans isomerization about the proline imide bond is believed to be the rate-limiting step in the denaturation of a number of proteins and may be a key step in protein folding in general.[3b,7] A widely accepted model is that

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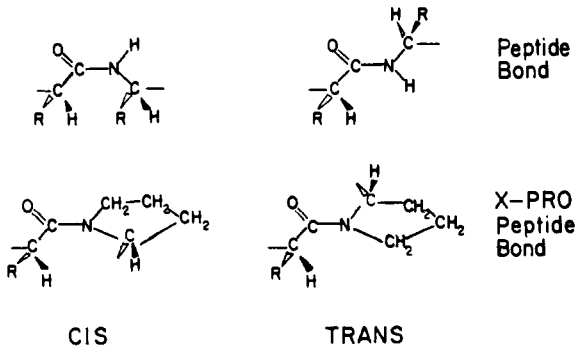

Figure 1. Cis and trans isomers for a peptide bond in general and an X-Pro peptide bond (where X is any amino acid).

the fast phase of protein folding involves the internalization of hydrophobic side chains into specific domains, which are then brought into proper juxtaposition via a slow phase involving proline isomerization.[7]

In this study we describe a signal for proline in ultraviolet resonance Raman (UVRR) spectra which is sensitive to the cis/trans conformation. It has recently become possible to obtain Raman spectra at high sensitivity by using laser excitation in the deep ultraviolet region.[8-10] For proteins[9b,10b,c] these spectra contain enhanced vibrational modes of aromatic residues, and also of the amide vibrations of the polypeptide backbone. Hudson and Mayne[11] have recently reported enhanced proline C–N modes near 1500 cm^{-1} with 218-nm excitation of proline dipeptides. We now report strong enhancement of a band at 1465 cm^{-1} in polyproline, which is assigned to the C–N stretch of the imide bond. This mode is analogous to the amide II′ mode of peptides in D_2O, occurring at essentially the same frequency. Enhancement of the proline–imide stretch is substantially greater at 218 nm, however, because of a significant red shift in the $\pi-\pi^*$ electronic transition of the imide relative to the amide bond. Thus 218-nm excitation provides discrimination in favor of proline. The proline band is a dominant feature in the spectrum of histidine-rich glycoprotein, in which proline constitutes 16.6% of the residues, and can also be seen for ribonuclease A, which contains four proline residues. When polyproline II is converted to polyproline I, involving a transition from trans to cis proline residues, a 1435-cm^{-1} band grows in at the expense of the 1465-cm^{-1} band, consistent with the expected downshift of the C–N stretch for the cis imide bond. Likewise when ribonuclease is denaturated at low pH an upshift in the proline frequency is seen, from 1458 to 1466 cm^{-1}, attributable to the cis → trans isomerization attendent on denaturation. The frequencies differ appreciably from those found by Hudson and Mayne,[11] ~1485 and ~1515 cm^{-1}, for linear and cyclic glycyl-L-proline, which are models for *trans*- and *cis*-proline linkages.

Experimental Section

Poly-L-proline, poly-L-lysine, and ribonuclease A from bovine pancreas (Type XII-A; No R-5500) were purchased from Sigma Chemical Co. Histidine-rich glycoprotein (HRG) was a gift from Dr. William T. Morgan, Department of Biochemistry, Louisiana State University Medical Center. The polypeptides and proteins were dissolved in 50 mM phosphate buffer at the concentrations and pH/pD's indicated in the figure captions. Ammonium sulfate was added to the peptides as an intensity standard for the measurement of Raman intensities.

