of **5**.[10,11] Use of the benzylidene protecting group is essential, as it serves as a masked form of the sensitive enol ether functionality present in **5**. Protection of **7** as its (2-methoxyethoxy)methyl (MEM) ether (**8**, 75%, mp 53.5–54.0 °C) and reaction of **8** with *N*-bromosuccinimide in carbon tetrachloride at reflux[12] affords the bromide **9** (87%, mp 57.0–57.5 °C), which undergoes smooth displacement with benzeneselenol and triethylamine to form the phenyl selenide **10** (96%, mp 44.0 °C). Oxidation/elimination of selenide **10** affords the benzoyl ester of **5** (99%, mp 44.0–44.5 °C),[13] which upon mild transesterification with potassium carbonate in methanol at 23 °C provides **5** (92%, mp 102.5 °C). The coupling partner **6** (mp 73–74 °C) is prepared by Swern oxidation of **11**,[14] available in high yield from uridine by a series of standard protection–deprotection steps.[15] As efforts to purify the labile aldehyde **6** lead to its deterioration, coupling reactions are performed directly on the crude material employing, in this case, a 2-fold excess of the aldehyde. Thus, crude **6** and alcohol **5** are transformed, as described above, to the crystalline coupling product **4** in 92% yield after purification by flash chromatography (1:1 mixture of diastereomers). For purposes of characterization, the diastereomers can be separated by careful column chromatography (mp 57–59 °C and 59.5–61 °C, respectively). Free-radical cyclization of diastereomers **4** under a variety of conditions proceeds smoothly to provide, after siloxane hydrolysis with potassium fluoride in methanol, the diols **12** and **13**. Cyclization of **4** in toluene at reflux leads predominantly to the undesired diol **13** (**12**:**13** = 1:3). At lower reaction temperatures, made possible with triethylborane initiation,[16] the selectivity toward formation of **13** increases (**12**:**13** = 1:6 at 0 °C, 74% combined yield). A variety of modified silicon linkers are similarly found to favor formation of **13** [$(CH_3O)_2Si$, 1:3; $(CH_2)_3Si$, 1:5; $(CH_3)_2SiOSi(CH_3)_2$, <5:95]. Though various C5′-epimerization schemes can be imagined, further experimentation reveals a striking solvent effect in the cyclization of **4**, leading to an inversion in selectivity. Reactions conducted in methanol, ethanol, 2-propanol, or acetonitrile preferentially form the diol **12** (**12**:**13** ~ 3–4:1).[17] As reactions in alcoholic media produce unacceptable levels of reduction product, acetonitrile has proven to be the optimum solvent. In a typical experiment (Bu_3SnH, Et_3B initiation, CH_3CN, −10 to 23 °C), **12** (mp 98.5–101 °C) is obtained in 62% yield after siloxane hydrolysis and radial chromatography. Diastereomer **13** (18%) and alcohol **5** (13%) are isolated in separate fractions. The identity of synthetic **12** is confirmed upon deprotection (ceric ammonium nitrate; 3 N HCl) and HPLC comparison with authentic tunicaminyluracil. In addition, synthetic and natural tunicaminyluracil[1d] are separately transformed to the α-peracetate derivatives, shown to be identical in all respects, including optical rotation.

In conclusion, an efficient method for carbon–carbon bond formation between an aldehyde and an allylic alcohol is described, forming the basis for a synthesis of the protected tunicaminyluracil derivative **12** or its C7′-epimer **13**. With regard to the latter application, a notable feature of this methodology is its almost certain compatibility with the problematic *N*-acetylglucosamine glycosidic linkage, thus allowing for a highly convergent synthesis of the tunicamycin antibiotics by late-stage carbon–carbon bond formation.

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Supplementary Material Available: High-resolution ^1H NMR, IR, and mass spectral data of all synthetic intermediates and synthetic and authentic tunicaminyluracil α-peracetate, ^{13}C NMR spectra of the latter, and HPLC comparisons of synthetic and authentic tunicaminyluracil and tunicaminyluracil α-peracetate (33 pages). Ordering information is given on any current masthead page.

