Supplementary Information

A. Figures

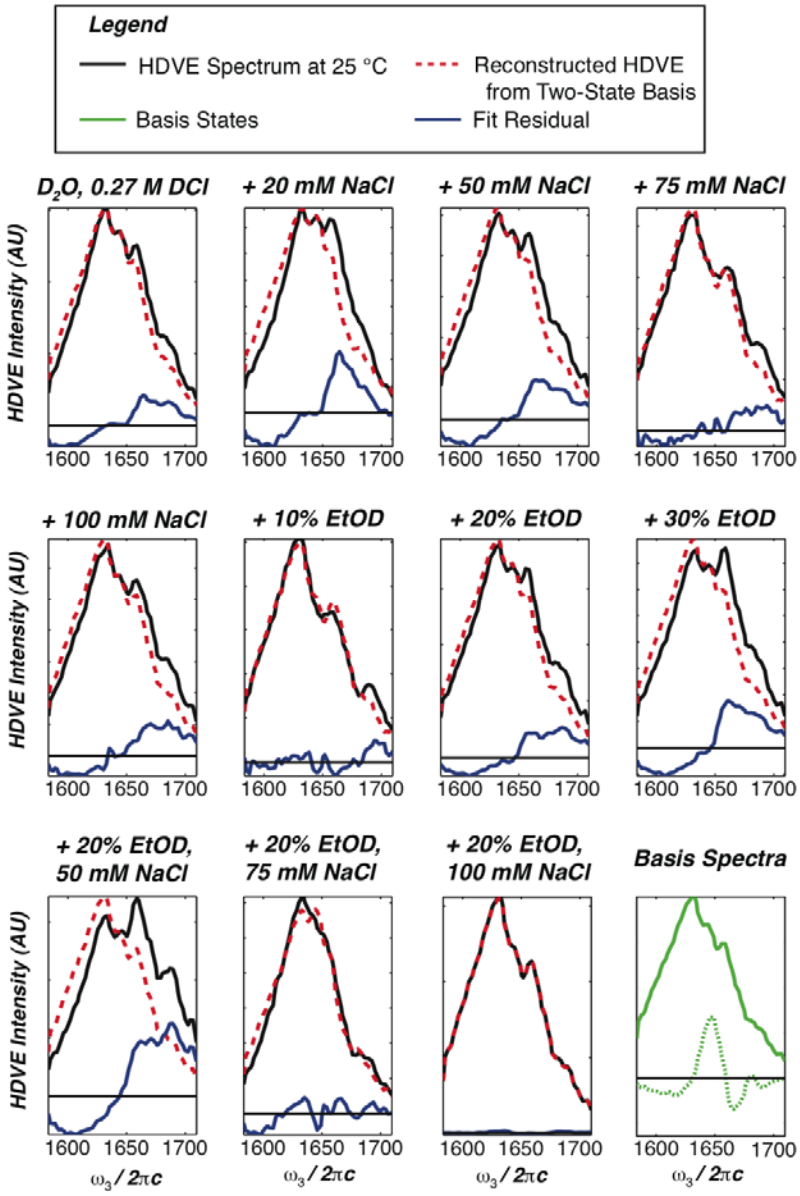

Figure 8 Solvent-dependent HDVE spectra are shown for 25 °C with reconstructed spectra using a two-state basis derived from 0.27 M DCl, D_2O:20% EtOD + 100 mM NaCl. The fit residuals are indicative of experimental irreproducibility, such as shifts in the spectrum of the laser or variations in scattering.

