Examination of the Solvent Perturbation Technique as a Method To Identify Enzyme Catalytic Groups†

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ABSTRACT: The present study was undertaken for the purpose of evaluating the solvent perturbation technique as a method to identify enzyme catalytic residues. For establishment of expected directions and sizes of pK_a perturbations for different types of acids in different classes of solvents, a study of the pK_a of a series of acids in mixed solvent systems was carried out. Consistent with previous findings, the presence of organic solvents (25% v/v) increased the pK_a values of neutral acids while it decreased or did not change the pK_a values of cationic acids. The size of the perturbation observed was dependent on the nature of the organic solvent and on the polarity of the neutral form of the acid. The solvent perturbation studies were then extended to the catalytic aspartate residue of yeast hexokinase. The pK_a of this residue was determined from the MgATP V/K profile measured in the presence and absence of organic solvents (25% v/v). While dimethylformamide and methanol induced small but perhaps significant increases in the observed pK_a, dimethyl sulfoxide and propylene glycol did not. The pK_a values, from the MgATP V/K profiles measured in the presence of fully saturating glucose, were not significantly increased by the organic solvents. The pK_i vs. pH profile for the competitive inhibitor lyxose was also measured in the presence and absence of organic solvents. While methanol (25% v/v), dimethylformamide (25% v/v), and dioxane (17.5% v/v) induced a large increase in the pK_a, propylene glycol and dimethyl sulfoxide (25% v/v) did not. The results from this investigation indicate that the solvent perturbation technique should not be relied upon indiscriminately.

The study of the pH dependence of an enzyme-catalyzed reaction is a popular approach to identifying substrate or activator moieties and enzyme active-site residues which play a role in substrate binding and catalysis [for reviews on this topic, see Cleland (1977, 1982) and Tipton & Dixon (1979)]. When properly applied, pH studies can provide a great deal of information concerning the mechanism of the enzyme-catalyzed reaction. In addition, they can be carried out quickly and inexpensively. The basic technique involves measuring the variation in the maximum velocity (V)[1] and V/K for the reaction of substrate and/or the K_i for the binding of an inhibitor or activator as a function of the reaction pH. The kinetic parameter that is being measured will begin to change as the reaction pH exceeds or falls below the pK_a, depending on whether the group must be protonated or deprotonated for proper function. The pH profile of the reaction is simply constructed by plotting the log of the kinetic parameter against the pH of the reaction at which the value of the kinetic parameter was measured. The number of ionizing groups is defined by the slope of the curve while the apparent pK_a[2] of the ionizing group(s) is defined by the intersection of the asymptotes to the curve. Typically, both the number of ionizing groups and their apparent pK_a values are determined by computer fitting the data to the rate equation which appropriately describes the type of ionization involved.

The enzyme catalytic groups which can be detected by pH studies include aspartate, glutamate, histidine, cysteine, lysine, arginine, and tyrosine. Precise identification of the catalytic residue is not a simple matter, however, as the pK_a of an amino acid side chain in the environment of an active site can differ dramatically from that of an amino acid in water. The range of pK_a values of active-site carboxyl residues, for example, often overlaps the range seen for histidine, making it impossible to differentiate between these two residues strictly on the basis of the measured pK_a value. In 1962, Findlay et al. proposed a method by which one could distinguish between the ionizations of neutral acid residues (carboxyl, sulfhydryl, and phenolic groups) and cationic acid residues (amine residues) on the basis of the difference in their behavior in mixed organic–aqueous solvent systems.

