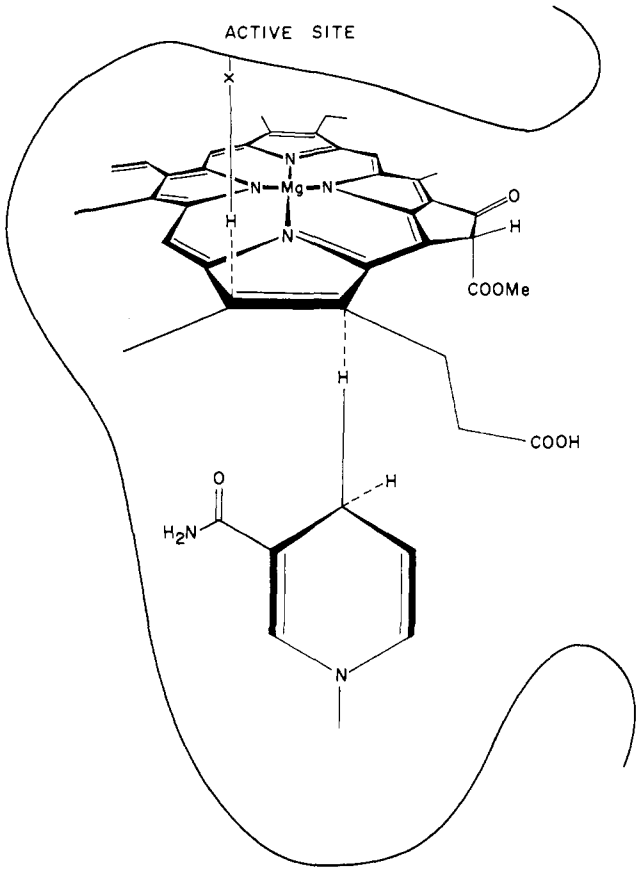

Figure 2. Model for the active site of protochlorophyllide reductase.

calculated relative to the control integration. When the enzymatic reaction was run in deuteriated assay buffer, the C18 proton resonance was almost completely depleted (Figure 1B). When the enzymatic reaction was run with [(4*S*-^{2}H)NADPH][20] as the cofactor, the C17 proton resonance was 70% depleted (relative integration 1:0.24, Figure 1C). Finally, when the reaction was run with [(4*R*-^{2}H)NADPH] as substrate, the NMR spectrum was indistinguishable from that of the Control[21] (Figure 1D). As the coupling between the C17 proton and the C18 proton is small (2 Hz[17]), deuteriation results in only minor changes in the splitting pattern. It has been previously demonstrated that the barley enzyme also catalyzes the transfer of the *pro-S* hydride from NADPH.[15] A possible rationale for the incorporation of only 70% deuterium at C17 is currently under investigation.

These studies suggest the model for the active site of protochlorophyllide reductase that is outlined in Figure 2. The fact that the enzyme catalyzes a trans reduction follows from the structure of chlorophyll. Regarding the origin of the hydrogen atoms, the NMR studies presented in this communication demonstrate that a hydride is delivered to the C17 position of protochlorophyllide from the *pro-S* face of NADPH and that the C18 position is protonated by water or an active site acid.[22]

Acknowledgment. This research was supported by grants from the donors of the Petroleum Research Fund, administered by the American Chemical Society, the Research Corporation, the Dreyfus Foundation, and the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, the US Army Research Office, and the National Science Foundation.

(20) The [(4*S*-^{2}H)NADPH] was 96% deuteriated as determined by NMR analysis.

(21) The [(4*R*-^{2}H)NADPH] was 92% deuteriated as determined by NMR analysis.

(22) The regiochemistry of the enzymatic reaction stands in contrast to the photochemical reduction of chlorophyllide by ascorbate, which gives the porphodimethene. Scheer, H.; Katz, J. J. *Proc. Natl. Acad. Sci.* **1974**, *71*, 1626.

