Nonequilibrium 2D-IR Exchange Spectroscopy: Ligand Migration in Proteins

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Abstract. 2D exchange spectroscopy maps networks of interconverting chemical species in dynamic equilibrium. We present the extension of ultrafast 2D-IR exchange spectroscopy to the nonequilibrium regime and its application to ligand migration in proteins.

1. Introduction

2D exchange spectroscopy (2D-EXSY) has been introduced in the field of nuclear magnetic resonance (NMR) already three decades ago [1]. Since then 2D-NMR-EXSY has grown into a powerful tool for mapping networks of chemical species that interconvert in equilibrium on a millisecond timescale. Recently, the concept of 2D-EXSY has been transferred to ultrafast vibrational spectroscopy, using 2D-IR pump probe [2] as well as 2D-IR echo techniques [3]. The sub-picosecond time resolution of 2D-IR-EXSY opens up a new range of exchange phenomena for real-time studies. While in an equilibrium 2D-IR-EXSY experiment exchange between species occurs spontaneously during the waiting time that separates the IR pulses, we present here an extension of 2D-IR-EXSY to triggered nonequilibrium systems.

2. 2D-IR exchange spectroscopy in and out of equilibrium

In pump-probe 2D-IR, a narrow-band IR_pump pulse is scanned across the absorption range of interest, defining the pump frequency axis of the 2D spectrum. After a waiting time τ, a broad-band IR_probe pulse measures the response of the sample, defining the probe frequency axis. Each vibration leads to a signal on the diagonal. If two chemical species x and y interconvert in dynamic equilibrium, and thereby change the frequency of a vibration during the waiting time τ, additional off-diagonal peaks are created by this exchange process. In this way, 2D-IR-EXSY maps connectivities by creating cross peaks between species connected by exchange. However, the 2D-EXSY concept is not limited to dynamic equilibrium, where exchange occurs spontaneously during the waiting time τ. Instead we can also trigger the exchange process by an additional UV/Vis_pump pulse applied during τ. In this fashion we can map interconversion of
species in a phototriggered nonequilibrium process, extending 2D-IR-EXSY to transient T2D-IR-EXSY.

3. Application to ligand migration in proteins

An exciting application where T2D-IR-EXSY can unfold its potential is the light triggered migration of ligands between different sites in a protein, such as the migration of the CO ligand in sperm whale myoglobin (sw-Mb). Mb is responsible for gas transport in muscle tissue. Besides CO it binds other small ligands like O₂ or NO. Associated with its function, the Mb:ligand complex features several conformational substates that coexist in solution. Different conformations show different bands in the ligand IR spectrum. CO at the binding site of sw-Mb displays two major bands that have been assigned to two conformations termed A₁ and A₃ [5, 6]. Upon photodetachment from the binding site, CO migrates to the primary docking site B, which mediates ligand transport to and from the binding site. Docked in B, the CO ligand again gives rise to several bands, B₂, B₁ and B₀ [5, 7, 8]. The issue we address here is the connectivity between A and B states, i.e. which A state dissociates into which B state. In the literature, B₂ and B₁ are attributed to reverse orientations of CO in the docking site of Mb in the A₁ conformation. B₀ has been assigned to the A₃ conformation [5]. An accompanying band due to a reverse CO orientation as for B₂ and B₁ has not yet been found for B₀. However, connections between A₁ and B₁/B₂ have been indicated [9].

To study the connectivities of A and B states by T2D-IR-EXSY, we selected the sw-Mb mutant V68Y featuring comparable populations of A₃ and A₁ [5]. As shown in Fig. 3a, 540 nm excitation detaches CO of both A substates from the heme iron. CO then enters the primary docking site B, populating B₂, B₁ and B₀ (Fig. 3b). From this 1D data no conclusions can be drawn about the connectivity between A₁/A₃ and B₂/B₁/B₀. The connectivity information can, however, be obtained from appropriate cuts through the cross-peaks of the T2D-IR-EXSY spectrum as shown in Fig. 3d. For the upper, the IRpump frequency has been set to the A₁ band, for the lower cut it has been set to A₃ (see arrows in Fig. 3c). The IRpump pulse tags the CO by vibrational excitation before it starts migration to the B sites. Thus we find in the cross peak cuts depletion at the ω₀₁ frequencies and an increased absorption at ω₁₂. The cuts for A₃ and A₁ look quite similar which would not be the case if A₃ converted exclusively to B₀ and A₁ to B₂ and B₁. Instead, the similar shape of the cuts at ω₁₂(B₂) and ω₀₁(B₁) shows that not only A₁ but also A₃ is connected to B₂. The horizontal brackets in the B-state spectrum Fig. 3e indicate the expected positions of the ω₁₂ bands. In Fig. 3f, the cuts are overlaid to highlight their differences. The negative signal around the ω₀₁ frequencies of B₁ and B₀ is blue shifted in the A₃ cut relative to the A₁ cut. This indicates that more B₀ is generated starting from the A₃ conformation. The positive ω₁₂ contributions of B₁ and B₀ partially cancel the negative ω₀₁ contribution of B₂ in both cuts. Also here, differences between the A₃ cut and the A₁ cut emerge. The amplitude of the A₁ cut is smaller around ω₀₁(B₂). This is another indication of the population of B₀ from A₃: ω₁₂(B₀) is closer to the ω₀₁
band of B2 than the $\omega_{12}$ contribution of B1, which leads to stronger cancellation in the A1 cut. The solid lines in Fig. 3f accompanying the A1 and A3 cuts are simulations assuming the connectivities $A_1 \rightarrow (B_1, B_2)$, $A_3 \rightarrow (B_0, B_2)$ and take into account the finite width of the IR$_{pump}$ pulse as well as the band overlap which limits the IR$_{pump}$ selectivity on A1 and A3. These connectivities are well supported by the data.

**Fig. 1.** (a) TRIR spectrum of sw-Mb$^{13}$CO-V68Y, 5 ps after 540 nm excitation. (b) TRIR spectrum of CO in the B states, 5 ps after 540 nm excitation. (c) Conventional 2D-IR spectrum of the A3 and A1 state. (d) Cuts through the T2D-IR-EXSY cross peaks. IR$_{pump}$ energies correspond to the arrows in (c). (e) Same as (c), rectangular brackets indicate the $\omega_{12}$ frequencies of the B2, B1 and B0 bands. (f) Overlay of the cuts in (d). Solid lines accompanying the cuts: signal expected for the connectivities $A_1 \rightarrow (B_1, B_2)$, $A_3 \rightarrow (B_0, B_2)$.

In summary, T2D-IR-EXSY allowed us to track the migration of the CO ligand from the A to the B states in Mb and to establish their connectivity. We found that B2 and B1 are populated when starting from the A1 substate. A3 is also found to be connected not only to B0 but also to B2. A possible explanation is that B2 actually consists of two bands, one being due to CO in the opposite orientation of B1 as already known, and one being the elusive counterpart of B0 featuring opposite CO orientation. This interpretation is supported by the finding of a splitting of B2 in low temperature spectra [5].

**References**