When proton loss generates an anionic conjugate base, as in the case of the neutral acids, ionization results in a net increase in the number of ions in the solution:

$$HA \rightleftarrows H^+ + A^-$$

Organic solvents shift the ionization equilibrium of neutral acids to the left, decreasing the number of protons in solution and increasing the pK_a of the solute (Bates & Pawlak, 1976; Bacarella et al., 1955; Danyluk et al., 1957; Douzou, 1977; Harned, 1939; Harned & Kazajian, 1936; Maurel et al., 1975; Shedlovsky, 1959; Spivey & Shedlovsky, 1967; Steel et al., 1967). When the unionized acid is a cation, loss of a proton merely alters the size of the cations in the solution without changing the number of ions to be solvated:

$$HA^+ \rightleftarrows H^+ + A^0$$

For these cationic acids, organic solvents, if they affect the pK_a at all, decrease it (Bacarella et al., 1955; Douzou, 1977;

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[1] Abbreviations: V, maximum velocity; K_m, Michaelis constant; V/K, ratio of the maximum velocity to the Michaelis constant; K_i, dissociation constant; Tris, tris(hydroxymethyl)aminomethane; THF, tetrahydrofuran; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; PG, propylene glycol; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); NADPH, nicotinamide adenine dinucleotide phosphate, reduced; NADP, nicotinamide adenine dinucleotide phosphate; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; MeOH, methanol; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EG, ethylene glycol; Me_2SO, dimethyl sulfoxide; DMF, dimethylformamide; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

[2] The pH profiles of functions which represent equilibrium binding (K_i for substrates or competitive inhibitors and K_i and K_m for metals) yield true pK_a values. In the case of V_{max} or V/K pH profiles, the apparent pK_a value may be displaced from the true pK_a value if under the conditions that the activity measurements were made the pH-dependent step is not rate limiting.

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Paabo et al., 1966; Maurel et al., 1975; Gutbezahl & Grunwald, 1953; Woodhead et al., 1965). The solvent perturbation method (as it is now called) used by Findlay et al. (1962) to identify the catalytic histidines of bovine pancreatic ribonuclease is based on the assumption that the effect of solvent on the ionization of the enzyme residue and on the ionization of the reaction buffer of the same acid type will be equivalent. The experimental protocol involves comparison of the pK_a values measured in both neutral acid and cationic acid buffers in the presence and absence of organic solvent. The reaction pH is measured in the absence of the organic solvent and is assumed in the calculation of the pK_a from the rate data measured for reactions in the mixed solvent system. Thus, the pK_a of a neutral acid enzyme group measured by using a cationic buffer would appear higher in the organic solvent system relative to the fully aqueous system while it would appear unchanged in the mixed solvent system buffered by a neutral acid. Similarly, a cationic enzyme group would show the same pK_a in either a pure water or a mixed solvent system when a cationic buffer is employed, while the pK_a would appear lower in the mixed solvent buffered by a neutral acid. A precaution that was taken in the original perturbation study and not in most recent studies is to measure the pH perturbation of the buffer systems.

In addition to the original study of bovine pancreatic ribonuclease (Findlay et al., 1962), the solvent perturbation technique has been applied to the identification of catalytic residues in *Escherichia coli* alkaline phosphatase (Krishnaswamy & Kenkare, 1970), glutamate dehydrogenase (Rife & Cleland, 1980), yeast inorganic pyrophosphatase (Knight et al., 1981), yeast hexokinase (Viola & Cleland, 1978), arginine esterase (Viljoen & Bates, 1981), dihydrofolate reductase (Williams & Morrison, 1981), rabbit muscle creatine kinase (Cook et al., 1981), pigeon liver malic enzyme (Schimerlik & Cleland, 1977), β-hydroxymethylglutaryl-CoA reductase (Veloso et al., 1981), mitochondria F-1 ATPase (Godinot & Penin, 1981), soybean β-amylase (Nitta et al., 1979), bovine brain hexokinase (Solheim & Fromm, 1980), and L-alanine dehydrogenase (Grimshaw et al., 1981). While the solvent perturbation method has become part of the working repertoire of some enzymologists, others have cautioned against its use (Laidler & Bunting, 1973) or rejected it (Fersht, 1977; Knowles, 1976). Is it reasonable to extrapolate the behavior of small solutes in mixed solvent systems to that of the active-site residues of enzymes in mixed solvent systems? The present study was carried out with two purposes in mind: (1) to quantitate pK_a perturbations of a wide variety of acids induced by organic solvents in order to evaluate the magnitude and consistency of the pK_a changes and (2) to examine and compare pK_a changes of a catalytic aspartate residue in the active site of yeast hexokinase as induced by the presence of various organic solvents in order to evaluate the similarity in behavior of an active-site carboxylic acid residue to a fully solvated carboxylic acid.

