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Solvent Effects on Stacking. A Kinetic and Spectroscopic Study of Thionine Association in Aqueous Alcohol Solutions

T. G. Dewey, Paulette S. Wilson, and Douglas H. Turner*

Contribution from the Department of Chemistry, University of Rochester, Rochester, New York 14627. Received November 21, 1977

Abstract: Rate constants for the self-association of thionine in aqueous solutions of methanol, ethanol, 1-propanol, urea, and formamide have been determined with the Raman laser temperature jump technique. The forward rates in aqueous ethanol and 1-propanol solutions are about a factor of 10 slower than the others. This is interpreted in terms of a specific alcohol–dye interaction. Absorption and fluorescence titrations are consistent with this interpretation. The results suggest that specific solvation properties are important in understanding stacking reactions.

Molecular associations via stacking are important in many areas of chemistry, ranging from dyes to nucleic acids.[1,2] At present, there is controversy over the driving forces for these reactions, particularly the role of solvent in determining stability.[1-6] One of the problems is a lack of experimental data with which to test various theories. The use of dyes as simple models for this process has been suggested by Hammes.[7] Thionine dimerization (see Figure 1) is a particularly attractive system because it combines a large spectral change with a high equilibrium constant.[8] There is also renewed interest in thionine solution chemistry because of its potential use in photogalvanic cells.[9] This paper reports the kinetics of thionine stacking as a function of solvent, and presents evidence for specific dye–solvent interaction.

Experimental Section

Materials. Thionine, 3,7-diaminophenazothionium chloride, was obtained from Eastman, and recrystallized from water as the perchlorate salt. The crystals contained 1 mol of water per mol of thionine, and concentrations were determined using a molecular weight of 344.6. Water was doubly distilled, and absolute ethanol was used. Methanol was spectral grade from Mallinckrodt, 1-propanol "distilled in glass" from Burdick and Jackson, formamide "Baker analyzed" 99%, and urea "ultrapure" grade from Schwarz/Mann.

Spectra. NMR spectra were taken on a 100-MHz JEOL PFT 100 Fourier transform spectrometer with a JEOL EC 100 computer. The WEFT pulse sequence was used to eliminate the residual HDO resonance.[10,11]

Absorption titrations were performed on a Cary 14 spectrophotometer, using a 10-cm path length cell. Fluorescence titrations were performed on the same solutions in 1-cm path length cells using a Perkin-Elmer MPF-2A or MPF-44A fluorimeter. The fluorescence was excited at 570 nm where the absorbance of the solutions is less than 0.05, and changes by less than 10% as the solvent composition is varied. A linear correction was applied for this absorbance change.

Kinetics. The self-association of thionine occurs in less than 1 μs, thus requiring use of the Raman laser temperature-jump method described previously.[12] The probe beam was filtered with a Corning

CS3-66 filter, and monitored at 605 nm, which is near the absorption peak of the monomer. As a control to check for photochemical effects, a 4.77×10^{-3} M solution in D_2O was tested. The temperature jump in D_2O is over 100 times smaller than in H_2O, and a negligible signal was observed. Relaxation curves were photographed on 35-mm film, projected and digitized with a Tektronix 4662 plotter and 4051 terminal. The points were then analyzed with a nonlinear least-squares fit to a single exponential. Each lifetime represents the average of at least 12 shots. The estimated error in the rate constants is ±25%. All solutions contained 0.01 M KH_2PO_4. As a control, rate constants were determined in 1 mol % ethanol with no added salt. They are 0.52×10^9 M^{-1} s^{-1} and 0.8×10^6 s^{-1} for k_1 and k_{-1}, respectively. These agree with those measured in the presence of salt, within experimental error.

Results

The stacking of planar dye molecules is known to result in an upfield shift in the NMR peaks of the ring protons, owing to increased shielding.[13-15] The best evidence for stacking is to follow this shift as the concentration of the dye is increased from a point where only monomers exist to a point where mainly associated species are present. Unfortunately, the high equilibrium constant for thionine dimerization, coupled with the low sensitivity of NMR, makes this experiment impractical. Instead, the NMR spectrum of 4 mM thionine has been measured at 25 and 50 °C. The higher association constant at 25 °C predicts that the proton resonance should shift upfield at the lower temperature if the equilibrium is due to stacking. Figure 2 shows that this is the case. This evidence combined with absorption spectra measured previously confirms the thionine association as a stacking equilibrium.[8]