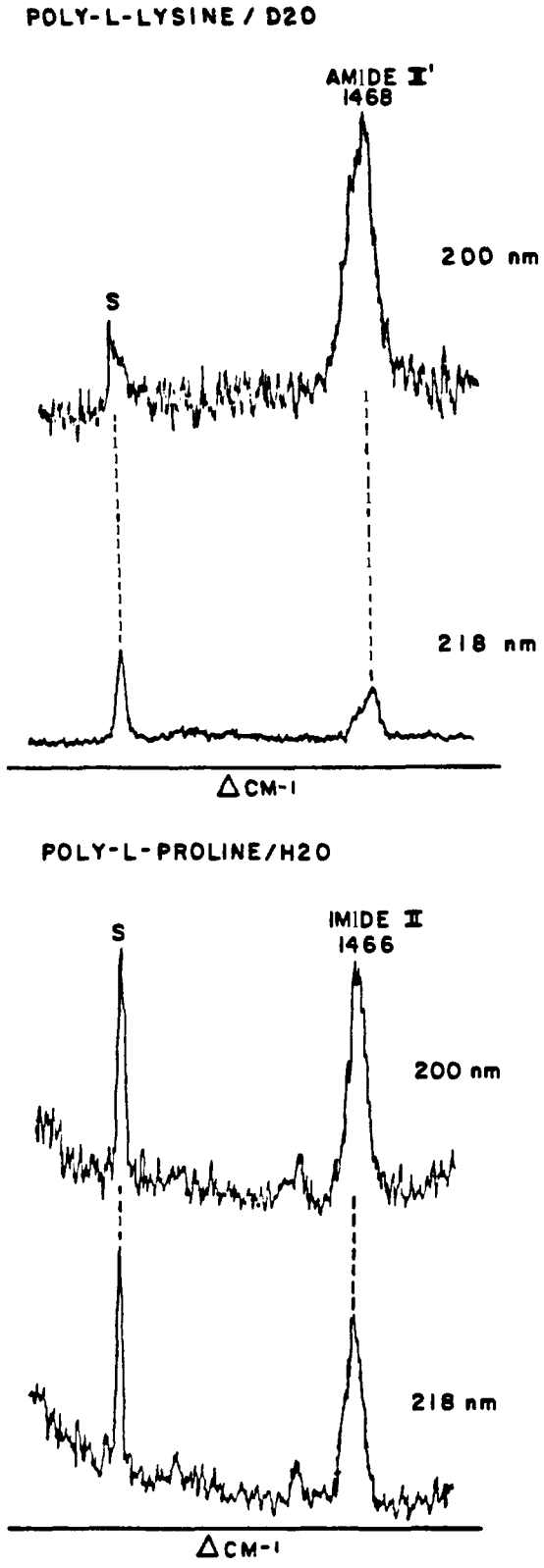

Figure 2. Raman spectra with 200- and 218-nm excitation for random coil poly-L-lysine (pD ~5) and poly-L-proline (pH ~7.5) in 50 mM phosphate buffer. Ammonium sulfate was added as an internal intensity standard.

The UV Raman apparatus is described in ref 10a. The fourth harmonic of a pulsed Nd:YAG laser with a hydrogen shift cell provided UV excitation at 200- and 218-nm excitation. The beam intersected the sample in the center of a wire-guided jet similar to that described by Reider et al.[13] The laminator was constructed by wrapping thin wire around a syringe needle and forcing the excess wire from each end to

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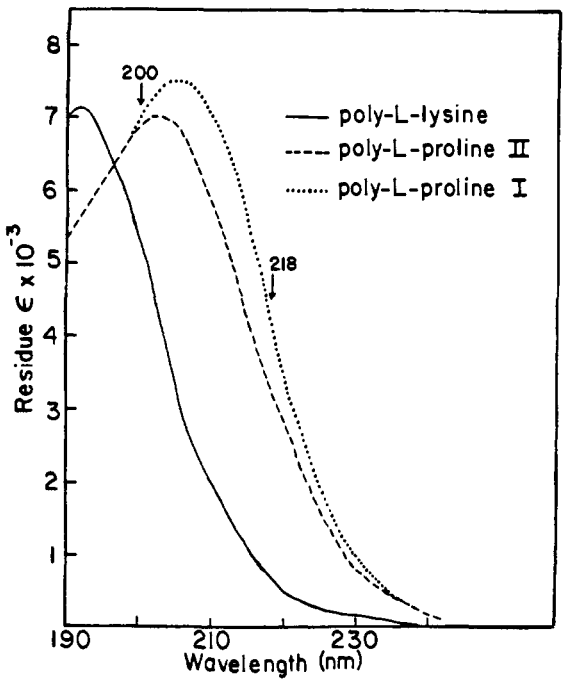