2D Chemical Exchange NMR Spectroscopy by Proton-Detected Heteronuclear Correlation

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Received August 15, 1988

We have developed new heteronuclear 2D NMR experiments for characterizing slow dynamic equilibria in macromolecules. These methods allow determination of both equilibrium constants and exchange rates from integration of 2D NMR cross peaks and are therefore in this respect superior to existing homonuclear 2D exchange spectroscopy techniques which require integration of diagonal peaks for determination of equilibrium constants. Chemical exchange NMR spectroscopy provides information about equilibrium constants and rates of conformational isomerization for chemical systems which are in slow dynamic equilibrium.[1-3] To date, most chemical exchange studies have utilized one-dimensional NMR methods, which can be more sensitive than transient 2D experiments if steady-state irradiation is applied. However, 1D techniques generally have insufficient resolution for extensive dynamic studies of macromolecules. Several two-dimensional chemical exchange NMR experiments are available, including NOESY[4] (2D nuclear Overhauser effect and exchange spectroscopy), Camelspin or ROESY[5] (rotating frame NOE spectroscopy), and 2D zz-spectroscopy.[6] However, NOESY and ROESY are of limited utility in studies of macromolecules if the chemical exchange crosspeaks are close to the diagonal or overlapped with crosspeaks arising from dipolar coupling. Homonuclear zz-spectroscopy[6] provides exchange crosspeaks well-resolved from the diagonal, but these peaks are antiphase with respect to homonuclear couplings. Since the proton line widths of proteins are comparable to or larger than the homonuclear coupling constants, crosspeaks in homonuclear zz-spectroscopy suffer from conformation-dependent cancellation artifacts and cannot be used for quantitative measurements of equilibrium constants and exchange rates. The new experiments described here provide exchange crosspeaks which are not overlapped with a diagonal and, due to their in-phase multiplet structure, peak volumes which can be related directly to equilibrium constants and exchange rates. By utilizing heteronuclear correlation, these experiments have better resolution of direct peaks arising from multiple conformational states and of exchange crosspeaks due to magnetization transfer between these states than their homonuclear counterparts. When performed at natural ^{13}C or ^{15}N abundance, these experiments are exclusively sensitive to chemical exchange and exclude dipole–dipole magnetization transfer pathways.

The pulse sequence of Figure 1A provides information about chemical exchange of I_zS_z spin states[7] between different molecular

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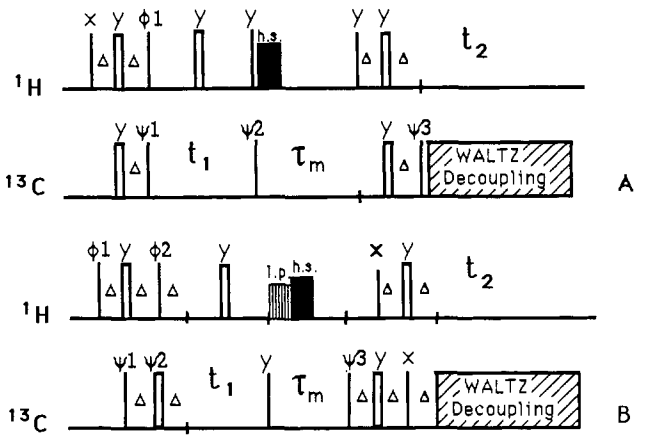

Figure 1. (A) 2D heteronuclear correlated I_zS_z chemical exchange spectroscopy. The phases are cycled: $\phi 1 = x, x, -x, -x$; $\Psi 1 = x$; $\Psi 2 = x, -x, x, -x$; $\Psi 3 = y, y, y, y, -y, -y, -y, -y$; receiver = $x, -x, x, -x$. In addition, the 180° pulses were cycled together along $\pm y$, resulting in a 16-step phase cycle. Quadrature detection in the ω_1 dimension was obtained by time proportional phase incrementation (TPPI) of $\Psi 1$ by 90°. The delays Δ are tuned to $1/(4J_{CH})$. Residual transverse magnetizations present during τ_m were reduced by a homogeneity-spoil (h.s.) pulse of ca. 1 ms. (B) 2D heteronuclear correlated S_z chemical exchange spectroscopy. The phases are cycled: $\phi 1 = x, x, -x, -x$; $\phi 2 = y, y, y, y, -y, -y, -y, -y$; $\Psi 1 = x$; $\Psi 2 = y$; $\Psi 3 = y, -y, y, -y$; receiver = $x, -x, -x, x, -x, x, x, -x$; and the 180° pulses are cycled together along $\pm y$ resulting in a 16-step cycle. TPPI of $\Psi 1$ and $\Psi 2$ by 90° provided quadrature detection in ω_1. The delays Δ are tuned to $1/(2J_{CH})$. Residual transverse and longitudinal magnetizations present during τ_m were reduced by ca. 1 ms homogeneity-spoil (hs) and long proton (lp) pulses, respectively. In both of these experiments, the hs and lp pulse lengths are adjusted to give optimum phasing of the spectrum corresponding to the first t_1 value. The DEPT transfer of sequence B could be replaced by a refocussed INEPT.

