Elucidation of the Stereochemical Course of Chemical Reactions by Magnetic Labeling

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There is a wealth of chemical and enzymatic reactions that stereoselectively affect one out of two diastereotopic groups in a molecule. Well-known examples include the hydride transfer reactions to NAD^+, $NADP^+$, or coenzyme F420.[1] In these reactions, a methylene group with two diastereotopic hydrogen atoms is created. Information about the stereochemical course of such reactions is usually derived from selective isotopic labeling of either of the diastereotopic groups involved in the reaction and analysis of the isolated reaction products.

Here we use proton exchange spectroscopy (EXSY)[2] to establish the stereoselectivity of enzymatic reactions involving diastereotopic groups (e.g., methylene protons) and to obtain valuable information about enzyme mechanisms. The stereochemistry of the reaction is unambiguously derived from the cross peaks between educt and product resonances observed in an exchange spectrum of the reaction under investigation (Figure 1). The different chemical shifts of hydrogen atoms in diastereotopic positions in t_1 and t_2 are used as "magnetic labels" to follow the fate of the protons during the mixing time τ_m (Figure 2). Isotopic labeling of either of the diastereotopic groups involved in the reaction is not needed, and isotope effects on reaction rates need not be taken into account in a quantitative analysis.

Biochemical applications of EXSY have previously concentrated on the elucidation of protein folding pathways by proton EXSY spectra[3] and on the determination of the *regiochemistry* of chemical reactions[4,5] (i.e., to address the question of which site in a molecule reacts) by heteronuclei ^{13}C and ^{31}P EXSY spectra. However, many enzyme-catalyzed reactions affect hydrogen atoms in diastereotopic sites, while non-hydrogen atoms in diastereotopic positions are not affected by the reaction. For these reactions, proton EXSY spectra are required for the elucidation of the stereochemical course. The recording of proton EXSY spectra is advantageous due to the higher sensitivity of proton NMR, which results in efficient usage of experiment time and enzyme activity.

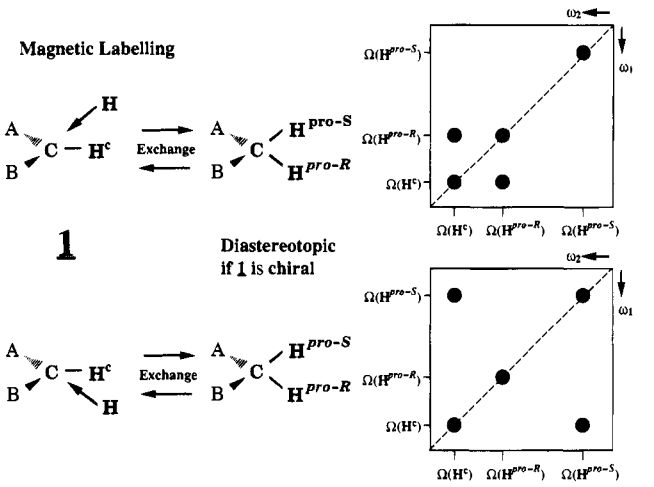

Figure 1. Elucidation of the stereochemical course of a chemical reaction involving a chiral compound **1** with magnetic labeling. Hydrogen atoms changing their molecular environment between t_1 and t_2 yield cross peaks with different chemical shifts in ω_1 and ω_2. Hydrogen atoms remaining in their molecular environment yield diagonal peaks. Depending on the stereochemical course of the reaction, either $H^{pro\text{-}S}$ or $H^{pro\text{-}R}$ originates from H^c and vice versa, resulting in the formation of a characteristic cross peak pattern in the 2D EXSY experiment.

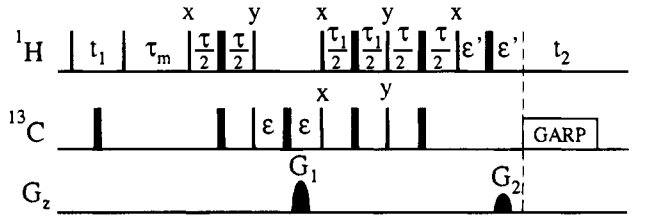

Figure 2. Pulse sequences of a ω_2 ^{13}C-filtered exchange experiment. The water resonance is suppressed directly before t_2 by a heteronuclear gradient echo[13] in conjunction with ^{13}C enrichment at C(14a). This allows only cross peaks originating from the water resonace to be observed (resulting in the asymmetry of the spectrum about the diagonal $\omega_1 = \omega_2$), but avoids saturation of the water, which would have eliminated any water cross peak.

