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Measurement and ab Initio Calculation of CSA/Dipole−Dipole Cross-Correlated Relaxation Provide Insight into the Mechanism of a H_2-Forming Dehydrogenase

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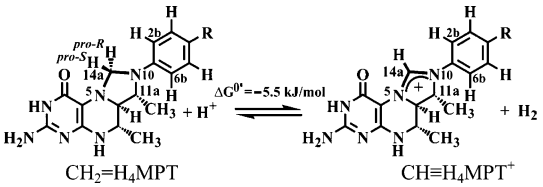
Cross-correlated relaxation (CCR) can be used to determine projection angles in molecules as has been shown in numerous examples.[1] Because CCR rates scale with the correlation time, transferred CCR can be used to determine the conformation of small molecules when bound to a macromolecule, provided the small molecule is in fast exchange with the unbound form.[2] Whereas strong drug/protein complexes usually do not fulfill this kinetic condition, efficient enzymes will always release their products fast to avoid product inhibition. Therefore, enzymes are the ideal candidates for the investigation of substrate and product conformations with transferred CCR.

Here, a new method for the measurement of CSA(^{13}C)/DD(^{13}C,^{1}H) CCR in CH_2 groups is applied to the hydrogenation of methenyltetrahydromethanopterin cation (CH≡H_4MPT$^+$) by molecular hydrogen with the enzyme H_2-forming N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase (Hmd) from *Methanothermobacter marburgensis*:[3,4]

Hmd catalyzes the reversible transfer of hydride from H_2 to the C14a-*pro-R* position of methylenetetrahydromethanopterin (CH_2═H_4MPT) (Scheme 1).[3a] The mechanism is interesting since the dehydrogenase appears to lack the redoxactive transition metals normally found in hydrogenases.[4] All available evidence indicates that the substrate, when bound to Hmd, is able to directly react with H_2.[4−6] In this communication the conformation of the enzyme-bound reduced substrate is defined by CCR measurements. This conformation can be studied at 40 °C and pH 7.5 where the reaction is blocked and only binding occurs.

Scheme 1



CH_2═H_4MPT can easily be ^{13}C-labeled in the C14a position.[3b] CCR between CSA(^{13}C) and the dipolar couplings (DD) between C14a and the two attached protons is given by eq S1 (Supporting Information),[1,7] where γ_H and γ_C are the gyromagnetic ratios of proton and carbon, respectively, r_{CH} is the *CH* bond length, θ and

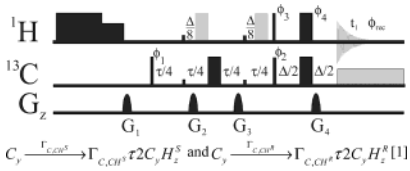


Figure 1. Pulse sequences for the measurement of CCR rates Γ_{C,CH^R} and Γ_{C,CH^S} by a cross (gray pulses omitted, $\phi_1 = y$) and a reference experiment (gray pulses applied, $\phi_1 = x$). Proton saturation is achieved with a low power MLEV16 pulse series and a $120_x/240_y$ pulse followed by a B_0 gradient. $\phi_1 = x,-x$; $\phi_2 = y,y,-y,-y$; $\phi_3 = x_4,y_4,-x_4,-y_4$; $\phi_4 = (\phi_3 + \pi)/2$; $\phi_{rec} = \phi_1 + \phi_2 + \phi_3$; x for all others pulse phases. Delays are set to: $\tau = 56.86$ ms, $\Delta = 3.2$ ms. The gradients G1, G2, G3, and G4 were set as 1:3:−5:2.

ϕ are the polar angles of the vector *CH* in the principal axis frame of the CSA tensor, and τ_c is the correlation time, σ_{ax} is the axial and σ_{rh} the rhombic part of the CSA tensor.