Kinetic experiments were performed in aqueous mixtures, since dye stacking has not been observed in nonaqueous solvents.[16] A typical relaxation is shown in Figure 3. The relaxations observed are due to the monomer-dimer equilibrium:
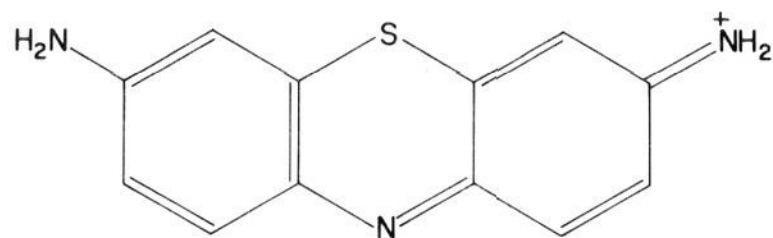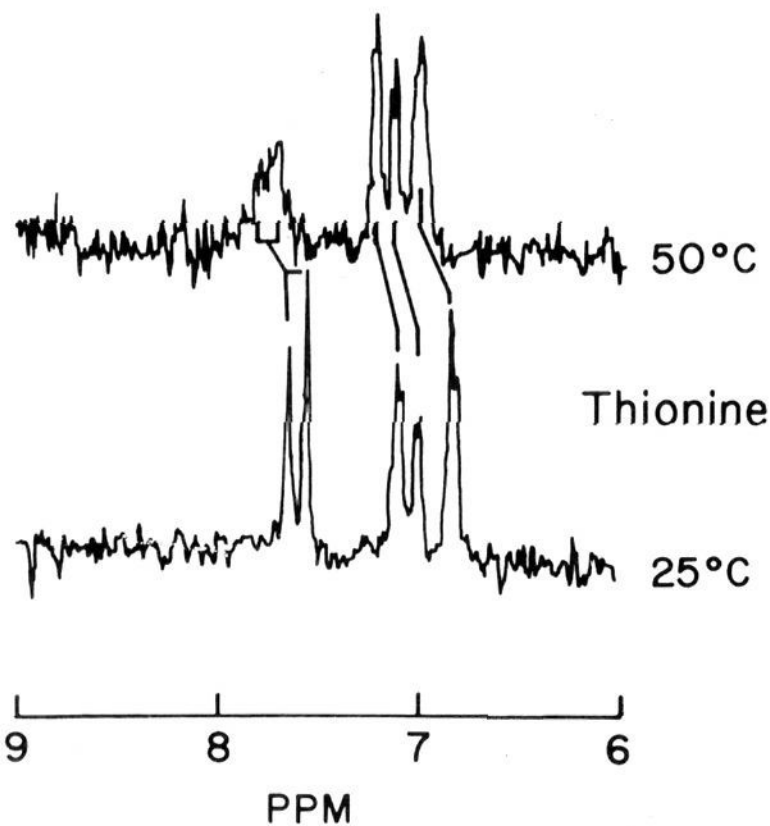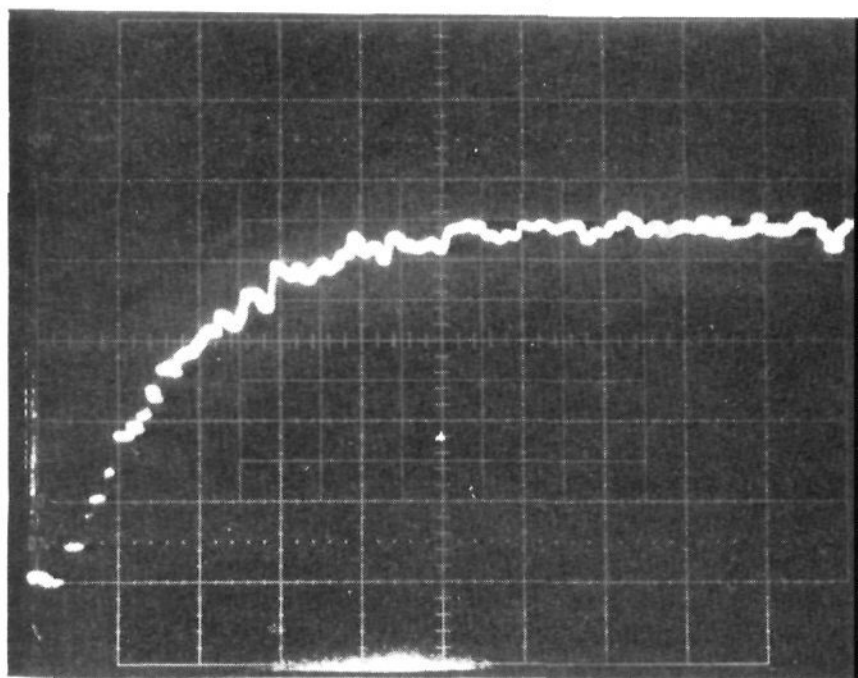
$$T + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} T_2 \tag{1}$$