Figure 3. UV absorption spectrum of random coil poly-L-lysine (from ref 13) and poly-L-proline I and II (from ref 16).

meet ~15 mm below the bottom of the needle. This permits a very thin sheet of liquid to form between the wires and allows the volume of the sample required to be decreased from 3–5 mL for the free jet sampling apparatus[10a] to 0.75–1.25 mL for the laminator, a valuable improvement when examining scarce proteins such as HRG. The flow rate is also decreased to as little as one drop/second, providing gentler treatment of the sample.

Sample integrity was monitored by the absence of time-dependent changes in the Raman spectra and of alterations in the UV absorption after Raman acquisition.

Results and Discussion

A. Proline C–N Enhancement. Figure 2 compares UVRR spectra, obtained with 200- and 218 nm excitation, for polyproline in H_2O and polylysine in D_2O. In both cases, the spectra are dominated by a single band at ~1465 cm^{-1}. For polylysine, this band is identified with the amide II′ mode. Its dominance is due to resonance with the amide $\pi-\pi^*$ transition at ~192 nm.[14] Dudik et al.[9b] showed that preresonance UV RR intensities of the *N*-methylacetamide amide II and III modes are consistent with A term enhancement via this transition. These two modes contain a mixture of N–C(O) stretching and N–H bending coordinates.[15] In D_2O solution, the N–H proton is replaced by a deuteron, and the N–H(D) bending frequency is shifted out of this region, leading to unmixing of the coordinates and leaving a nearly pure N–C(O) stretching amide II′ mode. This is demonstrated by the larger ^{15}N, ^{13}C isotope shift seen for amide II′ than for amide II′ of *N*-methylacetamide.[12] The amide II′ band is the dominant feature of 200-nm-excited RR spectra of *N*-methylacetamide[12] and of proteins[10c,d] in D_2O, establishing that C–N stretching is the main distortion coordinate in the $\pi-\pi^*$ excited state, as discussed by Mayne et al.[12] In proline, there is no N–H bending coordinate, and a nearly pure N–C(O) mode is therefore expected, which we label imide II. This mode was assigned by Hudson and Mayne[11] to the 1485-cm^{-1} band of glycylproline, and we assign it to the 1465-cm^{-1} band of polyproline from its close similarity in both frequency and enhancement pattern to the amide II′ band of polylysine.

Figure 2 also shows that the relative enhancements differ appreciably for polyproline and polylysine at 218 relative to 200 nm.

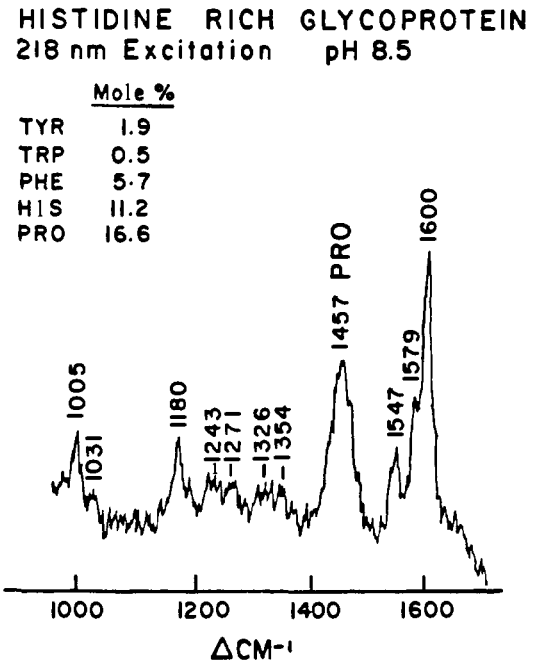

Figure 4. 218-nm-excited UVRR spectrum of histidine-rich glycoprotein ~2 mg/mL in 50 mM phosphate buffer (pH ~8.5). The spectrum is the sum of 6 scans collected over 0.05-Å increments. Mole percent contributions of the amino acids enhanced at this excitation wavelength are listed.