conformations. The resulting spectra exhibit direct peaks for each directly bound ^1H–^{13}C pair as in standard hetero COSY experiments. In addition, for interconverting conformations in slow dynamic equilibrium, the spectra also provide exchange crosspeaks arising from I_zS_z spin-order transfer during τ_m. The volumes of the direct peaks provide the relative populations of the multiple conformers and the volumes of the exchange crosspeaks are related to the corresponding rates of conformational isomerization. In fact, in a series of experiments recorded with incremented τ_m values, the initial slopes of the buildup curves are proportional to the rates of exchange and can be analyzed by methods available for homonuclear saturation transfer experiments.[1,2] Similar I_zS_z exchange experiments may be carried out with other evolution periods. For example, shifting the 180°$_y$ proton pulse at the center of the evolution period (Figure 1A) to the carbon channel yields a carbon-refocused two-quantum evolution period. This experiment is a ^1H–^1H correlated spectrum which contains only exchange crosspeaks. Removing the 180°$_y$ proton pulse from the center of the evolution period (Figure 1A) yields a heteronuclear double quantum experiment (with $\omega_1 = \omega\,(^1H) + \omega\,(^{13}C)$) which contains direct crosspeaks and exchange crosspeaks. An antiphase carbon chemical shift evolution period (with $\omega_1 = \omega\,(^{13}C)$) is obtained by changing the phase, $\phi 1$ (Figure 1A) to y.

The I_zS_z exchange experiments are suited for situations where both the I spin and a coupled S spin undergo the exchange simultaneously. If only the S spin undergoes exchange (for example, a metal hopping from one binding site to another) a different technique has to be applied. For this purpose, we have designed a second 2D NMR experiment in which chemical exchange can be monitored by the net transfer of S_z magnetization. This pulse sequence (Figure 1B) is related to the "double DEPT" heteronuclear 2D NMR experiment recently described for measurements

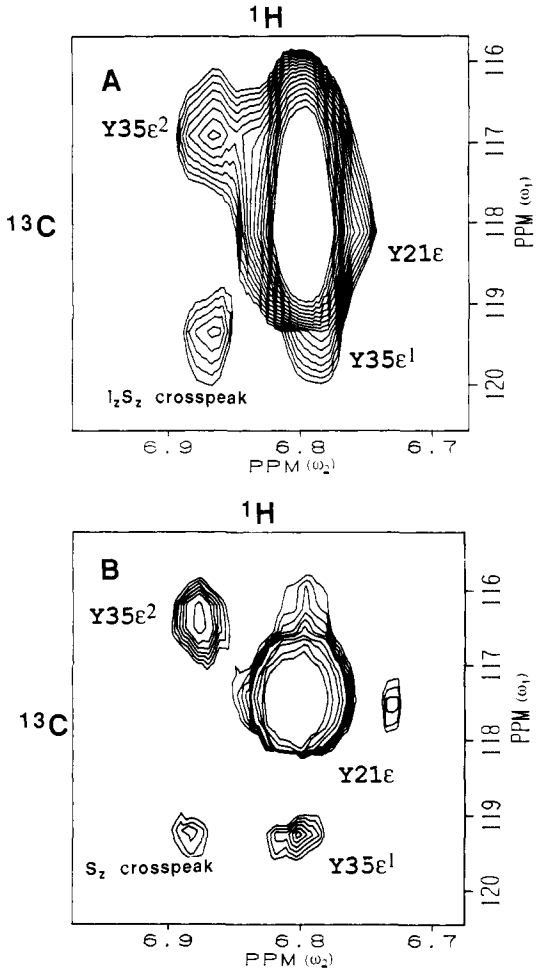

Figure 2. (A) Expansion from the aromatic region of a ^{13}C–^1H correlated I_zS_z chemical exchange spectrum of BPTI showing a "crosspeak" resulting from chemical exchange between two environments of the Tyr-35 $C^\epsilon H$'s. The I_zS_z mixing time τ_m = 100 ms. (B) Expansion of a ^{13}C–^1H correlated S_z chemical exchange spectrum[11] of the same BPTI sample recorded with mixing time τ_m = 125 ms. Both spectra shown were recorded from a 20 mM BPTI sample in ^2H_2O at 36 °C and pH 4.5 on a General Electric GN500 spectrometer at proton and carbon frequencies of 500 and 125 MHz, respectively. The 2D NMR data were collected with an ω_1 sweep width of 14 925 Hz using 200 and 240 t_1 values for the I_zS_z and S_z experiments, respectively. The spectra were processed with the software of D. Hare.

