Solvent Effects on Thiamine–Enzyme Model Interactions. 2,3,4-Trimethylthiazolium Iodide, a Model for Interaction with Negative Charges

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We have studied the solvent dielectric constant dependence of the ionic association process in 2,3,4-trimethylthiazolium iodide as a model for the interaction of the thiazolium ring of thiamine with a carboxylate or other negatively charged species in enzymic processes requiring thiamine pyrophosphate as a co-factor. The K_{ass} value for the ion pair (as measured by u.v. spectroscopy) increases as the solvent dielectric constant decreases from 3.4 in pure water to 175 in pure ethanol. The ion pair is subject to electrostatic stabilization as manifested by a linear log K_{ass} *vs.* (dielectric constant)$^{-1}$ plot. This type of interaction would be stabilized in a hydrophobic environment, and may have a multiple role in enzymic processes requiring the thiamine pyrophosphate coenzyme.

OF the enzymes requiring thiamine pyrophosphate (TPP)[1] as a co-factor, yeast pyruvate decarboxylase (PDCase) has been the most widely studied.[1] Model studies by Breslow[2] and Lienhard[3,4] have afforded a generally accepted mechanism for the TPP-mediated enzymic pyruvate decarboxylation. It was shown[3-5] that in model reactions all kinetically significant steps of the proposed reaction proceeded faster in ethanol than in water. Therein originated the proposal for the likelihood of a hydrophobic active site which, by virtue of the creation of a medium of low dielectric constant, would accelerate the enzymic reaction. This proposal is particularly attractive since creation of the 'appropriate' medium would then be a significant contribution to the enormous catalytic factors enzymic reactions are capable of exhibiting.

Primarily inspired by the above proposals and on account of the importance of TPP as a co-factor, we undertook a systematic study of the solvent dependence of the complexation of TPP with model amino-acid residues. Elsewhere we have discussed the TPP–tryptophan complex.[6] Here we present our results on 2,3,4-trimethylthiazolium iodide (TMTI), a model for the complexation of the thiazolium ring with a negatively charged (*e.g.* carboxylate) enzyme functional group. The model incorporates only the thiazolium portion of TPP, and has I on account of the desirable spectroscopic properties of the I complex (see below).

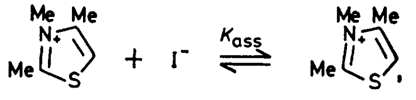
Potentially, such interaction between the positively charged thiazolium nucleus and a negatively charged ion is of three-fold value. It serves as a model for stabilizing the thiazolium ring near the active site, for attracting a carboxylate substrate (*e.g.* pyruvate) to the co-factor, and for stabilizing that co-factor conformation in which the pyrophosphate is folded back onto the thiazolium ring, as suggested by n.m.r. studies on TPP–Mn^{II} complexes.[7-9] We also present an approach to treating spectroscopic data for ionic association which leads to consistent results over a wide range of solvent dielectric constants.

EXPERIMENTAL

Synthesis of 2,3,4-*Trimethylthiazolium Iodide*.—A 2 : 1 v/v mixture of methyl iodide and 2,4-dimethylthiazole was refluxed overnight in 5 volumes of absolute ethanol (45 °C). After cooling the solid was filtered off and washed with cold ethanol. Recrystallization (three times) from ethanol afforded *crystals*, δ (D_2O; sodium 2,2-dimethyl-2-silapentane-5-sulphonate standard) 2.58 (3 H, s), 3.2 (3 H, s), 4.0 (3 H, s), and 7.65 (1 H, s) (Found: C, 28.3; H, 3.85; I, 49.7; N, 5.3. $C_6H_{10}INS$ requires C, 28.25; H, 3.95; I, 49.75; N, 5.5%). The solid was stored in an amber jar in a desiccator.

U.v. Studies of the TMTI Complex.—U.v. data were collected with a Cary 14 or Beckman Acta III spectrophotometer thermostatted *via* a circulating bath at 30 ± 0.1 °C (1 cm path 3 ml quartz cells). The ionic strength was maintained at 0.1 with added NaCl. Concentrations of TMTI were chosen so as to give a total optical density (OD) of 0.1—0.9. The samples were read against blanks containing all components except TMTI. The aqueous ethanolic solutions were made up as v/v mixtures whose dielectric constants at 30 °C were obtained by interpolation of the literature values for 25 and 35 °C.[10] TMTI solutions were made up immediately prior to use since they slowly turned yellow. For each solvent mixture on the average six TMTI concentrations were employed.