The 200/218 nm intensity ratio, measured relative to the 983 cm^{-1} breathing mode of SO_4^{2-}, present as an internal standard, is 5.6 for the amide II′ band of polylysine, but 1.2 for the imide II band of polyproline. This difference is directly attributable to a red shift of the imide vs. amide $\pi-\pi^*$ transition. Figure 3 compares UV absorption spectra for polylysine and polyproline, from ref 13 and 16. The 192-nm band of polylysine is red shifted to 202–205 nm for polyproline. This effect is attributable to the extra methylene substituent of the N atom of proline stabilizing the $\pi-\pi^*$ excited state.[17] There are two conformations for polyproline, I and II.[1] These are right- and left-handed helices containing cis and trans imide bonds, respectively. The absorption band of polyproline II, the form stable in H_2O, is slightly hypochromic and blue shifted relative to that of polyproline I (Figure 3). For both forms, 218 nm is half-way down the red side of the absorption band, while it corresponds to the foot of the polylysine absorption; 200 nm is close to the absorption maxima for both polylysine and polyproline. It therefore makes sense that the 218/200 nm intensity ratio is much higher for polyproline than for polylysine.

The absorption red shift makes 218 nm a good wavelength for proline enhancement, and the imide II band is in a region free from interference by modes of aromatic residues. This is illustrated by the 218-nm-excited UVRR spectrum of histidine-rich glycoprotein (HRG), shown in Figure 4. HRG is a plasma protein with a high content (11.2% of the residues) of histidine, which is believed to be involved in metal ion transport.[18] Its proline content is even higher (16.6%), and the strong band at 1457 cm^{-1} is assignable to the proline imide II. It is nearly as strong as the 1600-cm^{-1} band, which is a superposition of the ν_{8a} bands of phenylalanine and tyrosine[19] (5.7 and 1.9% of the residues). The proline band is much stronger than the amide II band at 1547 cm^{-1} (which also contains a contribution from the strong 1550-cm^{-1} band of tryptophan,[19] 0.5% of the residues) even though this band represents all the non-proline residues. (In contrast, histidine, which constitutes 11.2% of the residues, contributes only weakly, the histidine enhancement being quite modest,[20] and its spectrum

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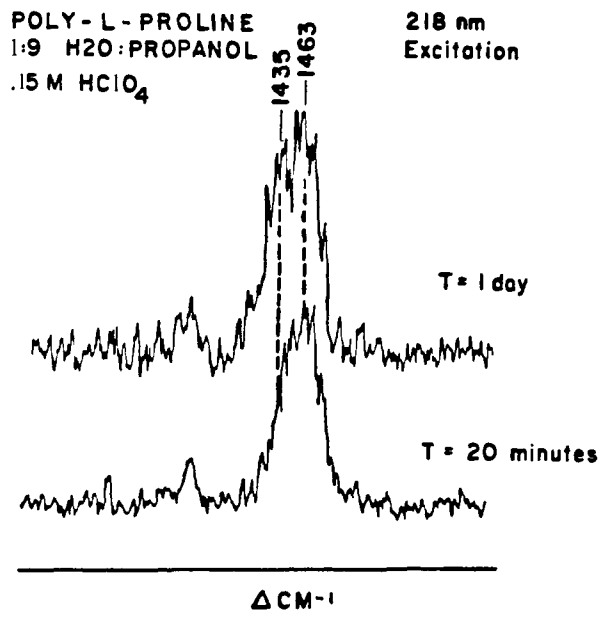

Figure 5. 218-nm-excited Raman spectra at various incubation times of poly-L-proline (~2 mg/mL) in 1:9 H_2O–propanol containing 0.15 M $HClO_4$ for catalysis.

is obscured by the bands from the remaining aromatic residues.[19]