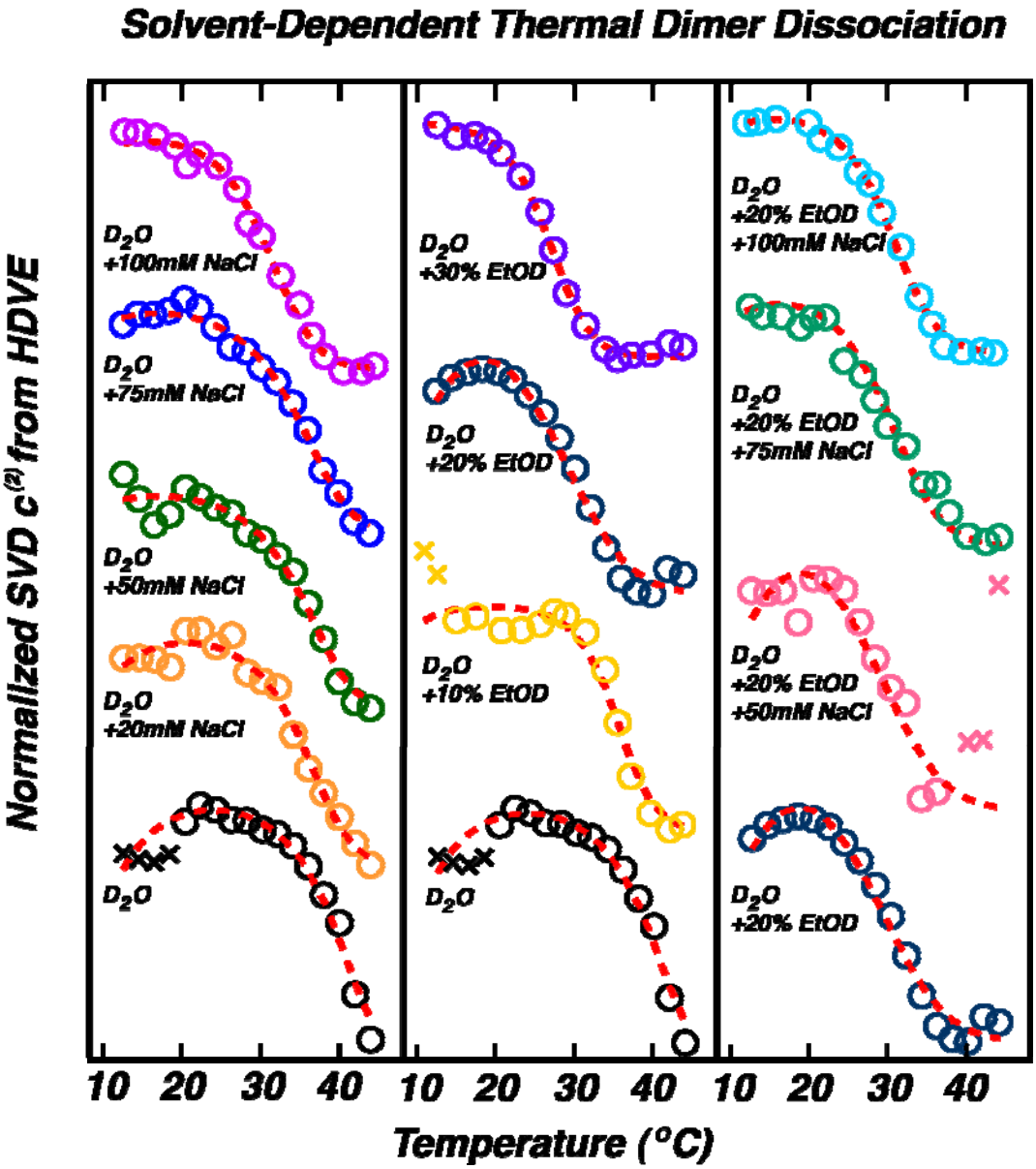

Figure 9. Melting and dissociation curves in all tested solvents along with two-state model fits, shown in Fig. 8. The total insulin concentration was 1.7 mM and all solvents included 0.27 M DCl. HDVE experiments were repeated 3-7 times in each solvent, and the sets of spectra chosen for analysis had the least aggregation. For solvents that caused persistent aggregatation, aggregate spectra (points indicated with an X) were excluded from the fit (see Fig. 10).

