Carbon-13 Nuclear Magnetic Resonance Comparison of the Crystalline and Solution States of Carbonyl Hemoglobin A

Sir:

There has been a great amount of progress over the last 4 decades in determining three-dimensional structure in proteins, including hemoglobins, primarily by the application of X-ray crystallography.[1] The X-ray-determined structures have then often been used to infer mechanisms of enzyme action and cooperative effects. Argument over the extent to which a crystalline structure can be applied to the function of a protein in solution centers on the effects of crystal lattice forces on protein structure and on protein conformational dynamics, factors that are not directly evaluated by X-ray crystallography. In order to determine what structural differences, if any, exist between the crystalline and solution states, polarized UV-visible spectroscopy has been applied to myoglobin,[2] azide binding to metHb,[3,4] and infrared spectroscopy to myoglobin and hemoglobin.[5,6] In this communication, we report a ^{13}C NMR study of HbACO in the crystalline and solution states.

Carbon-13 NMR spectroscopy of proteins has advanced greatly in the last 10 years, primarily through instrumentation improvements.[7] Recently, a technique known as "magic-angle spinning" for removing the line-broadening influence of chemical shift anisotropy[8] has been combined with high-power ^1H decoupling and cross polarization[9] to provide a method capable of yielding high-resolution ^{13}C NMR data on solid samples.[10] Based on the thesis that ^{13}C NMR can therefore serve as another useful probe of biomolecules in the crystalline state and as a means of comparing the crystalline and solution states, we have carried out ^{13}C NMR experiments on crystalline human carbonyl hemoglobin A and reported the results in this communication, along with a comparison of the corresponding solution data.

Crystals of HbACO were prepared[11] in the same way as reported for the X-ray crystallographic studies.[12] Crystals saturated with ^{13}CO were grown in a syringe containing a 95% isotopically labeled ^{13}CO atmosphere. Approximately 1 g of crystals was transferred to a specially designed magic-angle spinner, with effort being taken to minimize the amount of mother liquor cotransferred. Visible spectra of crystals dissolved in N_2-degassed distilled water before and after a 4-h magic-angle spinning experiment indicate that there is no detectable exchange of bound CO and O_2 in the 4 h. Microscopic examination of the sample after the experiment demonstrated the sample remained crystalline, although the buffer liquid was somewhat depleted.

The ^{13}C NMR results are shown in Figure 1. Part a of the figure is the CP/MAS spectrum of the crystalline HbACO sample described above. Parts b and c of the figure show the unaltered and artificially broadened solution spectra, respectively, of HbACO with enriched CO. Several distinct regions in the spectra are apparent. Resonances between 10 and 75 ppm to lower shielding than Me_4Si are due to aliphatic carbons; aromatic carbon resonances are found from about 110 to 150 ppm, diamagnetic heme resonances from 138 to 148 ppm, and peptide carbonyl resonances from 170 to 185 ppm.[13] The resonance at 206.7 ppm in the

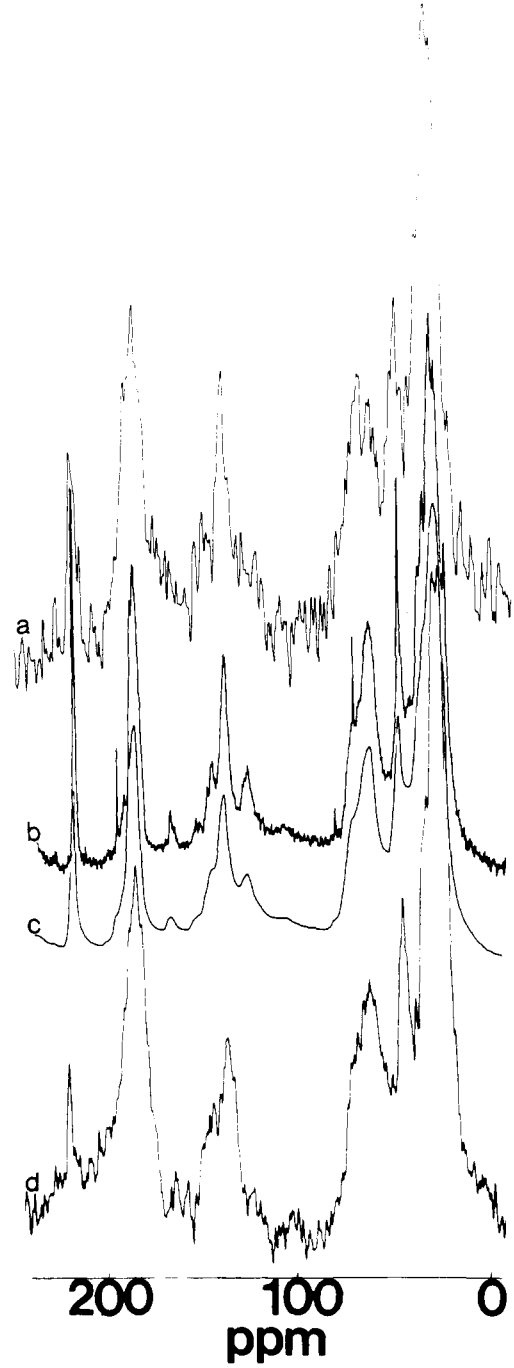

Figure 1. ^{13}C NMR spectra of HbACO. The scale is ppm with respect to Me_4Si. (a) Solid-state ^{13}C NMR spectrum of crystalline HbACO with ^{13}C-enriched CO in 2000 scans at 15.1 MHz with magic-angle spinning and cross polarization by using a modified JEOL FX-60Q spectrometer. (b) 25.1-MHz spectrum of 2 mM solution of HbACO with ^{13}C-enriched CO, obtained after 93 600 scans on a Jeol FX-100 spectrometer. (c) The same ^{13}C NMR spectrum of (b) with 40-Hz artificial broadening [to facilitate visual comparison with (c) and (d)]. (d) Solid-state spectrum of HbACO sample in which partial sample alteration occurred.