$\Gamma_{C,CH}$ can be measured similar to an approach described in ref 8 by a cross and a reference experiment as shown in Figure 1. Complete saturation of the protons yields pure carbon longitudinal magnetization which in the initial rate approximation during τ in the cross experiment evolves into the desired antiphase coherences involving both of the two diastereotopic protons H^{pro-R} (H^R) or H^{pro-S} (H^S) at C14a:

$$C_y \xrightarrow{\Gamma_{C,CH^S}} \Gamma_{C,CH^S}\tau 2C_yH_z^S \text{ and } C_y \xrightarrow{\Gamma_{C,CH^R}} \Gamma_{C,CH^R}\tau 2C_yH_z^R$$

Reverse INEPT transfer yields in-phase proton magnetization. The initial rate approximation is justified here since the cross-correlated relaxation rates turn out to be rather small compared to τ and the large cross-relaxation rate between the two geminal protons is efficiently quenched by the difference of the scalar couplings J(C14a, H^R) and J(C14a, H^S) of 10 Hz[3c] as explained in the Supporting Information. By contrast, in the reference experiment, cross-correlated relaxation does not evolve. Scalar coupling evolves during $\Delta/2 = (4J_{CH})^{-1}$. This gives rise to the following transfers, where the result for H^R is obtained by interchanging H^R and H^S.

$$C_y \xrightarrow{J_{CH}} -\sin(\pi J_{CH^S}\Delta/2)\cos(\pi J_{CH^R}\Delta/2)2C_xH_z^S \qquad (1)$$

Taking eqs S1 and 2 together, the cross-correlated relaxation rate for H^R and H^S, respectively, is found as:

$$\Gamma_{C,CH^S} = -I_{H^S}^{\text{cross}}/I_{H^S}^{\text{reference}} \sin(\pi J_{CH^S}\Delta/2)\cos(\pi J_{CH^R}\Delta/2)\tau \quad (2)$$

Measurements (Figure 2A−D) in the absence and presence of 25 μM Hmd yielded CCR rates for the two protons at (1mM) C14a of:

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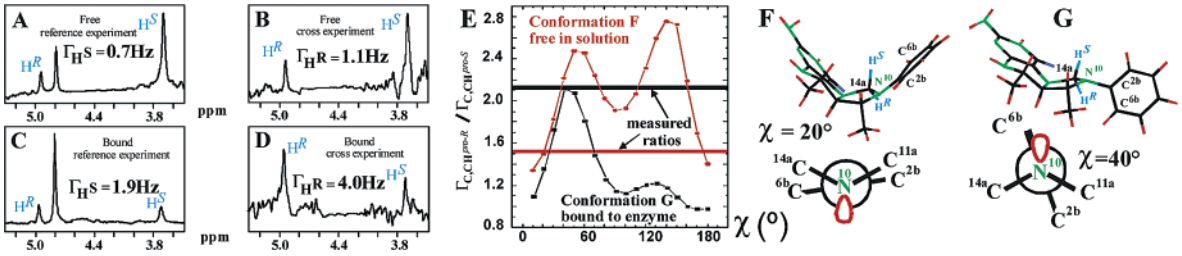

Figure 2. A (C) and B (D) show the reference and cross spectra of the two C14a proton resonances in the free (bound) form. Measurement times were 24 min for A and C and 5.4 and 2.7 h for B and D, respectively. The peak at 4.9 ppm is water. (E) Dependence of the CCR on the torsional angle χ of the phenyl ring between the $C^{11a}-N^{10}$ bond and the $C^{1b}-C^{2b}$ bond in the free (red curve, F) and bound (black curve, G) forms of $CH_2{=}H_4MPT$. Experimentally determined ratios of the CCR rates of the two C14a protons are shown as red and black bars, respectively. (F and G) The phenyl ring in the free form (F) conjugates with the free electron pair at N10, while conjugation is abolished in the bound form (G) of $CH_2{=}H_4MPT$.[7]

$$\Gamma^{free}_{C,CH^{pro\text{-}R}} = 1.1 \pm 0.1\ \text{Hz}, \quad \Gamma^{free}_{C,CH^{pro\text{-}S}} = 0.7 \pm 0.1\ \text{Hz}$$

$$\Gamma^{bound}_{C,CH^{pro\text{-}R}} = 4.0 \pm 0.2\ \text{Hz}, \quad \Gamma^{bound}_{C,CH^{pro\text{-}S}} = 1.9 \pm 0.4\ \text{Hz}$$