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Helix Formation in Apocytochrome b_5: The Role of a Neutral Histidine at the N-Cap Position

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In the last few years, NMR investigations have revealed that many proteins display discrete conformers under native conditions.[1] Little is known about the occurrence of conformational equilibria when a structural component such as a prosthetic group or a cofactor is removed. In what follows, we demonstrate that the deletion of heme–protein interactions in cytochrome b_5 increases the population of a nonnative conformer in the C-terminal region of the protein. The partial destabilization allows us to study one of the factors responsible for the formation of a helix and conclude that a single main chain/side chain hydrogen bond plays a key role in attaining the holoprotein secondary structure. The hydrogen bond involves a neutral imidazole group at the helix N-terminal boundary (N-cap position)[2] and satisfies the backbone amide H-bond requirement of the third residue in the helix.

The water-soluble fragment of cytochrome b_5 is a 98-residue protein containing a single heme group[3] which confers some rigidity and stability to the protein[4] and influences its fold.[5] We use the apoprotein of rat liver cytochrome b_5[6] as a model molecule for probing precursor states of the holoprotein and the relationship between sequence and structure. We have shown by NMR spectroscopy that the structural effects of heme removal are localized; apocytochrome b_5 retains native holoprotein features in the region remote from the heme binding site (β-sheet and helices I and VI).[7,8] Here we describe unique dynamic properties involving His 80, a residue located 20 Å from the heme site at the start of helix VI.

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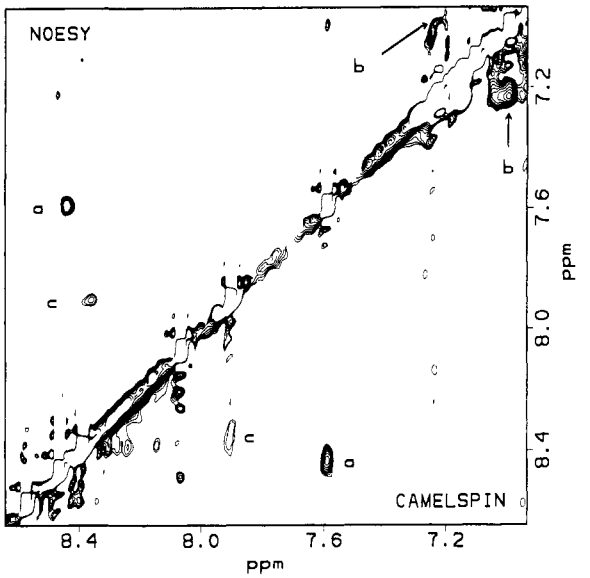

Figure 1. Histidine ring region of a NOESY spectrum[10] (upper half) and a CAMELSPIN spectrum[11] (lower half) recorded on 4 mM apocytochrome b_5 in ^2H_2O, pH 6.1 I (uncorrected for isotope effects) and 298 K. The mixing times were 75 ms for the NOESY data and 25 ms for the CAMELSPIN data. The strength of the locking field in the CAMELSPIN experiment was 2500 Hz. Marked a, b, and c are exchange cross peaks, a corresponding to His 80 $C^\epsilon H$, b to His 80 $C^\delta H$, and c to His 27 $C^\epsilon H$. All data were collected in the pure absorption mode[12] and processed with the program FTNMR[13] as reported elsewhere.[8]

In the holoprotein, the pK_a of His 80 is below 5.5, a value explained by a hydrogen bond between the N^δ of His 80 and the NH group of Asp 82.[9] The apoprotein NOESY[10] spectra contain resonances readily assigned to His 80 which display the same titration behavior.[8] Two strong cross peaks, marked a and b in Figure 1, correspond to these signals and cannot be interpreted in terms of the holoprotein structure. They also appear in TOCSY[14] spectra (not shown) and in CAMELSPIN[11] spectra (Figure 1, lower half). In the CAMELSPIN experiment, exchange cross peaks have the same sign as the diagonal peaks whereas those due to rotating-frame NOEs have the opposite sign.[11] Only the exchange cross peaks are plotted in Figure 1, which demonstrates that the ring of His 80 samples two conformations slowly on the NMR time scale, a "holo-like" major one (M) and a minor one (m) accounting for a few percent of the total protein. By repeating TOCSY or NOESY experiments at pH values ranging between 6 and 9, the $C^\epsilon H$ of His 80 in the minor form is seen to shift from 8.4 ppm to 7.7 ppm. An approximate pK_a value of 6.9 can be obtained.[15]

The holoprotein His 80 $C^\epsilon H$ is in dipolar contact with the deshielded amide proton of Asp 82,[16] as expected in the presence of the side chain/main chain hydrogen bond. Figure 2 shows that His 80 (M) of the apoprotein exhibits the same effect.[8] The amide NH of Asp 82 (M) is close to the amide NH of Asp 83, itself in contact with that of Arg 84. NH_i to NH_{i+1} NOEs are also observed from Lys 86 to Ala 88.[15] These sequential peptide NH connectivities exist in the holoprotein[15] and stem from the short

