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

Louis J. Romano* and Robert J. Levis*

Department of Chemistry, Wayne State University
Detroit, Michigan 48202

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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

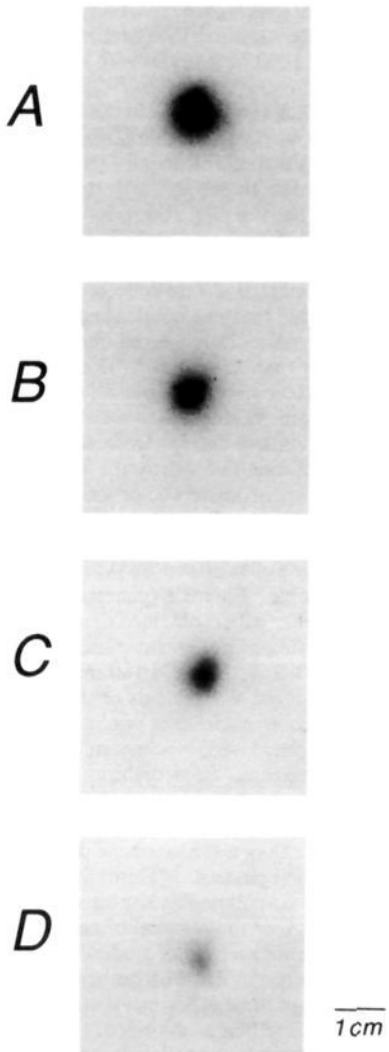

Figure 1. Autoradiograms of filters containing a vaporized dideoxy sequencing reaction. A dideoxy C sequencing reaction using [α-^{32}P]-dATP to label the DNA was carried out using Sequenase (United States Biochemical) under reaction conditions described by the manufacturer where short sequences were 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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short single-stranded DNA.[8] We report here the successful laser vaporization of single-stranded DNA molecules having strand lengths greater than 1000 nucleotides with no discernible strand degradation.

The laser vaporization strategy used in this investigation involves coupling pulsed laser energy into a thin film of rhodamine 6G dye molecules containing a single-stranded DNA sample.[9] Unlike prior studies,[5-7] we have chosen to use in this series of studies a chromophore which has a strong absorption (λ_{max} = 526 nm) in a region where the DNA does not. Following exposure of the dried mixture in vacuo to 532-nm photons from a doubled Nd:YAG laser, the vaporized materials were collected and analyzed. We have previously shown[9] that nucleotides and a 17-base-long oligonucleotide can be molecularly ejected into the vapor phase at laser fluences greater than 85 mJ/cm^2. This fact suggested that nondestructive vaporization of very long DNA strands might be possible using this same method.

To test this prediction, two dideoxy DNA sequencing reactions[10] were performed under conditions where the average chain lengths produced were either approximately 65 or 400 nucleotides long.[11,12] Each of these ^{32}P-labeled samples was vaporized from a rhodamine 6G thin film at various power levels, and the vaporized materials were collected on a piece of Whatman 3MM filter paper positioned 10 mm from the sample. Figure 1 (panels A–D) shows an autoradiograph of the filter papers obtained upon exposing the dried rhodamine 6G containing sequencing reaction to laser pulses having powers of 320, 260, 210, and 160 mJ/cm^2, respectively. The vaporization observed shares many of the features obtained from similar studies using nucleotides or oligonucleotides.[9] First, as the laser power is decreased, the amount of molecular vaporization product also decreases. Second, the images of the vaporized material on the filter paper reveal a highly directional vaporization process. The distribution is peaked in the normal direction and is considerably tighter than a simple cosine distribution expected for a thermal desorption process.[13] Third, the ^{32}P present on the filter paper is evenly distributed as expected for molecular vaporization. Spallation, or the removal of macroscopic pieces of the mixture, has been shown in prior studies[6] to lead to a spotted or speckled appearance.[14] Taken together, the images of the distribution obtained in these experiments strongly suggests that individual molecules are being vaporized.