CCR rates can be interpreted structurally provided the CSA tensor is known. The pucker conformation of the five membered ring of $CH_2{=}H_4MPT$ was previously determined by transferred NOESY spectroscopy to be $_{14a}T^{10}$ in the free and $^{14a}T_{10}$ in the bound form. This agreed with semiempirical quantum mechanical calculations (Figure 2, F and G).[5] CSA tensors for these conformations were calculated using Gaussian 98 (S1) employing the density functional theory, including atomic orbitals (DFT/GIAO method), the B3LYP functional, and the 6-311G* basis set. Potential influence of the protein on the CSA calculation was ignored due to lack of structural information. Insertion into eq S1 yields the dependence of CCR rates on the rotation state of the phenyl ring (Figure 2E). The ratio of the rates is on average larger for the free than for the bound form, probably because, for the first, both free electron pairs of N^5 and N^{10} are on the same side of the five-membered ring, while for the latter they are on opposite sides. The marked dependence of the rate on the conformation of the phenyl ring is due to the conjugation of the phenyl-π-electron system with the free electron pair of N^{10}. For the free form of $CH_2{=}H_4MPT$ (Figure 2F), the ratio of the two rates is consistent with a distribution of states for χ ranging between $-20°$ to $20°$ via $0°$, whereas in the bound form, χ is fixed at approximately $40°$ (Figure 2G). The CCR measurement allows a more detailed analysis of the conformation of the phenyl ring than NOE's due to the small variation of H,H-distances for the two conformations about the $N^{10}-C^{1b}$ bond.

The $\chi = 40°$ conformation of the phenyl ring does not allow conjugation of the free electron pair of nitrogen N^{10} with the aromatic ring in the bound form, whereas in the free form, this energetically favorable interaction exists. This supports the proposed mechanism in which preformation of the hydride[5,6] at C14a-H^{proR} is effected by the high electron density in the free electron pair of N^{10}. There is a striking similarity between this finding and the productive and nonproductive conformations of $CH_2{=}H_4F$ bound to thymidilate synthase (Figure 3).[8] In contrast to the hydride-transfer reaction catalyzed by Hmd, thymidilate synthase catalyzes the transfer of a methyl group of $CH_2{=}H_4F$ to uracil. A key step of this reaction is the protonation of $CH_2{=}H_4F$ at N^{10}, a reaction similar to the hydride activation in $CH_2{=}H_4MPT$, that is facilitated by high electron density in the free electron pair of the N^{10} nitrogen. While in the nonproductive conformation of $CH_2{=}H_4F$ the phenyl ring conjugates with N^{10}, therefore reducing the electron density at N^{10}, the phenyl ring is perpendicular to the free electron pair in the productive conformation ($\chi = -60°$), promoting protonation of N^{10}.

In conclusion, we have introduced a sensitive experiment to measure the cross-correlated relaxation rate of $CH_2{=}H_4MPT$ when bound to the enzyme Hmd. We have also shown that the combination of DFT calculation of CSA and transferred CCR yields useful information about the conformation of an enzyme-bound substrate. Because substrate off rates are generally in the fast exchange regime required for transferred cross-correlated relaxation measurements, the approach presented may be applicable to investigating the reaction mechanism in a wide variety of enzymes.

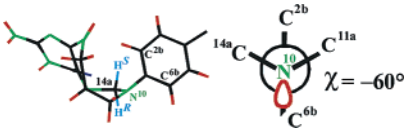

Figure 3. Productive conformation of $CH_2{=}H_4F$ bound to thymidilate synthase from *Lactobacillus casei*[8] abolishes conjugation between the phenyl ring and the free electron pair at N^{10}.

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Supporting Information Available: Reference S1(PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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