Figure 1. Structure of thionine.

Figure 2. The 100-MHz ^{1}H NMR spectra of thionine in D_2O at 25 and 50 °C. Chemical shifts are measured relative to an external standard of TSP (sodium 3-trimethylsilylpropionate-*2,2,3,3,-d_4*).

Figure 3. Relaxation in 4.05 × 10^{-3} thionine, 1 mol % ethanol, 0.01 M KH_2PO_4. Wavelength is 605 nm, horizontal scale 200 ns per division, and final temperature 22 °C.

The concentration dependence of the lifetimes for such a reaction is given by

$$\tau^{-2} = k_{-1}^2 + 8k_1 k_{-1}[\text{Th}]_\text{T} \qquad (2)$$

where $[\text{Th}]_\text{T}$ is the total concentration of dissolved thionine. Plots of τ^{-2} as a function of $[\text{Th}]_\text{T}$ are shown in Figures 4 and 5. A straight line is obtained for each solvent mixture in agreement with eq 2. The rate constants derived from these plots are listed in Table I. The ratio of k_1 and k_{-1} in water gives
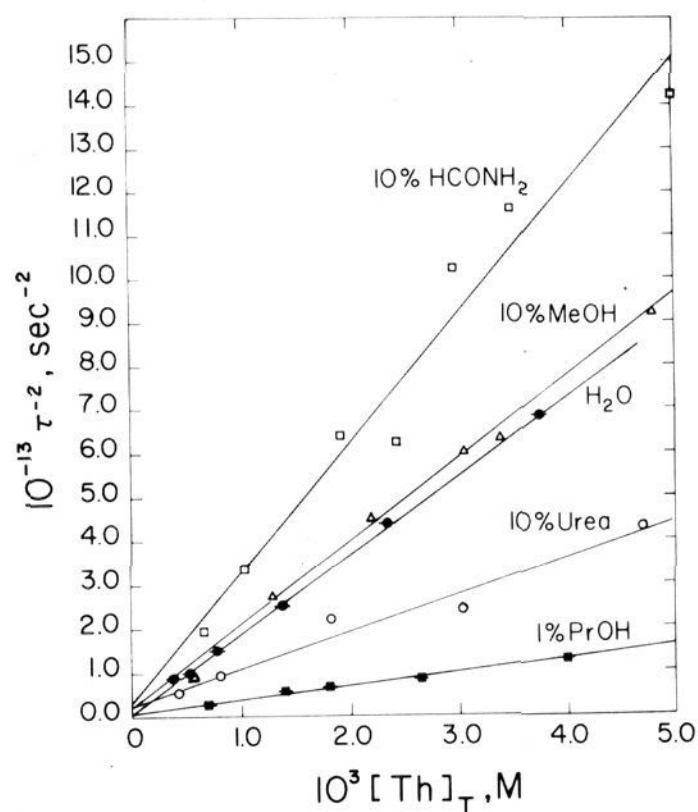
Figure 4. Plots of square of reciprocal relaxation time vs. total thionine concentration at 22 °C: ●, water; ○, 10 mol % urea; ■, 1 mol % 1-propanol; □, 10 mol % formamide; △, 10 mol % methanol. All solutions contained 0.01 M KH_2PO_4.
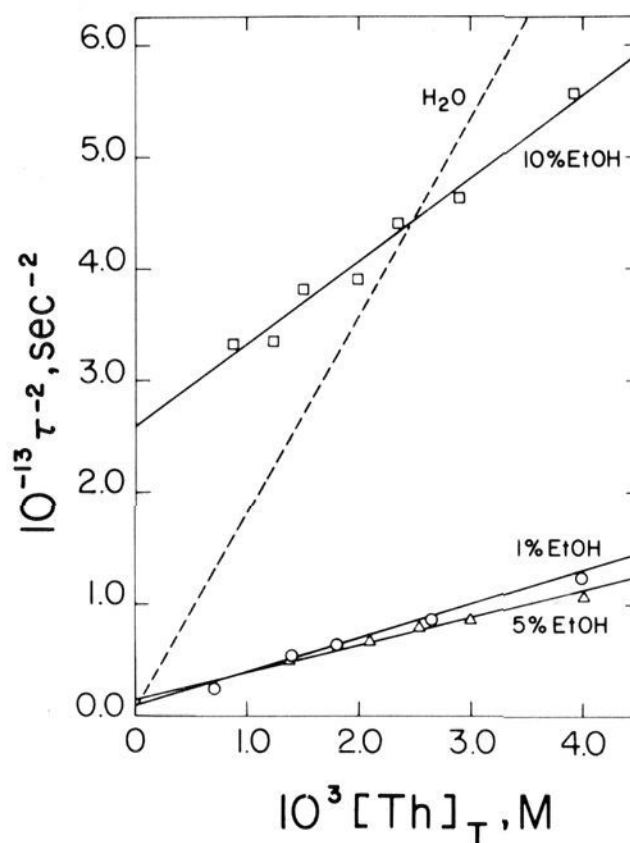
Figure 5. Plots of square of reciprocal relaxation time vs. total thionine concentration for ethanol–water mixtures at 22 °C: (- - -), 100% water; ○, 1 mol % ethanol; △, 5 mol %; □ 10 mol %. All solutions contained 0.01 M KH_2PO_4.