Determination of K_{ass}.—The u.v. spectra of solutions of TMTI at various concentrations (see Figure 1 for the behaviour in water; behaviour in other solvent mixtures is essentially the same) indicated non-linear concentration dependence in the long-wavelength region (above 285 nm). In accord with Kosower's interpretation of the spectra of *N*-methylpyridinium iodide solutions,[11] we assign this behaviour to the formation of an ion-pair type charge-transfer complex. Consider the simplified expression (1),



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$$K_{ass} = C_{eq}/A_{eq}B_{eq} \qquad (1)$$

where C_{eq} is the concentration of the complex and A_{eq} and

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B_{eq} are the concentration of the free ions. If the observed long wavelength absorption is due to the complex only, we obtain equation (2), where OD and ε are the observed

$$K_{ass} = \frac{OD/\varepsilon}{([TMTI]_0 - OD/\varepsilon)([TMTI]_0 - OD/\varepsilon)} \qquad (2)$$

optical density and molar extinction co-efficient of the complex, $[TMTI]_0$ is the intial concentration of TMTI, and $C_{eq} = OD/\varepsilon$. Rearrangement[12] gives equation (3). Plotting the

$$\frac{[TMTI_0]^2}{(2[TMTI_0] - C_{eq})OD} = \frac{1}{\varepsilon} + \frac{1}{\varepsilon K_{ass}} \cdot \left[\frac{1}{(2[TMTI]_0 - C_{eq})}\right] \qquad (3)$$

left hand of equation (3) against $(2[TMTI]_0 - C_{eq})^{-1}$ should give a straight line with ordinate intercept $1/\varepsilon$ and slope $1/\varepsilon K_{ass}$. C_{eq} is initially unknown but can be obtained by a series of iterations starting with $C_{eq} = 0$ and converging when further change in C_{eq} no longer changes the calculated value of K_{ass}.

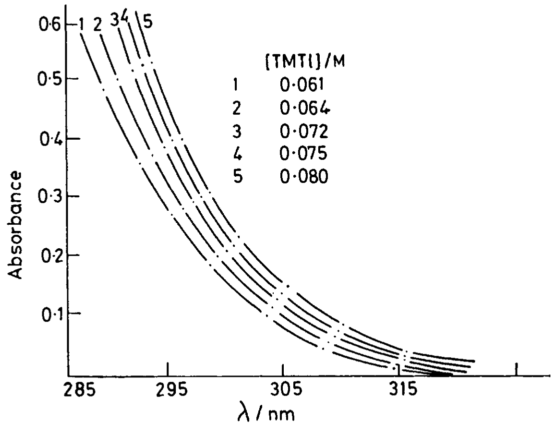

FIGURE 1 Long wavelength u.v. absorption behaviour of TMTI solutions in water at 30 °C

Since one is considering an ionic association process in media of various dielectric constant, D, the thermodynamic K_{ass} values can be calculated by using the extended Debye–Huckel equation[13] for the activity coefficient, F_i, where

$$F_i = \exp\left(\frac{2.303Z^+Z^-A\sqrt{I}}{1 + Ba\sqrt{I}}\right) \qquad (4)$$

$A = 2(1.290 \times 10^6)Z^2/(DT)^{3/2}$, $B = 35.56/(DT)^{1/2}$, and $I = 2[TMTI]_0\alpha_iZ^2$; I is the ionic strength, T is the absolute temperature, Z is the charge on cation or anion, a is the ion size parameter, and α_i is the degree of dissociation of the complex.

Combining equations, one arrives at the expression (5).

$$\frac{[TMTI]_0{}^2}{(F_i)(OD)} = \frac{(1 + \alpha_i)[TMTI]_0}{F_i} \cdot \frac{1}{\varepsilon} + \frac{1}{K\varepsilon} \qquad (5)$$

In the first iteration α is set equal to 0. An ε is calculated, then a new α is generated [equation (6)] and the process

$$\alpha_i = [TMTI]_0 - (OD/\varepsilon)/[TMTI]_0 \qquad (6)$$

is repeated (always employing a linear least-squares analysis to obtain the slope) with the convergence set at $|\varepsilon_i - \varepsilon_{i+1}|/\varepsilon_i \leqslant 0.001$.

RESULTS AND DISCUSSION

Table 1 presents a summary of data [K_{ass}, ε, and r (correlation coefficient)] for TMTI at 290 nm as a function of dielectric constant, with an ion size parameter a of 5 Å.