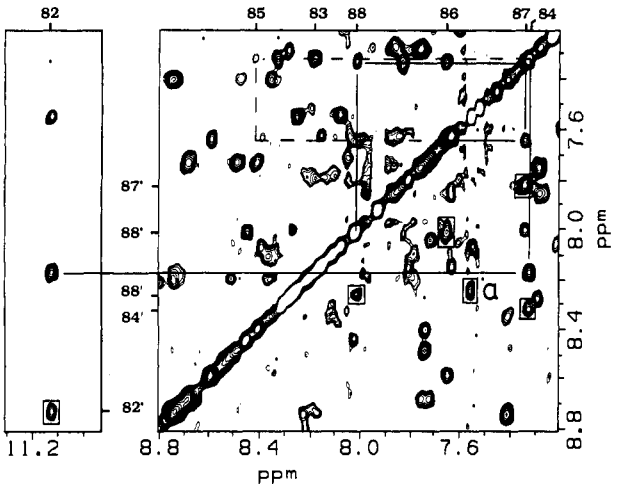

Figure 2. Peptide NH region of a NOESY spectrum recorded on a 4 mM sample of apocytochrome b_5 in 90% ^1H_2O/10% ^2H_2O at 298 K, pH 6.5. The mixing time was 75 ms. The left-hand panel illustrates connectivities to the shifted amide proton of Asp 82.[8,15] The right-hand panel contains the other NH–NH connectivities characteristic of helix VI.[3] The helix is traced in a solid line except for the connectivities involving residue 85 (dashed line); cross peaks 84/85 and 85/86 are detected at lower pH and lower temperature. Cross peaks that are framed arise from conformational exchange. Cross peak a designates the same peak as in Figure 1.

and irregular helix VI.[3] In addition, the apoprotein spectrum exhibits helix VI amide–amide cross peaks which are not seen in the holoprotein. They are present in TOCSY and CAMELSPIN apoprotein spectra and originate from exchange: the amide hydrogen of residues 82, 84, 86, 87, and 88 are found in two distinct conformations. The amide chemical shifts in the m form are closer to the random coil values[17] than in the M form; this supports a broken His 80–Asp 82 hydrogen bond and a fluctuating helix. We also note the exchange cross peak c in Figure 1 which arises from His 27 $C^\epsilon H$.[8] It is likely that His 27 (β-strand 3) witnesses the helix fluctuation because it is in contact with Arg 84 and its carbonyl group forms a hydrogen bond with the amide group of Leu 79 (β-strand 2).[3,8] TOCSY and CAMELSPIN data collected on the reduced form of the holoprotein show none of the exchange cross peaks discussed above.

The NMR data can be accounted for by the following model:[18]

$$\begin{array}{ccc} & \text{fast, } K_a^M & \\ M\text{-}H^+ & \rightleftharpoons & M + H^+ \\ \text{slow} \;\Updownarrow\; K_c^+ & & K_c \;\Updownarrow\; \text{slow} \\ m\text{-}H^+ & \rightleftharpoons & m + H^+ \\ & \text{fast, } K_a^m & \end{array}$$

where $M\text{-}H^+$ and $m\text{-}H^+$ denote the major and minor conformers with protonated His 80; K_a^M and K_a^m represent the acid dissociation constants; and $K_c = [M]/[m]$ and $K_c^+ = [M\text{-}H^+]/[m\text{-}H^+]$ are the equilibrium constants for the conformational change when His 80 is in the neutral and protonated forms, respectively.[19] The dynamics of the helix fluctuation are to be determined in order to assess whether a single process is responsible for all exchange manifestations. However, it is clear that the C-terminal segment

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is structurally labile. The smallest chemical shift difference between sites in exchange is 120 Hz (His 80 $C^{\delta}H$). On the basis of this information and preliminary buildup curves,[20] it appears that the lifetime in the holoprotein conformation is of the order of a second while, in the minor form(s), a few milliseconds.

Heme removal is manifested by both gross and subtle structural perturbations in cytochrome b_5. For the C-terminal region, it results only in moderately enhanced local unfolding into another conformation. Prosthetic group binding drives the equilibrium completely toward M and renders alternative forms undetectable. Interestingly, the local unfolding reveals that a side chain/main chain interaction is crucial to stabilize helix VI. The uncharged imidazole group of His 80 is an acceptor for the amide hydrogen of Asp 82, and we propose that His 80 participates in a pH-dependent N-terminal boundary as defined by Presta and Rose in their helix hypothesis.[21] The bond directs the folding at physiological pH by introducing a ca. 2 kcal mol^{-1} preference for the native helix[22] and survives in the final structure.