Experimental Procedures

Materials

All organic solvents were Baker Analyzed reagents, used without further purification. The benzoic acid derivatives used in the titration experiments were obtained from Aldrich. Other salts and buffers were purchased from Baker, with the exception of Tris, Taps, Bis-Tris, Hepes, Mes, and Pipes, which were obtained from Sigma. Lyxose, glucose, NADP, ATP, glucose-6-phosphate dehydrogenase, and yeast hexokinase were also from Sigma.

All pH measurements were made at room temperature by using an Orion 701 or Corning 112 pH meter equipped with a Corning glass combination electrode. The meters were standardized at the temperature of measurement with pH 4.00, 7.00, and 10.00 commercial calibration bufers.

Methods

Determination of δ. One milliliter of 0.01 M HCl and 2.5 mL of the test solvent were placed into a 10-mL volumetric flask, which was then filled to volume with water. Five of the flasks were prepared with only water and HCl; a mean (and standard deviation) for the aqueous pH was calculated from these readings. Two separate pH measurements were made on the contents of each flask; the average was used as the pH value for each solvent mixture. The constant δ was calculated for each solvent:

$$\delta = \text{average pH in solvent} - \text{mean pH in water}$$

Effect of Ionic Strength on δ in DMF. Test samples contained 0.14 mL of 0.5 M HCl and sufficient NaCl to bring the final solution to the desired ionic strength. Ten milliliters of DMF was added to one of each pair of test samples, and water was used to bring the total volume in each container to 40 mL prior to taking pH readings.

Solvent Effects on Mes Buffer. Exactly 0.1 M Mes buffer was prepared and divided into four portions, each of which was then adjusted with concentrated KOH to a pH within the buffering range. All test samples were contained in 10-mL volumetric flasks, in duplicate. To 5.0 mL of one of the Mes solutions was added 2.5 mL of the test solvent (water, methanol, DMF, or Me_2SO) followed by water to fill the flask to volume. The pH of each test sample was measured, and the readings for each set of duplicates were averaged. This average was corrected by the δ value determined experimentally for the solvent.

pK_a Determinations. Titration mixtures contained 30.0 mL of a 0.1 M aqueous solution of the test compound and 10.0 mL of the test solvent or water. The titrant was 0.75 M sodium hydroxide, standardized by titration against potassium hydrogen phthalate. Titrations were performed under nitrogen with constant stirring. A Dosimat automatic titrator, capable of incremental delivery of 0.001 mL, was used for the sodium hydroxide addition. pH readings were recorded following each addition once the solution had equilibrated. The pK_a and the standard error in the pK_a were calculated by using a Fortran program provided by W. W. Cleland. The data were fitted to the equation for the base titration of an acid:

$$Y = A + \frac{B}{1 + H/K}$$

where Y is the milliliters of base added, A is the total concentration of acid present, B is the stoichiometry, H is the proton concentration, and K is the dissociation constant of the acid.

Kinetic Assays in General. The hexokinase reaction was monitored at 25 °C by using the glucose-6-phosphate dehydrogenase couple. A Beckman DU Model 2400 spectrophotometer was used to detect the change in absorbance of the reaction mixture at 340 nm as NADP was converted to NADPH. Aluminum(III), which inhibits hexokinase by complexing ATP, was sequestered by including 0.5 mM citrate in each reaction mixture. All reaction mixtures also contained 0.075 M buffer, 1 mM NADP, and 10 mM $MgCl_2$, in a total reaction volume of 1 ml. Preliminary tests under each solvent–pH condition were performed to select an amount of hexokinase that would give a reasonable rate of reaction and

to determine the amount of glucose-6-phosphate dehydrogenase to be added to ensure that the hexokinase reaction, not the coupling reaction, was rate limiting. All reactions were initiated by the addition of hexokinase. The constancy of the initial velocity of each reaction was measured as a function of reaction time to determine whether or not hexokinase was losing activity in the reaction solution.