TABLE 1

Values of K_{ass} for TMTI as a function of dielectric constant (ion size parameter 5.0 Å; T 30 °C, 290 nm)

% Ethanol (v/v)	Dielectric constant [a]	K_{ass}/l mol^{-1}	ε [b]	r [c]
0.0	77.0	3.4	89	0.995
4.0	74.5	6.7	58	0.922
6.0	73.8	8.7	50	0.957
8.0	72.6	9.8	49	0.987
12.0	70.5	8.8	58	0.925
16.0	68.7	12.4	51	0.984
25.0	64.5	11.4	45	0.995
30.0	61.8	11.3	53	0.996
40.0	56.5	14.7	61	0.995
50.0	53.0	11.1	155	0.998
70.0	47.0	21.3	194	0.854
80.0	34.5	30.8	279	0.981
90.0	27.5	55.5	402	0.982
100	23.0	176.5	405	0.980

[a] Interpolated from values at 25 and 35 °C. [b] Molar extinction coefficient of complex at 290 nm. [c] Correlation coefficient from a linear regression analysis.

Whereas the data in low % ethanol mixtures show considerable scatter, in higher % ethanol content they are more reliable. Clearly, the association is stronger as the dielectric constant is lowered.

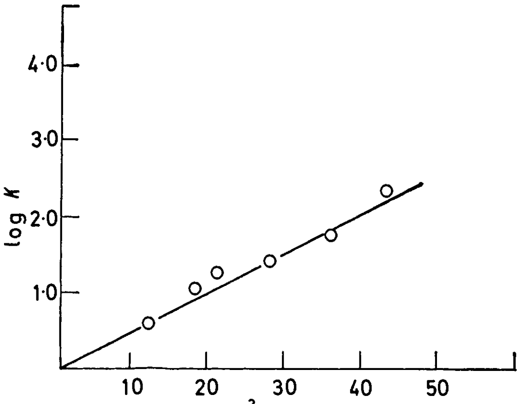

FIGURE 2 Test of electrostatic stabilization of TMTI; log K vs. (dielectric constant)$^{-1}$ plot at 30 °C

We tested the data by using the Fuoss[14] expression [(7) or (8)] for the association of a 1 : 1 electrolyte (where

$$K = \frac{4\pi Na^3}{3\,000} \exp\left(\frac{|Z_1Z_2|e^2}{akT} \cdot \frac{1}{D}\right) \qquad (7)$$

$$en\,K = en\,\frac{4\pi Na^3}{3\,000} + \frac{|Z_1Z_2|e^2}{akT} \cdot \frac{1}{D} \qquad (8)$$

N is Avogadro's number, k is the Boltzmann constant, e is the electronic charge, and other symbols are as defined before).

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A plot of log K *vs.* $1/D$ is presented in Figure 2 with the appropriate input in Table 2. These data represent final iterated values on the ion size parameter (from the slope of the log K *vs.* $1/D$ plots) which converged to *ca.* 4.87 Å. The linear behaviour in Figure 2 clearly implies the presence of a classical ion pairing process.

TABLE 2

Final K_{ass} and ε values calculated with consistent (4.68 Å) ion size parameter

% Ethanol (v/v)	K_{ass}/ l mol^{-1}	ε [a]	r [b]
0	3.6	88	0.995
50	11.1	155	0.998
70	23.3	182	0.861
80	31.7	275	0.985
90	54.8	407	0.981
100	175.8	407	0.980

[a] Molar extinction coefficient of ion-paired complex. [b] Correlation coefficient from linear regression analysis.

This report only provides results at 290 nm; however the results at other wavelengths (285—300 nm) are in substantial qualitative agreement, *i.e.* K_{ass} gets much larger as the dielectric constant is lowered.

Binding of the fluorescent dye 2-*p*-toluidinonaphthalene-6-sulphonate to PDCase suggested the presence of a hydrophobic binding site for this competitive inhibitor of TPP.[15] This hydrophobic binding site would enhance the possible interactions we are suggesting: thiazolium binding by a carboxylate on the enzyme, attraction of thiazolium for a carboxylate-containing substrate, and stabilization of that coenzyme conformation in which the pyrophosphate is folded back onto the thiazolium ring.

Finally in the related system *N*-methylpyridinium iodide, it was found that in a low dielectric medium both contact and solvent-separated ion pairs exist.[16] Presumably TMTI (and the co-factor when enzyme bound) can also exist as two types of ion pairs. In the other study [16] we have shown that the u.v. data treatment employed here gives K_{ass} values in close accord with conductance data.

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[6/2353 *Received*, 30*th December*, 1976]

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