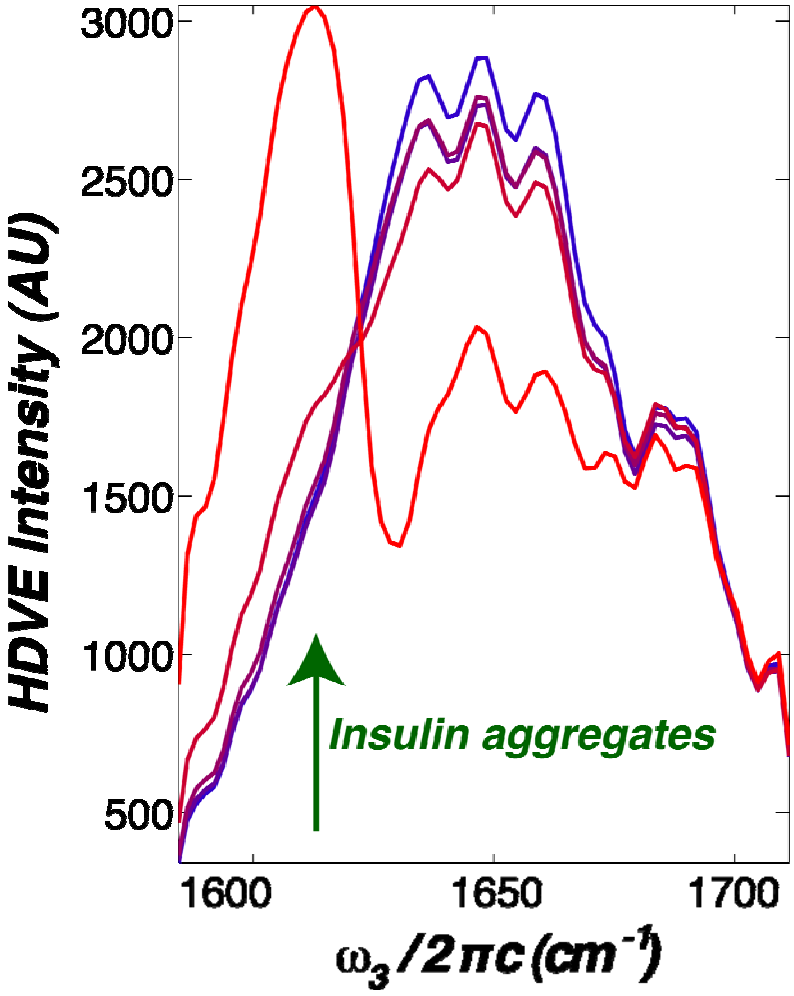

Figure 10. Temperature-dependent HDVE series showing the aggregate peak used to exclude spectra from two-state fitting. Spectra were candidates for exclusion from the fitting procedure if a high or low temperature endpoint in that series clearly displayed the aggregate peak (>20% of the signal). In these sets, the endpoint spectra and adjacent temperatures displaying the aggregate peak (>3% of the signal) were excluded. Aggregation was inhibited by salt concentrations ≥ 50 mM and ethanol fractions ≥20%.