K_i Determinations. For the K_i experiments, glucose concentration was varied from 0.2 to 2.0 mM while ATP concentration was held constant at 5 mM. Lyxose concentration was held constant at a value at least twice its K_i under the reaction conditions.

Determinations of V/K. V/K_{MgATP} was determined with glucose concentration held constant at 2.0 mM, and also with glucose concentration at 50 mM in two separate series of experiments. ATP was varied at four to six concentrations ranging from $0.5K_m$ to $10K_m$.

Although the pH of every reaction mixture was measured, the values used to calculate the pK_a of the kinetic parameters were those determined in the fully aqueous systems. The potassium salts of the following buffers were used for pH control: Mes, pH 5.2–6.3; Pipes, pH 6.4–7.0; Hepes, pH 7.0–8.1. The initial velocity data were computer fitted to eq 1, the pH data to eq 2, and the inhibition data to eq 3 by using Fortran programs (Cleland, 1979):

$$v_0 = \frac{VA}{K + A} \quad (1)$$

$$\log Y = \frac{\log C}{1 + H/K_A} \quad (2)$$

$$v_0 = \frac{VA}{K(1 + I/K_i) + A} \quad (3)$$

where v_0 is the initial velocity, V is the maximum velocity, K is the Michaelis constant for the substrate, I is the inhibitor concentration, A is the substrate concentration, H is the proton concentration, C is the pH-independent value of the parameter, Y is the observed v_0, V/K, or K_i, and K_A is the acid dissociation constant for the group that must be deprotonated for maximum activity.

Results

Determination of δ for 25% (v/v) Organic Solvents. The value of δ was calculated by taking the difference between the mean pH reading in water and the average pH reading in the 25% solvent mixtures. δ was found to be 0 for 25% v/v ethanol, methanol, dioxane, and THF. For 25% v/v solutions of DMF, Me_2SO, propylene glycol, and ethylene glycol, δ was determined to be +0.17, +0.14, −0.07, and −0.10, respectively. The relationship between the δ for DMF and the molar ionic strength of the mixed solvent solution is as follows: (M, δ) 0.001, +0.19; 0.046, +0.21; 0.091, +0.25; 0.101, +0.29; 0.121, +0.28; and 0.151, +0.38.

pH Perturbation of Mes Solutions. The sensitivity of the magnitude of the pH change of a Mes buffer solution induced by an organic solvent was examined as a function of the hydrogen ion concentration of the Mes solution. The pH of the Mes solution measured in the presence of 25% (v/v) organic solvent was corrected by using the δ for the solvent. The results obtained are as follows: (water, MeOH, Me_2SO, DMF), 5.50, 5.45, 5.46, 5.39; 5.98, 5.91, 5.92, 5.82; 6.31, 6.24, 6.24, 6.16; and 6.67, 6.62, 6.60, 6.52. The differences in the pH of the fully aqueous Mes and the organic solvent containing Mes solutions are as follows: (pH of fully aqueous Mes, ΔpH MeOH, ΔpH Me_2SO, ΔpH DMF) 5.50, −0.05, −0.04, −0.11; 5.98, −0.07, −0.06, −0.18; 6.31, −0.07, −0.07, −0.15; and 6.67, −0.05, −0.07, −0.15.

Change in the pK_a of Small Molecules Caused by Addition of Organic Solvents. The appropriate value of δ was subtracted from each of the pK_a values calculated from the titration data. These corrected pK_a values were then used to determine the change in pK_a for each acid, where the change in pK_a is the pK_a in the mixed solvent minus the pK_a determined in water. The change in pK_a for the tested acids in each solvent is shown in Table I. The average change in pK_a values for acids classified by charge type and polarity is shown for each solvent in Table II. The standard error in the pK_a values was $\leq$0.03 pH unit.