The ^{32}P-labeled materials were then eluted from the filters and run on a high-resolution polyacrylamide sequencing gel (Figure 2). It is clearly evident from this analysis that extremely large DNA molecules can be efficiently vaporized without any noticeable strand cleavage or degradation. In the case of the sequencing reaction containing products having an average length of 65 nucleotides (Figure 2A), bands up to 85 nucleotides in length were visible. Extended exposures (not shown) indicate the presence of longer strands (in the 120–140-nucleotide-long range). The banding pattern for the samples generated at each of the laser powers (Figure 2A, lanes 2–5) is as sharp as that for the starting material (Figure 2A, lane 1). This strongly suggests that no strand degradation occurred. Although we cannot rule out the possibility of trace amounts of strand scission at random positions on these long strands, it is clear that the majority of the vaporized strands were recovered intact.[15]

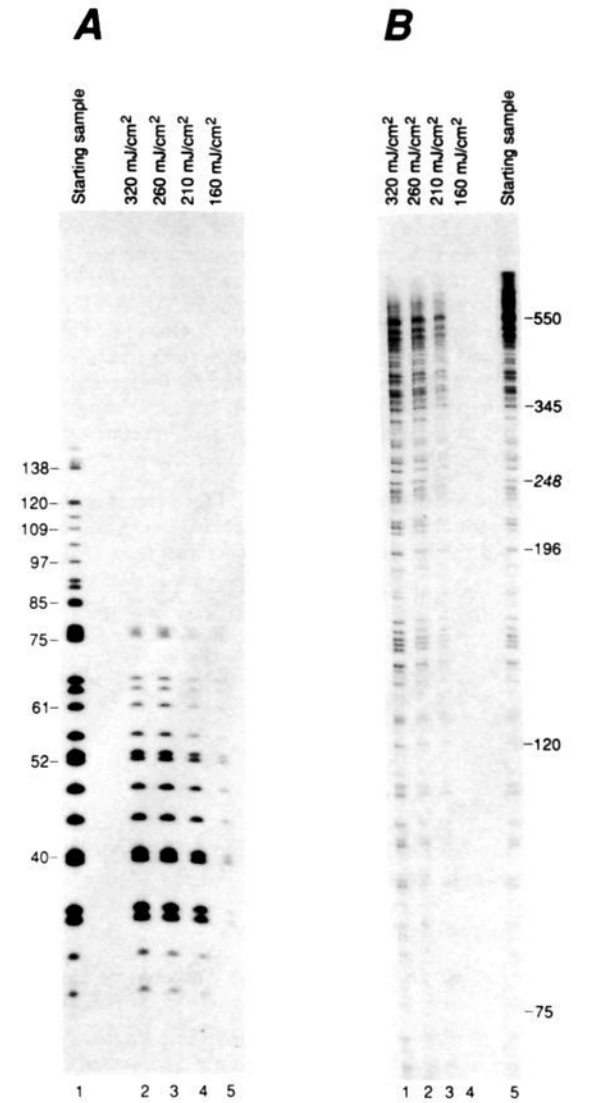

Figure 2. Polyacrylamide gel electrophoretic analysis of the vaporized DNA sequencing reactions. The vaporized radioactive materials were excised and extracted from the filter papers as described in the caption below Figure 1 and the resulting concentrated solutions loaded and electrophoresed on a denaturing 8% polyacrylamide gel: panel A, lane 1, 0.02 μL of the starting short-chain sequencing reaction prior to vaporization; panel A, lanes 2–5, samples recovered following vaporization at 320, 260, 210, and 160 mJ/cm^2, respectively. Panel B, lanes 1–4, correspond to long-chain DNA sequencing reaction samples recovered following vaporization at 320, 260, 210, and 160 mJ/cm^2, respectively; panel B, lane 5, contains 0.006 μL of this starting sequencing reaction prior to vaporization. For both panels A and B, standard G, A, T, and C sequencing reactions are run in parallel in order to precisely determine the lengths of the indicated bands.

The intensity distributions of the vaporized samples were substantially different from that of the starting sample. For example, a comparison of the ratio of the 20-mer to 75-mer for the starting sample and vaporized materials (Figure 2A) by densitometric scanning of the autoradiogram indicated that the intensities of the 75-mer bands are reduced in intensity relative to the 20-mer bands by 90% for the vaporized sample. This is

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further strong support for molecular vaporization since spallation would be expected to generate materials on the filters having band intensity distributions identical to that of the starting sample.[6] Similar results were obtained from the sequencing reaction carried out to give very long labeled DNA strands (Figure 2B). Careful analysis of this gel reveals that DNA strands in excess of 1000 nucleotides long have been vaporized.