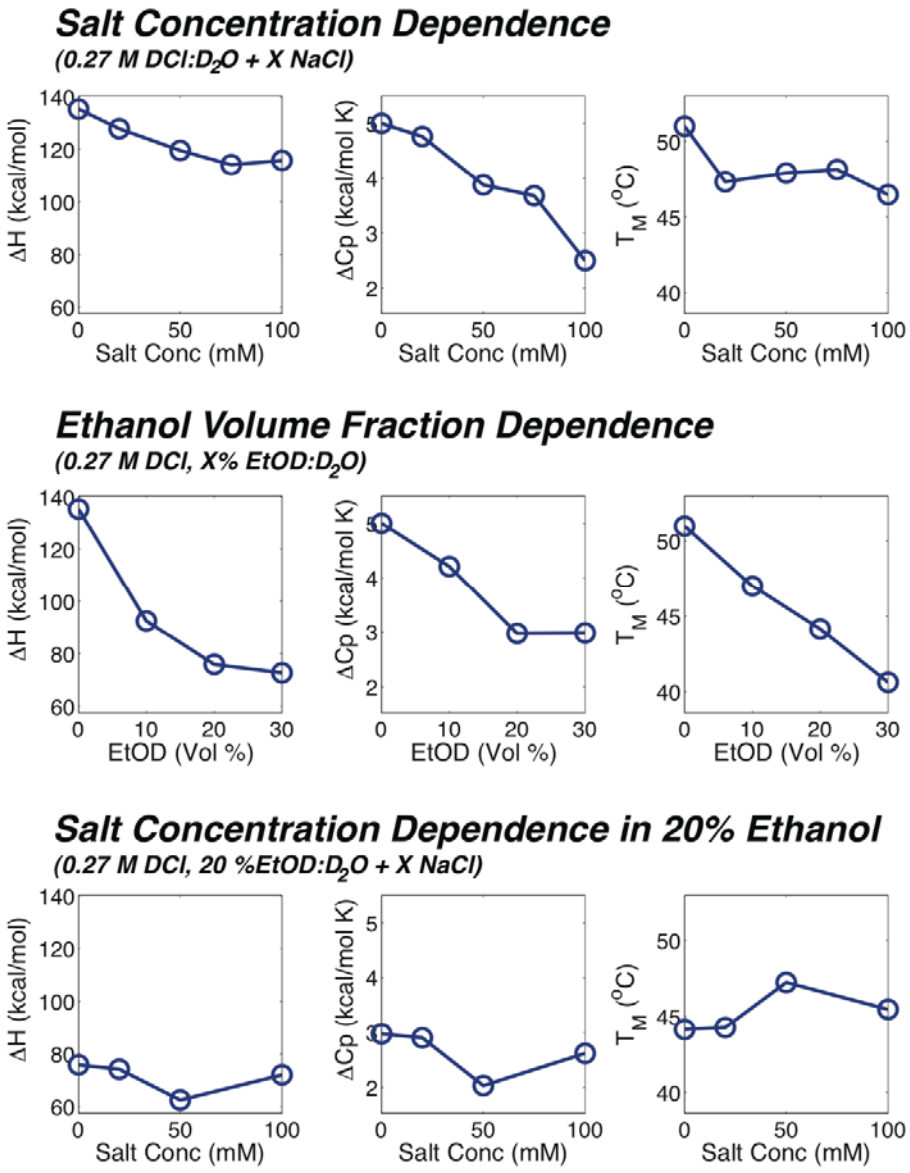

Figure 11. Extracted parameters for the two-state models appearing in Figure 9.

B. Window Treatment

At insulin concentrations of 10 mg/mL, the conditions for temperature-dependent spectra without using ethanol co-solvent were aggregation prone and necessitated treating the ordinarily hydrophobic CaF_2 glass sample cell windows with a hydrophilic coating of a polyethyleneglycol (PEG) silane. The window was pretreated by rinsing with 4M NaOH and distilled H_2O. The silane solution was prepared by diluting methoxy(ethyleneoxy)$_{9\text{-}12}$ propyltrimethoxysilane, obtained from Gelest, Inc. (Morrisvila, PA), to 2% in a 10 mM acetic acid solution in 95% ethanol and 5% water. The entire window was dipped in the silane solution for 2-3 min, rinsed with ethanol, and cured for 10 min at 110 °C in a hot plate oven. As the coating degraded (typically after 2-3 experiments), the windows were cleaned with NOCHROMIX (Cabin John, MD), and recoated using the aforementioned procedure.

C. Acquiring 2D IR Data

All experiments performed are third-order nonlinear spectroscopies that were acquired using IR pulses resonant with the amide I band at ~1660 cm^{-1}. HYPERLINK \l "Chu071" [1], HYPERLINK \l "Jon09" [2], HYPERLINK \l "Kha03" [3] The FWHM bandwidths were 90 fs in time and 165 cm^{-1} in energy as measured by TG FROG. The IR pulses were split into three replicas of equal intensity and focused in the sample in the boxcar geometry. The emitted third-order signal was spatially and temporally overlapped with a local oscillator (LO) and characterized using spectral interferometry. In consecutive shots, one excitation beam was mechanically chopped to allow differential or gain detection. Both transmitted and reflected signals overlapped with the local oscillator were collected on separate stripes (S1 and S2) of a 2x64 element MCT array equipped with a 2 cm^{-1} resolution spectrometer. For K_D determination,