Many of the tested acids were insoluble in the THF mixture, limiting the data obtained in this solvent. Pyrophosphate was insoluble in most of the solvent mixtures. Benzoic acid, anthranilic acid, and *p*-hydroxybenzoic acid were titrated as saturated solutions, because they are not soluble in water to the extent of 0.1 M.

All of the solvent mixtures contained the organic component as 25% (v/v). As a result, the mole fraction of the organic component varies from 0.06 for Me_2SO to 0.13 for methanol. The magnitude of a solvent effect would (in a general way) be expected to be proportional to the mole fraction of the solvent in the mixture. In Table II is also shown the solvent-induced change in pK_a expected had the organic solvent been present as 0.10 mole fraction of the mixture. The adjustment was made by dividing the observed pK_a change (Table II) by 10 times the mole fraction of the organic component actually present in the solvent mixture. This conversion allows within reasonable approximation direct comparison of the relative amounts of pK_a change caused by different solvents.

Solvent Perturbation of pH Profiles. The rather puzzling results of the kinetic experiments are summarized in Table III. Some solvents clearly perturbed the pH profile of the lyxose K_i; others did not. No solvent shifted the pH profile of V/K_{MgATP} greater than 0.25 pH unit when the reaction mixture contained 50 mM glucose. There is more perturbation observed in the pK_a determined from the profile of V/K_{MgATP} when glucose was 2 mM in the reaction mixture, but only in the case of the methanol and dimethylformamide mixtures is it large enough to be considered a perturbation.

The irregularity of the kinetic results led to a study of the reproducibility of the measurement technique. The pH dependence of V/K_{MgATP} was determined 5 times in water and 5 times in 25% DMF, with glucose 2 mM in the reaction mixture. The results of these repetitions are as follows: $pK_a(H_2O)$ 6.15, 6.11, 6.14, 6.14, and 6.17 (mean ± 2 SD = 6.14 ± 0.04); pK_a(25% DMF) 6.55, 6.59, 6.57, 6.56, and 6.57 (mean ± 2 SD = 6.57 ± 0.03). The data from which the pK_a values were calculated are shown in Figures 1–4.

Discussion

δ. All pH measurements were made with a combination glass electrode which had been standardized with aqueous commercial buffers. The glass electrode responds to proton activity in aqueous dioxane (Van Uitert & Haas, 1953), aqueous alcohol (Bacarella et al., 1955; Bates et al., 1963), nonaqueous Me_2SO (Ritchie & Uschold, 1967), and nonaqueous DMF solutions (Demange-Guerin & Badoz-Lambling, 1964). However, the pH meter reading of mixed solvents does not have the same meaning as those of fully aqueous solutions. First, the activity coefficient of the proton in a mixed solvent need not be the same as its activity coefficient in water. In addition, the aqueous standard buffers used to calibrate the pH meter differ substantially in composition from the mixed

Table I: Solvent-Induced Change in pK_a of Acids Grouped by Charge Type and Polarity[a]