an equilibrium constant of 2660 as compared with the value of 1260 determined spectrophotometrically.[8] Discrepancies of this order are not uncommon for dye systems. The value of k_1 in water, 2.4 × 10^9 M^{-1} s^{-1}, is the same as the rate constant for disproportionation of the radical cation semithionine in 0.05 M aqueous sulfuric acid and 0.01–0.1 M aqueous trifluoromethylsulfonic acid at 25 °C.[9,17] This suggests that the forward rates of both reactions are similarly controlled.

It was not possible to determine accurate rate constants in 10 mol % 1-propanol. This is because the very low equilibrium constant in this mixture results in a poor signal-to-noise ratio for the relaxation. However, if the trend observed in going from
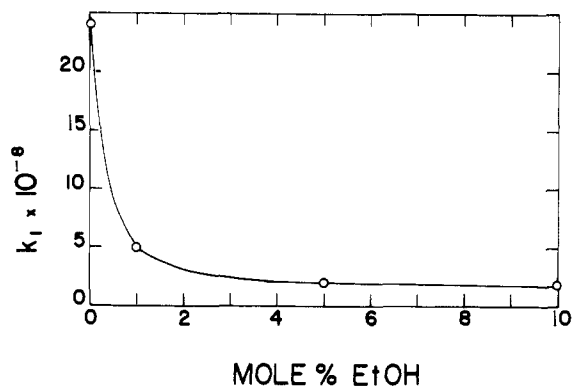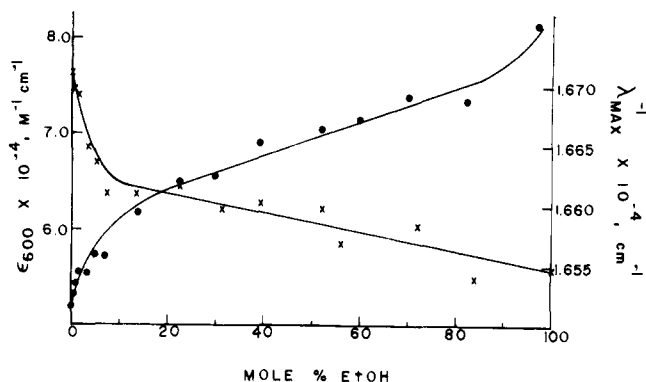
Figure 6. Plot of k_1 vs. mol % ethanol.

Figure 8. Absorption titration of thionine with propanol: O, extinction coefficient at 600 nm; □, energy of absorption peak.

Figure 7. Absorption titration of thionine with ethanol: ●, extinction coefficient at 600 nm; x, energy of absorption peak.

Figure 9. Fluorescence titration of thionine. λ_{ex} 570 nm: O, ethanol as cosolvent; x, propanol as cosolvent.