These experiments suggest that it is now feasible to perform gas-phase analysis of long-chain nucleic acids. Moreover, nondestructive molecular vaporization of DNA is a crucial requirement for mass spectral analysis of this class of macromolecule. Coupling this vaporization with laser ionization theoretically allows mass analysis of these species with single nucleotide resolution. Such a method would have an enormous impact on DNA and RNA analysis. The potential even exists to develop this technology into a time-of-flight, mass spectral based, dideoxy DNA sequencing method that would obviate the need for a polyacrylamide gel electrophoresis step and would thus be several orders of magnitude faster than current technology.

Electrophilic Catalysis Can Explain the Unexpected Acidity of Carbon Acids in Enzyme-Catalyzed Reactions

John A. Gerlt* and John W. Kozarich

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

George L. Kenyon

Department of Pharmaceutical Chemistry
School of Pharmacy, University of California
San Francisco, California 94143

Paul G. Gassman*

Department of Chemistry, University of Minnesota
Minneapolis, Minnesota 55455

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Many enzymes catalyze the abstraction of a proton from a carbon adjacent to a carbonyl/carboxylic acid group (α-proton of a carbon acid). However, the rate of proton abstraction is much greater than that predicted from the ΔpK_a between the substrate in solution and the active-site base; turnover numbers are frequently $\approx 10^2$–10^5 s^{-1} (Table I),[1-28] implying a ΔpK_a of ≤ 2–5.[29] Entropic contributions to the observed rate accelerations could be important in increasing rates, since an active site will both approximate the base to the carbon acid and align the C–H σ-bond with the π-system of the carbon acid. However, measurements of both the effective molarities in intramolecular base-catalyzed enolization reactions[30] and the effect of conformational restriction on the pK_a values of ketones[31] indicate that these effects cannot explain the observed rates. Transiently stable intermediates are observed in many reactions, suggesting that the pK_a values of the carbon acid and the base are similar. Electrophilic catalysis is often used as a *qualitative* explanation for the ability of bases to abstract protons from weakly acidic substrates. In fact, the available X-ray structures of active sites which catalyze proton abstraction (Table I) reveal that electrophilic catalysts are *always* proximal to the carbonyl/carboxylic acid groups of the substrates. We herein demonstrate that electrophilic catalysis is *quantitatively* sufficient to explain the observed rates of the enzymatic reactions.

The pK_E (where K_E relates the concentrations of keto and enol tautomers of a carbon acid) is the difference between the pK_a values of the α-proton of the keto tautomer and the hydroxyl group of the enol tautomer.[18] We now point out that the pK_E is also the difference between the pK_a values of the α-proton and the carbonyl group bound proton of the carbonyl-protonated acid. On the basis of the available X-ray structures (Table I), we suggest that the pK_a value of the α-proton of the carbonyl group protonated acid and not the pK_a value of the substrate in solution is critical to understanding the kinetics of proton abstraction.

The pK_E of mandelic acid, 15.4,[2] relates the pK_a of the α-proton of the keto tautomer, 22.0, to the pK_a for the enol tautomer, 6,6; it also relates the pK_a value of the α-proton to that of the carbonyl group bound proton of *protonated* mandelic acid (Scheme I). Assuming that the pK_a of the carbonyl group bound proton of protonated mandelic acid is ≈ -8,[32,33] then the pK_a of the α-proton must be ≈ 7.4. This value is similar to the pK_a values of ≈ 6.4 recently assigned to the lysine and histidine bases in the active site of mandelate racemase.[5]

pK_E values are not yet available for aliphatic carboxylic acids which are substrates for α-proton abstraction. However, we assume that the pK_E values for these will be ≈ 3 units greater than that measured for mandelic acid, i.e., the enol tautomer is relatively less stable.[34] Since the pK_a values of the carbonyl group bound protons of these protonated acids are ≈ 2 units higher than that of mandelic acid,[33,35] the pK_a values of the α-protons of the

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