We demonstrate the magnetic labeling approach on the reaction catalyzed by H_2-forming methylenetetrahydromethanopterin dehydrogenase (E.C. 1.12.99), a new hydrogenase found in most methanogenic archaea. In one step of methanogenesis,[6] the enzyme catalyzes the reversible reduction of N^5,N^{10}-methenyltetrahydromethanopterin ($CH{\equiv}H_4MPT^+$) with H_2 to N^5,N^{10}-methylenetetrahydromethanopterin ($CH_2{=}H_4MPT$) and H^+ (Figure 3a). In contrast to all hydrogenases known to date, no prosthetic group responsible for the activation of elemental hydrogen by this enzyme could be identified,[7] which makes the reaction mechanism of this enzyme of considerable interest. The absolute prochirality of the diastereotopic methylene protons at C(14a) ($H^{pro\text{-}R}$ = 4.96 ppm, $H^{pro\text{-}S}$ = 3.5 ppm) was determined by NMR[8] and by chiroptical methods.[9]

A proton exchange spectrum of the enzyme-catalyzed reaction is shown in Figure 3b. This spectrum was recorded in H_2O solution since the methenyl hydrogen (H^c in Figure 3) exchanges

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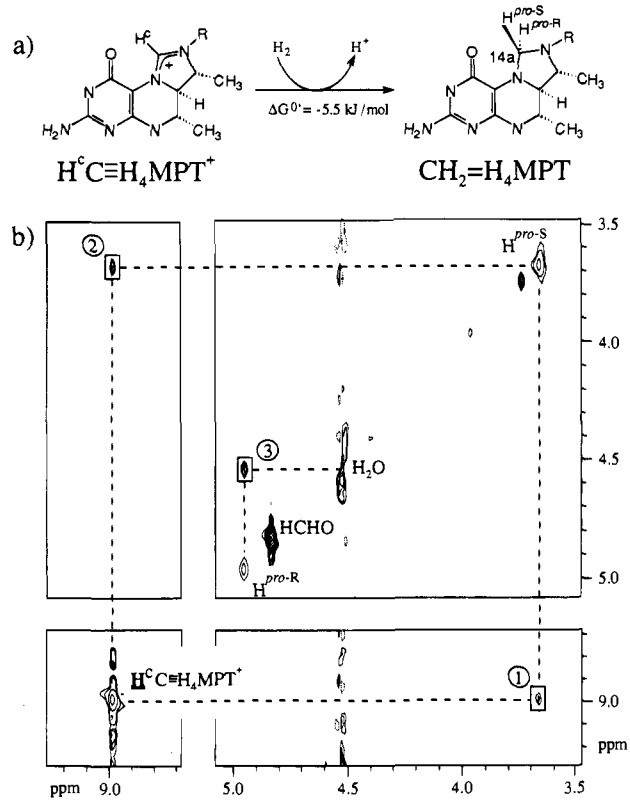

Figure 3. (a) Hydrogenation reaction catalyzed by H_2-forming methylenetetrahydromethanopterin dehydrogenase. For the structure of R and the stereospecific assignment of the hydrogen atoms of the C(14a) methylene group, see refs 14 and 8, respectively. (b) Exchange spectrum of the reaction catalyzed by H_2-forming methylenetetrahydromethanopterin dehydrogenase obtained using the pulse sequence described in Figure 2. The three numbered cross peaks indicate exchange between the methenyl hydrogen atom of $CH{\equiv}H_4MPT^+$ (H^c, cf. Figure 1) and $H^{pro\text{-}S}$ of $CH_2{=}H_4MPT$, the reverse exchange, and the exchange between water and $H^{pro\text{-}R}$ of $CH_2{=}H_4MPT$. The sample contained 2 μmol of both $^{13}CH_2{=}H_4MPT$ and $^{13}CH{\equiv}H_4MPT^+$ (isotopic enrichment 99%) and 25 units (micromoles of substrate conversion per minute) of H_2-forming methylenetetrahydromethanopterin dehydrogenase from *Methanobacterium thermoautotrophicum* in 700 μL of potassium phosphate buffer (120 mM, pH 7.0). The spectrum was recorded at 50 °C on an AMX 600 spectrometer equipped with gradient accessories; 144 t_1 increments of 16 scans each were collected in an experiment of 1 h duration, τ_m was 400 ms, and τ and τ_1 were set to $1/(4J_{CH})$ and $1/(8J_{CH})$, respectively. Gradient pulses (1 ms duration) were sine-shaped with a maximum amplitude of 0.275 T m^{-1} for G_1; G_2 was adjusted for maximal signal ($G_1/G_2 \approx \gamma_H/\gamma_C$, γ gyromagnetic ratio of the respective nucleus). GARP[15] decoupling was applied during t_2.