Table I. Rate Constants for Thionine Dimerization at 22 °C

Solvent	$10^{-9}k_1$, M^{-1} s^{-1}	$10^{-6}k_{-1}$, s^{-1}	K, M^{-1}
H_2O	2.4	0.9	2660
10% MeOH	1.6	1.4	1125
1% EtOH	0.49	0.8	635
5% EtOH	0.20	1.4	140
10% EtOH	0.18	5.1	35
1% PrOH	0.36	1.0	360
10% PrOH		(18)	
10% urea	0.61	1.7	360
10% $HCONH_2$	2.1	1.8	1140

water to methanol to ethanol is followed for propanol, then the relaxation time is expected to be dominated by the k_{-1}^2 term of eq 2. The following lifetimes were measured in 1-propanol (concentrations in parentheses): 49 (9.52×10^{-3} M), 54 (6.81×10^{-3} M), 48 ns (5.28×10^{-3} M). Within experimental error, these relaxations are independent of concentration, and they have therefore been averaged to give an approximate value for k_{-1} of 1.8×10^7 s^{-1}.

The most dramatic result of this work is the large decrease in k_1 upon adding ethanol or 1-propanol to water. This is demonstrated in Figure 6 where k_1 is plotted as a function of mol % ethanol. The kinetics of stacking has been studied for several dyes in water.[18-22] Typically the measured forward rates are very similar, and changes in stability constant primarily indicate changes in reverse rates.[18] In the present work, the decrease in equilibrium constant in going from water to water-ethanol (propanol) reflects mainly a large decrease in forward rate. In fact, the reverse rate is rather insensitive to added cosolvent except at high concentrations of ethanol and
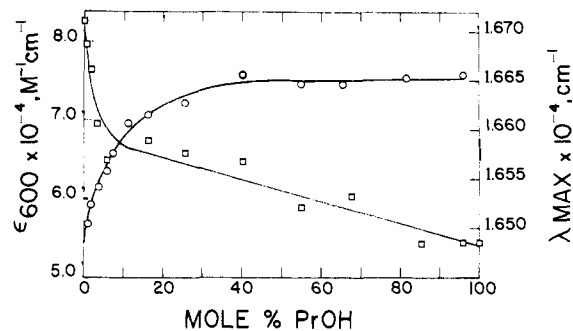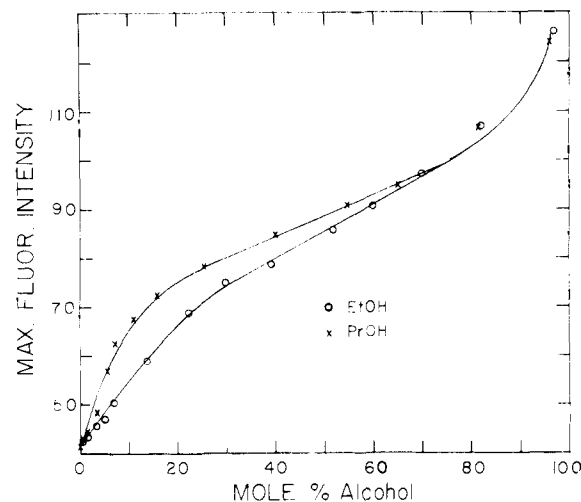
propanol (see Table I). An interpretation of solvent effects on thionine dimerization must be able to explain these effects.

The spectroscopic changes produced by placing thionine in various solvent environments were studied by measuring absorption and fluorescence spectra as a function of the cosolvent. These experiments were performed at a thionine concentration of approximately 10^{-6} M, where dimer formation is negligible (≤0.5%). Figures 7 and 8 show the extinction coefficient at 600 nm and the energy of the absorption maximum as a function of the mole fraction of ethanol and propanol at 23 °C. Titrations of the maximum fluorescence intensity are shown in Figure 9. Equivalent titrations for methanol and urea are available in the microfilm edition. In general, the shapes of the absorption and fluorescence spectra do not change with added cosolvent, but the peak positions do move several nanometers. The titrations can be divided into three regions. The initial low concentration region always exhibits some curvature which probably indicates preferential solvation. The higher concentration region is always linear as might be expected for a nonspecific solvent effect. Finally, at extremely high alcohol concentrations there is often a deviation from linearity. All of the titrations were repeated at 5 and 50 °C, and the curves were very similar.