Although the proline band is unusually strong in HRG, we note that it is discernable in other protein UVRR spectra that have been published. For example, a ~1460-cm^{-1} band of significant intensity can be seen[21] in 218-nm-excited spectra of cytochrome *c* which contains three (tuna) or four (horse) proline residues in a 13 kD polypeptide chain. This band was assigned to CH_2 bending (plus a very small tryptophan contribution) as have other bands in this region which have been seen in visible excitation Raman spectra of proteins and peptides,[15] including polyproline.[22] It seems unlikely, however, that CH_2 bending vibrations are significantly enhanced with 218-nm excitation, while the sensitivity for proline residues is high. Evidence is presented below in ribonuclease A spectra that a small number of proline residues can be detected.

B. Cis/Trans Proline Frequencies. When polyproline is placed in 1:9 H_2O–propanol containing 0.15 M $HClO_4$, it converts from polyproline II to polyproline I.[23] The interconversion rate follows zero-order kinetics, indicating a "zipper" mechanism, and depends on polymer length (many hours for long chains[24]). Figure 5 shows the 218-nm-excited polyproline RR spectrum in this solvent. With increasing incubation time, a band at 1435 cm^{-1} gradually grows in at the expense of the 1465-cm^{-1} band. While the signal-to-noise ratio in these spectra is not as high as one would like, the increase in the 1435/1465 cm^{-1} scattering ratio is unmistakable and has been confirmed in repeated experiments. We attribute the new band to polyproline I. The conversion is not quantitative; the position of the equilibrium as well as the rate depends on the solvent.[23] The approximately equal 1435/1465 cm^{-1} band intensities seen after 1 day do not change significantly with longer incubation times. Since polyproline I and II contain cis and trans imide bonds, respectively, we associate the 1435- and 1465-cm^{-1} frequencies with the imide II vibration for cis and trans conformations, at least in the polyproline structure. It is known that the amide II frequency of cis peptides is ~100 cm^{-1} lower than that of trans peptides.[15] Consequently the shift observed for polyproline I is in the right direction. The smaller extent of the shift for proline suggests a smaller C–N stretching force constant

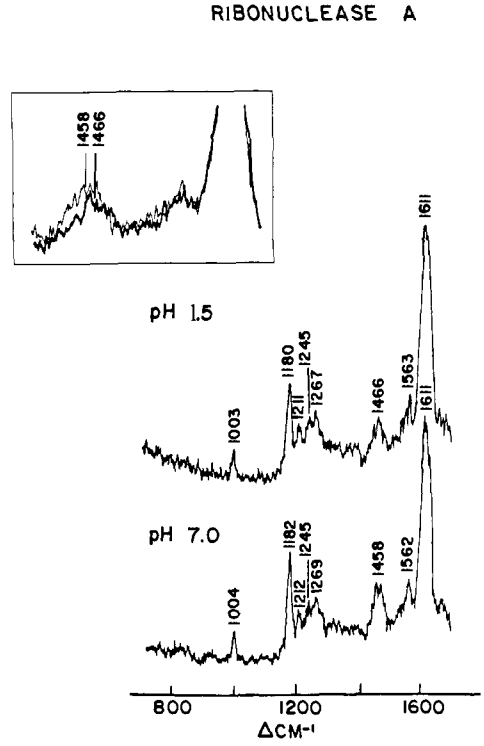

Figure 6. 218-nm-excited UVRR spectra of ribonuclease A (~2 mg/mL) in 50 mM phosphate buffer at the indicated pH's. The spectra are each the sum of 7 scans collected over 0.05-Å increments.

change, consistent with the smaller thermodynamic destabilization of the cis bond in proline relative to other peptides (see introduction).

This result differs, however, from that of Hudson and Mayne,[11] who reported strong bands at ~1485 and ~1515 cm^{-1} in the 218-nm-excited Raman spectra of linear and cyclic glycyl-L-proline, respectively. These frequencies are substantially higher than those that we observed for polyproline, or HRG, and to the extent that the cyclic gly-pro structure models a cis proline linkage, the shift is in the wrong direction. We have no explanation for this discrepancy, which merits further study.