	pK_a measured in water	solvent at 25%							
		methanol	ethanol	EG	PG	Me_2SO	DMF	dioxane	THF
polar carboxylic acids									
glycylglycine, pK_a 1	3.15	+0.32	+0.31	+0.28	+0.29	+0.40	+0.31	+0.48	+0.36
acetic acid	4.59	+0.31	+0.36	+0.23	+0.27	+0.37	+0.36	+0.69	+0.71
anthranilic acid	4.85	+0.34			+0.40	+0.29	+0.39	+0.76	
succinate, pK_a 1	4.12	+0.32	+0.37	+0.27	+0.28	+0.40	+0.36	+0.61	
succinate, pK_a 2	5.29	+0.35	+0.42	+0.31	+0.32	+0.44	+0.39	+0.59	
citrate, pK_a 2	4.47	+0.27	+0.30	+0.26	+0.28	+0.38	+0.32	+0.56	
citrate, pK_a 3	5.60	+0.38	+0.33	+0.27	+0.30	+0.44	+0.33	+0.45	
nonpolar carboxylic acids									
2,5-dihydroxybenzoic acid	2.44	+0.42			+0.50		+0.47	+0.73	
3,5-dihydroxybenzoic acid	3.98	+0.50			+0.57	+0.51	+0.63	+0.99	
p-hydroxybenzoic acid	4.34	+0.57				+0.63			
benzoic acid	3.98	+0.55			+0.53	+0.54	+0.61	+0.97	
nonpolar cationic acids									
nicotinic acid	4.73	−0.21			−0.10	−0.37	−0.46	−0.21	
nicotinamide	3.37	−0.31			−0.25	−0.44	−0.58	−0.39	
2-methylimidazole	8.13	−0.17	−0.17	−0.07	−0.11	−0.39	−0.51	−0.30	−0.38
imidazole	7.13	−0.25	−0.25	−0.11	−0.21	−0.41	−0.55	−0.34	
piperazine	10.03	−0.27	−0.30	−0.11	−0.15	−0.23	−0.34	−0.18	
polar cationic acids									
Hepes, pK_a 1	2.94	−0.10	−0.06	+0.16	+0.02	−0.07	−0.08	0	−0.06
Hepes, pK_a 2	7.54	−0.08	−0.07	+0.12	+0.02	−0.08	−0.16	−0.03	−0.09
glycylglycine, pK_a 2	8.27	−0.09	−0.11	+0.10	−0.05	−0.12	−0.16	−0.06	
Bis-Tris	6.60	−0.06	−0.08	+0.07	+0.01	−0.12	−0.21	−0.03	−0.16
Mes, pK_a 2	6.14	−0.07	−0.10	+0.16	+0.04	−0.10	−0.17	−0.06	
Tris	8.23	−0.07	−0.11	+0.08	+0.01	−0.09	−0.19	−0.03	
Taps	8.46	−0.01	−0.03	+0.08	+0.02	−0.09	−0.14	+0.06	−0.03
miscellaneous acids									
boric acid	9.29	+0.13	+0.61	−1.11	−1.31	+1.21	+0.94	+0.83	
cacodylic acid	5.92	+0.30	+0.38	+0.24	+0.30	+0.52	+0.41	+0.55	
KH_2PO_4	6.91	+0.45	+0.49	+0.33	+0.34	+0.73	+0.53	+0.58	
$Na_3HP_2O_7$	8.19			+0.26					

[a] Change in pK_a is the pK_a measured in the solvent less the pK_a in water.

Table II: Average Solvent-Induced Change in pK_a for Four Categories of Acids[a]

acid category (polarity–charge type)	methanol	ethanol	EG	PG	Me_2SO	DMF	dioxane	THF
nonpolar carboxylic	+0.51 (+0.40)			+0.54 (+0.72)	+0.56 (+1.0)	+0.57 (+0.69)	+0.90 (+1.34)	
polar carboxylic	+0.33 (+0.26)	+0.35 (+0.38)	+0.27 (+0.27)	+0.31 (+0.41)	+0.39 (+0.70)	+0.35 (+0.42)	+0.59 (+0.88)	+0.54 (+0.75)
nonpolar cationic	−0.24 (−0.19)	−0.24 (−0.26)	−0.10 (−0.10)	−0.16 (−0.21)	−0.37 (−0.66)	−0.49 (−0.59)	−0.28 (−0.42)	
polar cationic	−0.06 (−0.05)	−0.08 (−0.09)	+0.10 (+0.10)	+0.01 (+0.01)	−0.10 (−0.18)	−0.17 (−0.20)	−0.02 (−0.03)	−0.09 (−0.12)

[a] Average pK_a change normalized to 0.1 mole fraction of organic component in solvent is shown in parentheses.