of ^{13}C T_1 relaxation times of proteins by one[8]- and two[9]-dimensional NMR techniques. The resulting spectra are similar to those of the I_zS_z chemical exchange experiment, with crosspeaks due to chemical exchange of S_z magnetization during τ_m.

Aromatic crosspeaks from a ^{13}C–^1H correlated I_zS_z chemical exchange spectrum of basic pancreatic trypsin inhibitor (BPTI) are shown in Figure 2A. The assignments given on the figure were obtained previously.[10] Under the conditions of these measurements, the reorientation rate of the Tyr-35 side chain is slow compared to the chemical shift time scale[12] and the two C^ϵ–H

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(11) The weak peak at ω_1 = 117.5 ppm, ω_2 = 6.71 ppm is tentatively attributed to strong proton–proton coupling between the $C^\delta H$ and $C^\epsilon H$ resonances of Tyr-21 at 6.71 ppm and 6.81 ppm, respectively. This results in crosspeaks between the ϵ-carbons and the δ-protons of Tyr-21 due to mixing of wave functions between the strongly coupled proton spins. Weaker artifact peaks also occur in other tyrosine spin systems with strongly coupled protons.

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direct peaks are individually resolved[10] and labeled in Figure 2A. In addition, a crosspeak arising from chemical exchange of I_zS_z two-spin order from the Tyr-35 C^cH^1 to the C^cH^2 environment is also present. The symmetric Tyr-35 crosspeak, due to exchange from C^cH^2 to the C^cH^1 environments, is partially overlapped with the Tyr-21 C^cH direct peak. The same expanded region of a $^{13}C-^1H$ correlated S_z chemical exchange spectrum recorded with the pulse sequence of Figure 1B is shown in Figure 2B. In this slightly higher resolution spectrum, both the C^cH^1 to C^cH^2 and the symmetric C^cH^2 to C^cH^1 S_z exchange crosspeaks are resolved.

These results demonstrate the utility of proton-detected heteronuclear correlated chemical exchange spectroscopy for identifying slow conformational isomerization in proteins. In these experiments, the ratio of direct $^{13}C-^1H$ correlation peak volumes provides information about the equilibrium constant for the isomerization, while exchange crosspeak volumes are related to the rates of the forward and reverse exchange processes. These methods should be applicable generally to studies of slow conformational isomerization and folding/unfolding dynamic equilibria in small proteins.

Acknowledgment. We thank S. G. Hyberts, N. R. Nirmala, J. Peng, and V. Thanabal for helpful discussions. The work was supported by the National Institute of General Medical Sciences, N.I.H. (1GM38608), the National Science Foundation (DMB86-16059), and by the Damon Runyon-Walter Winchell Cancer Fund (DRG-920).

Valence Isomerization of Hexamethyl(Dewar benzene) Radical Cation. Pulse Radiolytic Investigation

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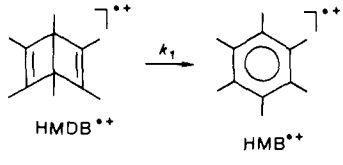
Organic radical ions are important intermediates in a wide variety of electron-transfer reactions.[1,2] Both the steady-state and time-resolved techniques have been extensively applied to probe various aspects of their chemistry. We have recently established that low-temperature pulse radiolysis can be successfully applied to the kinetic study of radical ion transformations with very low activation barriers.[3] The target of the present investigation is the hexamethyl(Dewar benzene) (HMDB)-hexamethylbenzene (HMB) system.[4] Studies by the CIDNP technique indicated the presence of two distinguishable radical cations in the system HMDB-HMB.[5] This view has not been supported by a nanosecond spectroscopic observation which failed to reveal any evidence for a radical cation other than $HMB^{\bullet+}$.[6]

The aim of this work is to present spectroscopic evidence for two different radical cations $HMDB^{\bullet+}$-$HMB^{\bullet+}$ and the activation barrier for their interconversion (reaction 1).