with H_2O and as a result a cross peak involving the water resonance was expected. Using the pulse sequence of Figure 2 in conjunction with ^{13}C labeling of C14(a) allows the observation of cross peaks involving the water resonance in ω_1 while at the same time suppressing the very strong signal of the water in ω_2. The resulting asymmetry of the spectrum[10] with respect to the diagonal ($\omega_1 = \omega_2$) does not lead to a loss of information. Although ^{13}C labeling proved very helpful in this particular study for water suppression and the observation of the water exchange, no isotopic labeling is required for the technique in general. The cross peaks correlating the signals of the methenyl hydrogen atom (H^c in Figure 3) and $H^{pro\text{-}S}$ of $CH_2{=}H_4MPT$ (marked (1) and (2) in Figure 3b) show that these protons are interconverted during the reaction. No cross peak connecting the methenyl proton of $CH{\equiv}H_4MPT^+$ and $H^{pro\text{-}R}$ of $CH_2{=}H_4MPT$ is observed, indicating that the reaction is stereospecific to more than 90% given the signal-to-noise ratio of 22:1 of the cross peak. Thus, the methenyl hydrogen atom of $CH{\equiv}H_4MPT^+$ is transformed into the $H^{pro\text{-}S}$ of $CH_2{=}H_4MPT$, while $H^{pro\text{-}R}$ originates from H_2. The corresponding cross peak cannot be observed due to the large line width of the H_2 resonance.[11] Since the reaction products need not be isolated, this information could easily be obtained in spite of the fact that the methylene protons in $CH_2{=}H_4MPT$ epimerize under the reaction conditions of the experiment ($k = 0.01$ s^{-1}).[9]

The cross peak connecting the water resonance (its diagonal peak is only visible as a noise band at $\omega_2 = 4.5$ ppm) and $H^{pro\text{-}R}$ of $CH_2{=}H_4MPT$ (marked (3) in Figure 3b) proves that the enzyme catalyzes an exchange of this hydrogen atom with water. This cross peak was quantified in a separate experiment (not shown) and was found to be 2.4 times stronger than the cross peak between the methenyl hydrogen and $H^{pro\text{-}S}$. This shows that the exchange with water occurs by direct exchange but not by a relay mechanism with the hydrogenation reaction taking place first. It is therefore concluded, in perfect agreement with other mechanistic studies,[9,12] that the enzyme catalyzes an exchange of $H^{pro\text{-}R}$ with both water (to be understood as an H^+ exchange) and H_2 (formally described as an H^- exchange). This unexpected finding gives important information about the unprecedented mechanism of this enzyme.

The technique described establishes the stereochemical course of chemical reactions involving diastereotopic groups in a single two-dimensional NMR experiment. Extraction of reaction rates is straightforward since isotopic labeling is not required and the influence of isotope effects is eliminated. The method should also be useful for the study of reaction mechanisms in organic chemistry (i.e., in the development of stereoselective reactions). For the practical application of the method, certain sensitivity requirements must be met. To achieve good sensitivity in a reasonable measurement time (ca. 1 h), a substantial fraction (on the order of 10%) of the educts must turn over in each mixing time (ca. 300 ms). At a typical concentration of 2.5 mM of each of the interconverting species (1.2 μmol in a sample volume of 500 μL), this corresponds to a minimum turnover of ca. 0.4 μmol/s, which equals 25 units (μmol/min) of enzyme activity, as used in these experiments. For this reason, the technique can currently best be employed for reactions which operate near equilibrium as in the example presented here.

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