Figure 6 shows 218-nm-excited RR spectra of ribonuclease A in H_2O at pH 7.0 and 1.5. This protein (12.6 kD) contains four proline, three phenylalanine, six tyrosine, and no tryptophan residues. The 1611-cm^{-1} band is again a superposition of phe and tyr ν_{8a} modes. There being no tryptophan we can attribute the 1004-cm^{-1} band to phe, the 1182- and 1212-cm^{-1} bands to phe and tyr, the 1269-cm^{-1} band to tyr, and the 1245- and 1562-cm^{-1} bands to amide III and II modes of the non-proline residues.[21] In the native protein, two of the four proline residues have cis-imide bonds,[25] but when the protein is denatured, a small percentage (~20%) is believed, on the basis of isomer-specific proteolysis, to convert to trans (while at least one of the *trans*-prolines remains 100% trans).[25] At pH 7 there is a clear proline imide II band at 1458 cm^{-1}, which shifts up to 1466 cm^{-1} at pH 1.5. This shift can be seen in the Figure 6 inset showing superimposed high-resolution scans at pH 7 (light trace) and pH 1.5 (heavy trace). The band is broad, due to the differing proline configurations, but the shift of the band contour is clear, in comparison with the constant 1611-cm^{-1} band. The shift is in the right direction for cis → trans isomerization, and it is significant considering the small percentage of prolines believed to undergo isomerization.

The 1466-cm^{-1} frequency observed at pH 1.5 is the same as that of polyproline II, but the 1458-cm^{-1} frequency at pH 7 is 23 cm^{-1} higher than the polyproline I frequency, although native ribonuclease is known to contain 50% *cis*-prolines. This difference may be due to a different C–N force constant in polyproline I than for an isolated cis protein in a polypeptide chain, or it may

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be due to vibrational coupling between adjacent proline residues in polyproline. It is interesting that the proline band in HRG is at 1457 cm^{-1}, the same frequency as in native ribonuclease, although the proline bonds in HRG are suggested on the basis of circular dichroism measurements to be trans.[26] Thus there appears to be some variability for the proline frequency among residues with the same imide isomer. This may also contribute to the breadth of the band in protein spectra. It is evidently not possible to associate proline conformation uniquely with fixed Raman frequencies. Nevertheless the distinct frequency difference observed for ribonuclease A at pH 7 and 1.5 indicates that UVRR spectroscopy may be quite useful in monitoring specific proline isomerization processes.

Acknowledgment. We thank Dr. William T. Morgan of the Louisiana State Medical Center for a generous gift of histidine-rich glycoprotein. This work was supported by NSF Grant CHE 79-09433 and NIH Grant GM25158.

Registry No. Proline, 147-85-3; polyproline, 25191-13-3; ribonuclease A, 9001-99-4; polylysine, 25104-18-1.

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Phosphate Ester Hydrolysis Catalyzed by Metallomicelles

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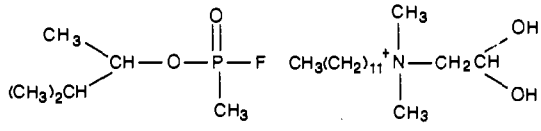
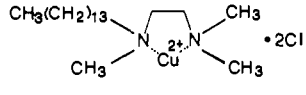
Abstract: Two long-chained cupric ion complexes were synthesized and found to possess remarkable catalytic activity toward phosphate triesters, diesters, and other phosphorus(V) compounds (including some particularly toxic and persistent materials). The complexes form "metallomicelles" which bind substrates with enzyme-like efficiency ($K_{assoc} > 10^5$ M). Rate accelerations approach the 10^5-fold level with turnover behavior. Possible reasons for the huge rate accelerations include enhanced electrophilicity of the micellized metal (demonstrated by polarography) and enhanced acidity of copper-bound water (demonstrated by rate vs. pH studies).