Discussion

The effect of alcohols on stacking associations has been attributed either to changes in solvent properties, especially surface tension, or to specific solvent–solute interactions.[2-6] The macroscopic properties of surface tension, dielectric constant, and viscosity for the solvent systems used in this work are listed in Table II. There appears to be no correlation be-

Table II. Physical Constants of Aqueous Solvent Systems at 25 °C[a]

Co-solvent	Mol %	Viscosity, cP	Dielectric constant	Surface tension, dyn/cm
H_2O	100	0.89	78.5	72.0
MeOH	10	1.24	71.5	50.0
EtOH	1	1.00	77.2	63.2
EtOH	5	1.40	72.0	46.4
EtOH	10	1.90	65.5	36.6
PrOH	1	1.05	76.4	48.8
PrOH	10	2.15	59.7	26.4
Urea	10	1.15[b]	90.7	
Formamide	10	1.04	87.9[c]	

[a] Obtained by linear interpolation of tables in ref 29, except as indicated. [b] K. Kawahara and C. Tanford, *J. Biol. Chem.*, **241**, 3228 (1966). [c] P. Rohdewald and M. Möldner, *J. Phys. Chem.*, **77**, 373 (1973).

tween these bulk properties and the rate constants measured for thionine association. There is also no correlation with the Y values, which is a measure of solvent polarity.

To understand the observed kinetic effects, it is necessary to consider the general mechanism for the reaction:

$$T + T \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} T, T \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} T_2 \qquad (3)$$
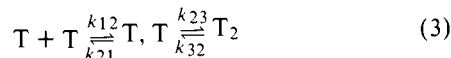
The first step is the diffusion-controlled formation of an outer sphere complex in which the reactants are separated by one or more solvent molecules. The second step is the activation-controlled collapse of the encounter group to form a stable dimer. Using the steady-state assumption for the outer sphere complex results in the following expressions for the measured rate constants:

$$k_1 = \frac{k_{12}k_{23}}{k_{21} + k_{23}} \qquad (4)$$

$$k_{-1} = \frac{k_{32}k_{21}}{k_{21} + k_{23}} \qquad (5)$$

The diffusion-controlled rate, k_{12}, can be estimated with the Smoluchowski equation:[23]

$$k_{12} = 4\pi NRD/1000 \qquad (6)$$

where R is the encounter distance, D is the diffusion coefficient of the monomer, and N is Avogadro's number. The charge of the thionine is neglected here since it is delocalized over the ring system. If the two thionines are separated by one solvent molecule in the encounter complex, then $R = 2r_{Th} + 2r_{solvent}$. Reasonable estimates for the radii of thionine and water are 4×10^{-8} and 1.4×10^{-8} cm, respectively. The diffusion coefficient of acridine orange, 5×10^{-6} cm^2 s^{-1},[24] provides an approximate value for thionine. The calculated k_{12} is thus 4.1×10^9 M^{-1} s^{-1}. This value will not change drastically for the solvent mixtures employed in this work. Theoretically, the diffusion coefficient will change inversely with viscosity. Actual determinations of diffusion rates in aqueous ethanol mixtures indicate that the variation is somewhat less than this.[25,26] The forward rate constant measured in water, 2.4×10^9 M^{-1} s^{-1}, is close to the diffusion-controlled limit. However, the rates determined in the ethanol and propanol mixtures are substantially slower.

Inspection of eq 4 indicates that if k_{12} is relatively constant, a large decrease in k_1 implies an increase in k_{21} and/or a decrease in k_{23}. The ratio, k_{12}/k_{21}, is the association constant for the outer sphere complex, K_0, and can be approximated by[27,28]

$$K_0 = 2\pi NR^3/3000 \qquad (7)$$

The value of K_0 is also relatively independent of solvent. Using a radius of 2.3×10^{-8} cm for ethanol, K_θ is calculated to be 1.6 and 2.5 M^{-1} in water and ethanol, respectively. This suggests that if ethanol is involved in the outer sphere complex, k_{21} will actually decrease rather than increase.

These calculations lead to the conclusion that the large decrease observed for k_1 in ethanol and propanol mixtures must reflect a large decrease in k_{23}.