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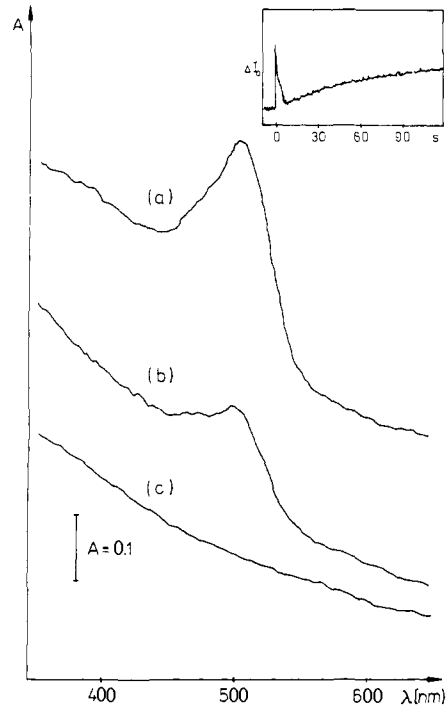

Figure 1. Absorption spectra of γ-irradiated (a) HMB (0.02 M) and (b) HMDB (0.02 M) in glassy matrices containing 3-methylpentane and 1-butyl chloride (1 M) at 77 K. The samples were 2 mm thick and received a radiation dose of 10^4 Gy. (c) Base line prior to γ-irradiation. Inset: scope trace from pulse radiolytic measurements of HMDB solution at 510 nm (T = 77 K).

The steady-state spectra of radical cations generated by γ-radiolysis of glassy solutions of HMDB and HMB at 77 K are presented in Figure 1.[7] In both cases an absorption with a maximum at 510 nm was seen, but at the same radiation dose the absorbance was higher for the HMB solution.[8] It is conceivable that the absorption at 510 nm represents the same species in both cases, i.e., $HMB^{\bullet+}$.[6] This view is compatible with a former proposition indicating a very fast interconversion process of $HMDB^{\bullet+} \rightarrow HMB^{\bullet+}$, which effectively proceeds even at 77 K.[1]

In order to verify unambiguously that the absorption at 510 nm observed for γ-irradiated HMDB solution has been correctly assigned to $HMB^{\bullet+}$ we have undertaken time-resolved studies hoping to monitor a delayed formation of $HMB^{\bullet+}$ in this case.

The transient absorption spectra of HMB at 93 K are presented in Figure 2. The spectrum determined shortly after the pulse shows a maximum at 510 nm.[9] At 2.5 s after the pulse this absorption is still present in the spectrum. The decay of this absorption presented as the oscilloscope trace is shown as inset in Figure 2. The rate constant of this decay at 93 K was found to be 0.11 s^{-1} treating the geminate recombination, responsible for the decay, as a complex first-order process (reaction 2).[10]

$$HMB^{\bullet+} \xrightarrow{k_2} HMB \qquad (2)$$

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(4) Radical cations of the solute molecules were observed in glassy solutions containing 3-methylpentane and a small addition of 1-butyl chloride acting as an electron scavenger. HMDB and HMB purchased from Aldrich were purified by standard laboratory procedures. The description of the pulse radiolysis system and other details of experimental procedure are given in ref 3.
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(7) The radical cations were generated with a ^{60}Co γ-source and measured with a Beckman Acta IV spectrophotometer 5 min after the irradiation was completed. The yield of radical cations was not linear with the radiation dose for both γ-rays and electrons, showing a saturation type dependence at high doses.
(8) Following the referee's suggestion we have photolyzed (200-W high-pressure mercury lamp, 365-nm interference filter) the sample of γ-irradiated HMDB. A few minutes of irradiation caused an increase of the absorption at 510 nm to the same level as observed for the HMB solution. Analysis of γ-irradiated HMDB solution after warm up to room temperature indicated a substantial conversion of HMDB to HMB (18% at the dose of 10^4 Gy). The conversion yield did not change when the sample was photolyzed with UV light.
(9) Taking into consideration the conversion yield of HMDB to HMB, the absorption of $HMB^{\bullet+}$ (curve a in Figure 2), and assuming a quantitative neutralization of $HMB^{\bullet+}$ to HMB we have estimated extinction coefficient for $HMB^{\bullet+}$ at 510 nm (ϵ = 2300).

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