Table III: pK_a Values Determined from the Lyxose pK_i Profile and MgATP V/K Profiles of Hexokinase[a]

solvent	pK_a from K_i in lyxose	ΔpK_a[b]	pK_a from V/K(MgATP), 50 mM glucose	ΔpK_a[b]	pK_a from V/K(MgATP), 2 mM glucose	ΔpK_a[b]
25% PG	6.56 ± 0.05	+0.28	5.96 ± 0.02	+0.13	6.36 ± 0.05	+0.21
25% methanol	7.47 ± 0.06	+1.19	5.99 ± 0.03	+0.16	6.53 ± 0.04	+0.38
25% Me_2SO	6.36 ± 0.03	+0.08	6.08 ± 0.02	+0.25	6.36 ± 0.02	+0.21
25% DMF	6.80 ± 0.05	+0.52	6.00 ± 0.02	+0.17	6.51 ± 0.04	+0.36
17.5% dioxane	7.32 ± 0.04	+1.04			6.26 ± 0.03	−0.02
water	6.28 ± 0.02		5.83 ± 0.02		6.15 ± 0.03	

[a] In addition to lyxose and/or ATP, reaction mixtures contained 75 mM buffer, 0.5 mM citrate, 10 mM $MgCl_2$, 1 mM NADP, and glucose-6-phosphate dehydrogenase. [b] ΔpK_a is the following difference: pK_a in solvent mixture minus pK_a in water.

solvent solutions; the meter reading in the mixed solvent may contain a large residual liquid junction potential. The net result of these two factors is that the pH meter reading of the mixed solvents may not reflect their proton activities.

If pH readings are to be made in a number of different solvent systems, there is a need to establish some relationship that will allow them to be compared. It has been shown experimentally that such a relationship can be established for acids in water–alcohol mixtures (Bates et al., 1963). For these mixtures, $\bar{E}_j$, the residual liquid junction potential, was found to depend only on solvent composition. This allows the derivation of a constant, δ, to correct pH readings obtained in

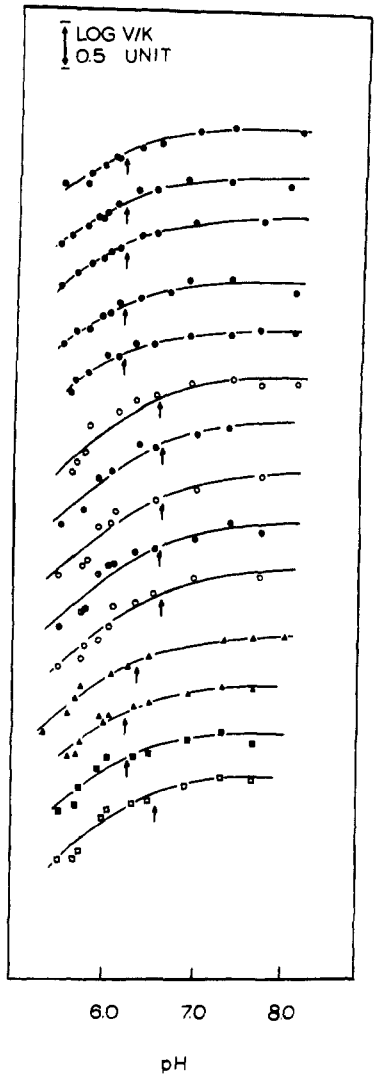

FIGURE 1: MgATP V/K vs. pH profile measured in the presence of 2 mM glucose and in the presence of (●) H_2O, (○, ◑) 25% (v/v) DMF, (■) 25% (v/v) propylene glycol, (▲) 25% (v/v) Me_2SO, (□) 25% (v/v) methanol, and (△) 17.5 (v/v) dioxane. The pK_a is indicated by (↑). The curves were computer fitted.

methanol–water solutions using a meter standardized with aqueous buffers, specifically $\delta \equiv \bar{E}_j - {}_m\gamma_H$, where ${}_m\gamma_H$ is the medium effect on the activity of the proton (also a solvent-dependent constant). At constant ionic strength, subtracting δ from the pH meter reading of the mixed solvent gives $pa_H{}^*$, where $pa_H{}^*$ is the proton activity referred to the standard state in that solvent. The $pa_H{}^*$ values for different solvent mixtures are comparable with each other and with pa_H, the proton activity in water referenced to the standard state in water.