^{13}CO-saturated crystalline sample can be assigned to CO bound to the heme iron, because the resonance disappears upon exchange with natural-abundance CO, and free CO is found at 184.6 ppm.[14] Resonance positions for the crystalline samples are calculated by reference to the aliphatic carbon resonance at 39.9 ppm from Me_4Si, because this is a well-resolved peak that does not shift appreciably between solution and solid-phase experiments.

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Comparison of the spectra in parts a, b, and c, especially the resonance of CO bound to heme iron, shows that they are essentially equivalent. The resonance due to CO bound to iron is at 206.7 ppm for the crystalline sample and 206.3 ppm for solution spectra.[14-18] This agreement is well within the experimental uncertainties, and one can conclude that the structure of the HbACO, especially in the bound CO region, is the *same in the solution and crystalline states of this sample*. This conclusion is in agreement with infrared results that give the same CO stretching frequency for crystalline and solution samples of HbAcO.[6]

Reproducibility of the solid-state ^{13}C NMR results described above is not always achieved. Part d of Figure 1 shows the spectrum of a crystalline HbACO sample with which the same care had not been exercised in transfers and in obtaining the ^{13}C CP/MAS spectrum (e.g., air driving the spinner instead of N_2, additional depletion of buffer). The visible spectrum taken from the ^{13}C CP/MAS experiment indicated that about 25% of the bound CO had been exchanged by O_2. For this sample, the resonance position of bound ^{13}CO is found to be at 212.3 ppm, approximately a 6-ppm shift from the values found for the other samples. There were other, smaller shifts in the ^{13}C resonance positions for this partially altered sample. The detailed reason for the significant difference between the CO resonance positions in the partially altered sample in comparison to the unaltered HbACO in the solution and crystalline states is not clear, but certainly reflects a significant difference in the local structural environment of the bound CO remaining in the partially altered crystals, which have experienced a significant depletion of buffer as well as partial O_2/CO exchange.

The magic-angle spinning ^{13}C NMR spectrum of a protein has recently been reported.[19] However, to our knowledge, this technique has not been applied to a crystalline protein or one in which the structure has been determined by X-ray diffraction. We believe that the technique holds great promise for comparing the crystalline and solution states, and such work is under way in these laboratories in the hemoglobin system and other systems.

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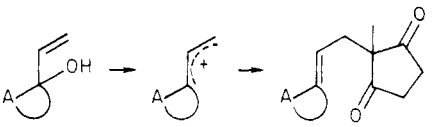
Silicon in Synthesis: An Exceptionally Short Synthesis of *dl*-11α-Hydroxyestrone Methyl Ether

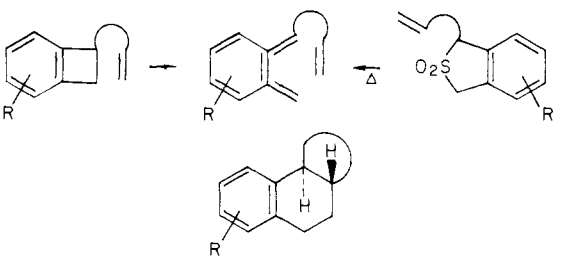
Sir:

The isolation of estrone[1] in 1929 paved the way for many ingenious total syntheses, each in its own way reflecting, to some extent, the state of the art of synthesis at that time.[2] The early routes culminated in the Torgov method for connecting ring D



torgov method

to the A-ring system via a vinyl carbinol and 1,3-diketone.[3] Recently, the use of *o*-xylylenes has further simplified the synthesis of estrone. The methods for generating the *o*-xylylene (or *o*-quinodimethane) intermediate are usually based upon thermolysis of benzocyclobutenes,[4] or cheletropic extrusion of sulfur dioxide from a benzo[*c*]thiophene 2,2-dioxide.[5] A particularly innovative



xylene method

way of constructing *o*-xylylenes by using a cobalt-mediated co-oligomerization of bis(trimethylsilyl)acetylene with a 1,5-hexadiyne has been used to synthesize estrone.[6]

Here we report a six-step synthesis of 11-oxygenated estrones that features in its key steps specific uses of organosilicon chemistry[7] and makes use of the Torgov and xylylene strategies mentioned above. The (*p*-methoxyphenyl)oxazoline **1**[8] was treated with *n*-butyllithium in ether at 0 °C to give **1a** which was quenched with methyl iodide, providing **1b** (96%)[9] [bp 107–110 °C (0.02 mm)]. When **1b** was further treated with *n*-butyllithium in ether at 0 °C, **1c** was formed, which on quenching with chlorotrimethylsilane gave **1d** [bp 127–129 °C (0.025 mm), 92%]. Unmasking **1d** was accomplished by treatment of **1d** in nitromethane with methyl iodide, removal of the nitromethane, and reaction with sodium borohydride in ethanol,[10] followed by workup with 90% aqueous acetic acid to give the aldehyde **2**, bp 96–103 °C (0.01 mm) (97%). The aldehyde **2** was converted into the vinyl carbinol **3** (95%) on treatment with vinylmagnesium bromide. The synthesis of **3** proceeds in three steps since isolation of **1b** is unnecessary, and the conversion of **1d** into **2** can be carried out

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