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Nondestructive Laser Vaporization of High Molecular Weight, Single-Stranded DNA

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The application of mass spectroscopy to DNA sequencing requires that methods be developed to transfer high-mass, single-stranded DNA into the gas phase.[1] Small proteins or oligonucleotides and stable biomolecules have been successfully desorbed and ionized by high energy particle bombardment for subsequent mass spectral analysis.[2] These methods are not amenable, however, to nondestructive desorption of very fragile molecules, such as high molecular weight, single-stranded DNA. One strategy for laser desorbing biomolecules, first reported by Hillenkamp,[3] involves irradiation of a mixture of a biomolecule and chromophore (or "matrix") so rapidly that both chromophore and biomolecule are transported into the gas phase before thermal degradation can occur. These techniques have demonstrated some success for desorbing large and relatively stable macromolecules such as proteins[4] or short single-stranded oligonucleotides.[5] Employing a slightly different methodology, Williams[6] demonstrated vaporization of duplex DNA up to 622 base pairs long from a frozen water matrix by coupling the pulsed laser energy into a copper substrate. Recently, these studies were extended to time-of-flight mass spectrometry of a similarly desorbed single-stranded 8-mer and a duplex 27-mer.[7] Ion spray techniques have also shown some promise for gasification and ionization of

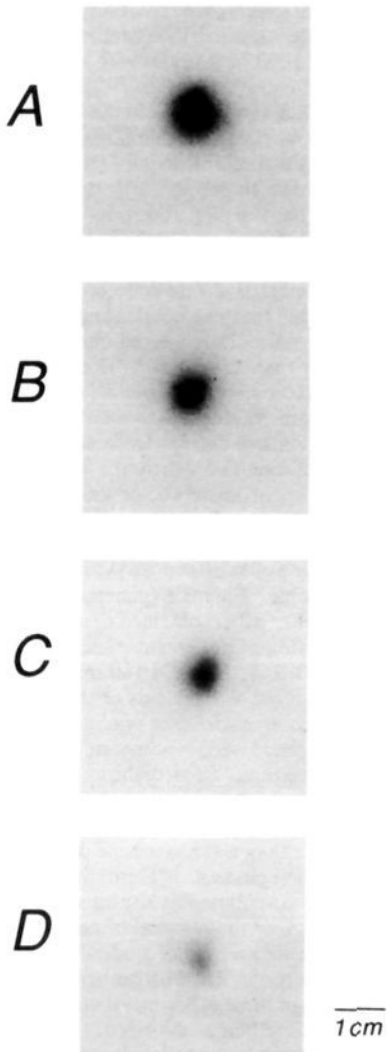

Figure 1. Autoradiograms of filters containing a vaporized dideoxy sequencing reaction. A dideoxy C sequencing reaction using [α-^{32}P]-dATP to label the DNA was carried out using Sequenase (United States Biochemical) under reaction conditions described by the manufacturer where short sequences were generated (Mn^{2+} buffer).[11] Immediately prior to vaporization, the DNA was denatured and mixed with a large excess of rhodamine 6G (approximate molar ratio of sequencing product DNA nucleotide to rhodamine 6G was 1:3000), and then 2 μL was spotted onto a glass cover slip. The dried sample was placed in a vacuum chamber, and one pulse of 532-nm photons was directed at the sample at 320 mJ/cm^2 so that the vaporized materials were entrapped on a piece of filter paper positioned 10 mm from the sample. Inspection of the sample on the glass plate indicated that approximately 25–50% of the rhodamine 6G had been vaporized by the laser pulse. The filter containing the putative vaporized DNA was removed, and the laser vaporization process was repeated on fresh samples at 260, 210, and 160 mJ/cm^2. Each filter was then exposed to Kodak XAR-5 X-ray film to obtain the distributions shown: panel A, 320 mJ/cm^2; panel B, 260 mJ/cm^2; panel C, 210 mJ/cm^2; panel D, 160 mJ/cm^2. Identical vaporization results (not shown) were obtained using a sequencing reaction performed so as to produce long product strands (Mn^{2+} buffer was not used, and the termination mix contained a 3:2 ratio of normal dideoxy C termination mix and extension mix[12]).

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