the LO was passed through the sample away from the excitation beam focal spot, and a balanced gain signal was detected, $S_{Det} = \left\langle \frac{S1_o}{\langle S1_c \rangle} - \frac{S2_o}{\langle S2_c \rangle} \right\rangle$, where □ □ indicates an average over 500 – 5000 laser shots and the subscript *o* and *c* indicate an open or closed chopper. In all other spectra, the LO did not pass through the sample cell and the collected signal was either $S_{Det} = \langle (S1_o - S1_c) - (S2_o - S2_c) \rangle$ or $S_{Det} = \left\langle \frac{S1_o - S2_o}{S1_c + S2_c} \right\rangle \sqrt{\langle S1_c + S2_c \rangle}$. The signal spectral interferogram was acquired at an LO delay (τ_3) of zero as a function of the delay between the first two pulses (τ_1) for rephasing and non-rephasing experiments, which were scanned in 4 fs steps to 4 ps and 2.5 ps, respectively. The waiting time, t_2 was zero in all spectra. The τ_1 and τ_2 origins were set within ± 10 fs by SHG autocorrelation, the LO origin was set within ±10 fs by comparison to a pump-probe. The 2D IR correlation spectrum was obtained from a sum of the 2D rephasing and 2D non-rephasing contributions. Each one of these spectra is a complex quantity and both absorptive and power spectra are analyzed in this manuscript. Depending on the relative polarization between the first two excitation beams and the third/LO, the resulting spectra were either collected for parallel (ZZZZ) or pependicular (ZZYY) conditions. Heterodyne-detected dispersed vibrational echo (HDVE) spectra were acquired using the Fourier transform spectral interferometry method under conditions identical to the 2D IR spectra, except τ_1 was fixed at 0 fs and τ_3 was set to ~4 ps.

D. Block Diagonalization

The purpose of the block diagonalization procedure is to break up a large Hamiltonian into smaller Hamiltonians that can be diagonalized separately while ignoring the minimum

amount of information. The assumption is that sufficiently small couplings lead to peak splittings that are inherently unresolvable due to the short lifetime of amide I modes, ~1 ps.

The algorithm is run on each time step separately. Initially, each site, i, comprises its own block. A list is made of all the off-diagonal elements b_{ij} that coupling sites i and j, which are larger in magnitude than the cutoff. This list of off-diagonal elements is used to join blocks that contain sites i and j. The list is iteratively used to join blocks until the block definitions converge. Different cutoffs were tested; the current value of 4 cm^{-1} was used because the resulting spectra appeared identical to those from the full calculation. Physically, a 4 cm^{-1} coupling corresponds to energy transfer rate between sites on a timescale, $(1.3\ ps)^{-1}$, that is slower than the dephasing time, and is similar to the experimental resolution for distinguishing two peaks. The time-averaging approximation requires two time-averaged Hamiltonian trajectories: one that is forward-averaged and one that is backward-averaged. The trajectory is split into the two time-averaged trajectories, then separated into blocks according to the breakdown of the forward-averaged Hamiltonian trajectory. Since the couplings vary little over the 170 fs window (the dominant effect of time-averaging is on the site energies), the obtained blocks would be nearly identical for both trajectories.

E. Table

Table 1. **Reported values of K_D for bovine insulin**

Species	Year	Researchers	Technique	pH	Ionic Strength (M)	T(°C)	K_{12} (M^{-1})	K_D (= $1/K_{12}$) in μM
Bovine	1966	Jeffery, PD et al[4]	Sedimentation	2	0.05	25	7950	126
				2	0.1	25	10200	98
				2	0.15	25	3830	261
				2	0.2	25	6640	151
	1973	Lord, RS[5]	Concentration-Difference UV-Vis Absorption	2	0.1	15	109000	9
				2	0.1	25	40000	25
				2	0.1	30	18000	56
				2	0.1	35	19000	53
				2	0.1	44	6100	164
				3.5	0.1	26	32000	31
				2	0.01	26	11000	91
				3.5	0.01	26	7500	133
	1980	Pocker, Y.[6]	CD	2	0.005	25	750000	1.3
	1985	Strazza, S et. al[7]	Concentration-Difference UV-Vis Absorption	2	0.1	17	27000	37
					0.1	21	16000	63
					0.1	25	12000	83
					0.1	30	10000	100
					0.1	34	6900	144
					0.1	38	5100	196
				7	0.01	25	20000	50
	1976	Jeffrey, PD et al[8]	Sedimentation	7	0.2	25	111000	10
	1971	Goldman, J et al.[9]	Sedimentation	8	0.1	25	222000	4.5
	2000	Nettleton, EJ et al[10]	Mass Spectrometry	3.3		22	10000	100

F. Works cited

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