In Reston, VA, 1980, a gathering of American chemists[1] was challenged to devise methods for destroying some of the most noxious compounds known to man, compounds which a saner world would never produce. These are the phosphate esters and related phosphorus(V) materials known as nerve agents (e.g., GD or "Soman") and used in chemical weaponry. Owing to our interest in catalysis,[2] especially in reactions of biologically important systems such as phosphate esters, we undertook the challenge and began developing catalysts that hydrolyze phosphorus(V) substrates. The first of these,[3] dubbed Atlanta-1 or A1, operates by (a) binding noncovalently a phosphate ester, (b) accepting the phosphoryl group on one of its hydroxyls, and (c) dephosphorylating to produce an aldehyde that immediately regenerates the original A1. Thus, true catalysis or "turnover" was



GD A1

achieved, one of our major goals. The overall rate enhancement with 8 mM A1 was substantial (1800-fold), yet we set our sights on attaining even greater reactivity. This led to the synthesis of an entirely different catalyst, A2, whose remarkable properties are described below.



A2

Experimental Section

Synthesis. Preparation of *N,N,N'*-Trimethyl-*N'*-tetradecylethylenediamine. *N,N,N'*-Trimethylethylenediamine (12.0 g, 0.12 mol) and 1-bromotetradecane (14.0 g, 0.05 mol) in 70 mL of absolute ethanol were refluxed for 10 min. A solution of NaOH (2.4 g in 4 mL of water) was added, and the mixture was further refluxed for 5 h, cooled, and shaken with a solvent mixture consisting of 20 mL of 1-butanol, 20 mL of benzene, and 100 mL of water. The organic layer was removed and washed with 120 mL of water (creating an emulsion that required several hours standing to separate). After removing the volatiles from the organic layer with the aid of a rotary evaporator, we distilled under reduced pressure the residue at 150-170 °C and 0.25 mmHg. An initial cut of 4.7 g (containing considerable amounts of alkyl bromide) was dissolved in 100 mL of ether and treated with dry HCl gas to generate a hydrochloride salt that precipitated from solution. The salt was removed by filtration and washed with ether. Free amine was isolated with use of aqueous base and an ether extraction in the usual manner. A second cut from the vacuum distillation (3.7 g) was virtually pure amine. The combined product, 7.7 g, had satisfactory ^1H and ^{13}C NMR spectra. No attempt was made to optimize yields. Anal. Calcd for $C_{19}H_{42}N_2$: C, 76.43; H, 14.18; N, 9.39. Found: C, 76.52; H, 14.11; N, 9.29.

$CuCl_2$ Complex of *N,N,N'*-Trimethyl-*N'*-tetradecylethylenediamine ("A2"). A solution of diamine prepared above (0.99 g, 3.3 mmol) in 10 mL of absolute ethanol was added slowly with magnetic stirring to a filtered solution of anhydrous $CuCl_2$ (0.55 g, 4.1 mmol) in 15 mL of absolute ethanol. The resulting precipitate was washed with four portions of cold absolute ethanol (30 mL each), recrystallized from absolute ethanol or methanol-ether, and dried in a desiccator at 72 °C for 1 h to give material with mp 104-105 °C dec. Anal. Calcd for $C_{19}H_{42}Cl_2CuN_2$: C, 52.70; H, 9.78; N, 6.47. Found: C, 52.66; H, 9.81; N, 6.44.

***N,N'*-Dimethyl-*N,N'*-ditetradecylethylenediamine.** *N,N'*-Dimethylethylenediamine (2.0 g, 0.023 mol) and 1-bromotetradecane (18 g, 0.065 mol) dissolved in 50 mL of absolute ethanol were refluxed for 15 min. A solution of NaOH (2 g in 3 mL of water) was then added dropwise over a period of 5 min after which the solution was further refluxed for 6 h. TLC analysis indicated that after this time period most of the initial diamine had reacted. A mixture composed of 40 mL of benzene, 40 mL of 1-butanol, and 200 mL of water was next added to the filtered reaction mixture. The resulting organic layer was separated, reduced in volume

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(1) Sponsored by the DOD.
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