It has been theoretically predicted that surface tension and "solvophobic" forces will induce stacking because of the change in cavity size in going from two monomers to a dimer.[3,4] The addition of alcohols lowers the surface tension of aqueous solutions. It is difficult to envisage a mechanism by which this would affect k_{12} or k_{23}. On the other hand, a decrease in the surface tension forces holding the dimer together would be expected to decrease the lifetime of the dimer, and possibly the intermediate T, T. This is essentially measured by k_{-1}, which should increase (see eq 5). Thus the effects expected if surface tension is important are opposite to those actually observed. It should also be noted that the kinetic effects observed in 10 mol % urea are similar, albeit smaller, to those observed in ethanol and propanol solutions. This is difficult to reconcile since the surface tension of water is increased by addition of urea.[29] It therefore appears that surface tension cannot explain the solvent effects on thionine stacking.

Much has been written about the effects of cosolvents on water "structure".[30-34] Three lines of evidence suggest that changing solvent structure is not responsible for the observed kinetic effects. In general, alcohols are thought to increase structure at low concentrations, whereas urea disrupts it.[31-34] However, the kinetic effects observed with alcohols and urea are similar. Furthermore, Figure 5 indicates the effect of ethanol in k_1 plateaus somewhere between 1 and 5 mol %. The maximum "structure" in ethanol-water mixtures is typically placed at about 10 mol %.[31] The magnitude of the decrease in k_1, and hence k_{23}, also argues against solvent structure. Increased structure could decrease k_{23} by preventing release of the solvent molecule trapped in the outer sphere complex. The duration of this exclusion process can be estimated by calculating k_{23}. Using the data for 5 mol % ethanol, assuming that the diffusion rate goes with viscosity, and employing eq 4, 6, and 7 yields $k_{23} = 9 \times 10^7$ s^{-1}. This would imply that the solvent structure responsible for this rate must be stable for about 10^{-8} s, which seems to be an unreasonably long time period.[31]

A more plausible explanation for the kinetic data is that thionine forms a specific solute-solvent complex with ethanol and propanol. In this interpretation, k_{23} represents the rate of solvent exchange on the thionine. The value of 9×10^7 s^{-1} calculated above indicates a significant interaction between thionine and ethanol. For comparison, the rates of water exchange on Zn^{2+} and Cd^{2+} ions are about 3×10^7 and 3×10^8 s^{-1}, respectively.[35] The forward rates measured with formamide, methanol, urea, and water suggest more rapid exchange for these molecules. This formulation can also account for the increase in reverse rate observed at high ethanol and propanol concentrations. The greater solvation of thionine by these alcohols may make alcohol attack on a dimer more effective than that of water. With the limited data available, a plot of k_{-1} in aqueous ethanol solutions vs. the ethanol concentration squared is approximately linear. This may suggest that collisions with two or more ethanols are necessary to dissociate the dimer. While the data are not sufficient to establish this kind of detail, it is clear that the trend agrees with that expected for preferential solvent interaction.

The absorption and fluorescence titrations of the thionine monomer are also consistent with the concept of preferential solvation. While no good theory exists for treating spectral properties in mixed aqueous solvents, it is clear that preferential

solvation should result in substantial initial curvature. Almost all the titrations show this behavior. (The exceptions are the fluorescence intensities of methanol and urea solutions.) The spectral changes with added ethanol, however, do not titrate the same way as k_1 (see Figures 6, 7, and 9). The forward rate constant changes much faster and reaches a plateau sooner. Evidently, the rate constant is sensitive to the most tightly bound ethanol molecule(s), whereas the spectroscopic properties are also influenced by secondary solvation.

The effect of solvent on stacking reactions is the subject of some controversy.[1-6] The present work demonstrates that by partitioning the equilibrium constant into forward and reverse rates, it is possible to gain insight into the microscopic mechanism for changes in equilibrium constant. The major conclusion of this study is that ethanol and 1-propanol decrease thionine stacking by forming relatively strong solvation complexes. In a sense, this solvation process competes with stacking. The forces responsible for this strong solvation are also of interest. Grunwald and co-workers have shown that dispersion forces are important for solute–solvent interactions when the solute exhibits a strong visible absorption and the solvent is relatively large.[36,37] This is surely the case here. It might also be expected that hydrophobic effects will play a role. Future experiments involving additional dyes and cosolvents should help determine the relative importance of these contributions.

Supplementary Material Available: Absorption and fluorescence titrations (3 pages). Ordering information is given on any current masthead page.

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