It was subsequently shown that δ is constant for aqueous mixtures of dioxane, acetonitrile, DMF, Me_2SO, and acetone. For THF mixtures, δ varies because $\bar{E}_j$ varies. However, the variation extends over a range of only a few hundredths of a pH unit (Douheret, 1967, 1968).

The values for δ determined in the present study are in agreement with those published previously (Bates et al., 1963; Douheret, 1967, 1968). No δ values for propylene glycol and ethylene glycol were found in the literature for comparison.

The constant δ makes no correction for ionic strength effects, which may alter $\bar{E}_j$ and γ_H. The values obtained in the present study were derived from pH measurements of 0.01 M HCl solutions. The variation of δ as a function of ionic strength was examined in 25% (v/v) DMF to assess the legitimacy of using these δ values to correct pH readings made at higher

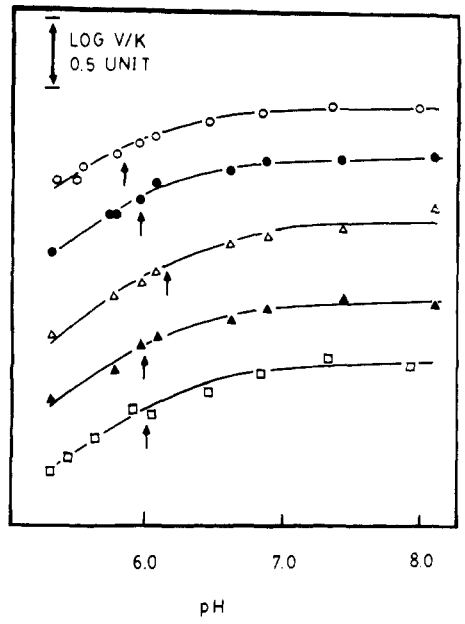

FIGURE 2: MgATP V/K vs. pH profile measured in the presence of 50 mM glucose and in the presence of (○) H_2O, (▲) 25% (v/v) DMF, (□) 25% (v/v) methanol, (●) 25% (v/v) propylene glycol, and (△) 25% (v/v) Me_2SO. The pK_a is indicated by (↑). The curves were computer fitted.

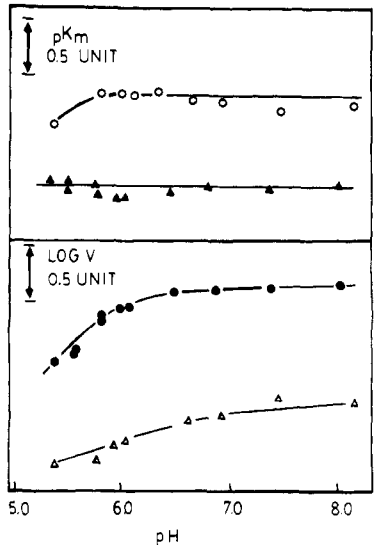

FIGURE 3: V (●) and MgATP K_m (▲) profiles measured in the presence of 50 mM glucose, and V (△) and K_m (○) profiles measured in the presence of 50 mM glucose and 25% (v/v) DMF. The K_m for MgATP at neutral pH was 0.09 mM in the absence of DMF and 0.06 mM in the presence of DMF. The curves were hand drawn.

ionic strength. The results indicate that pH readings vary only about 0.05 unit as ionic strength increases from 0 to 0.08 M. The δ values determined in dilute HCl were therefore deemed acceptable for correcting the pK_a values of acids titrated at 0.075 M concentration.

Enzyme solvent perturbation studies are usually carried out in the manner recommended by Cleland (1977) wherein the pH is measured only for aqueous reaction mixtures. This technique avoids correcting pH readings made in mixed solvent, but it is appropriate only if the buffered solution is uniformly perturbed throughout the range of use. In addition, the pH change induced in the reaction mixture by an organic solvent should be dictated by the solvent-induced change in the pK_a of the buffer, and therefore predictable. Accordingly, organic solvents were added to several Mes solutions which

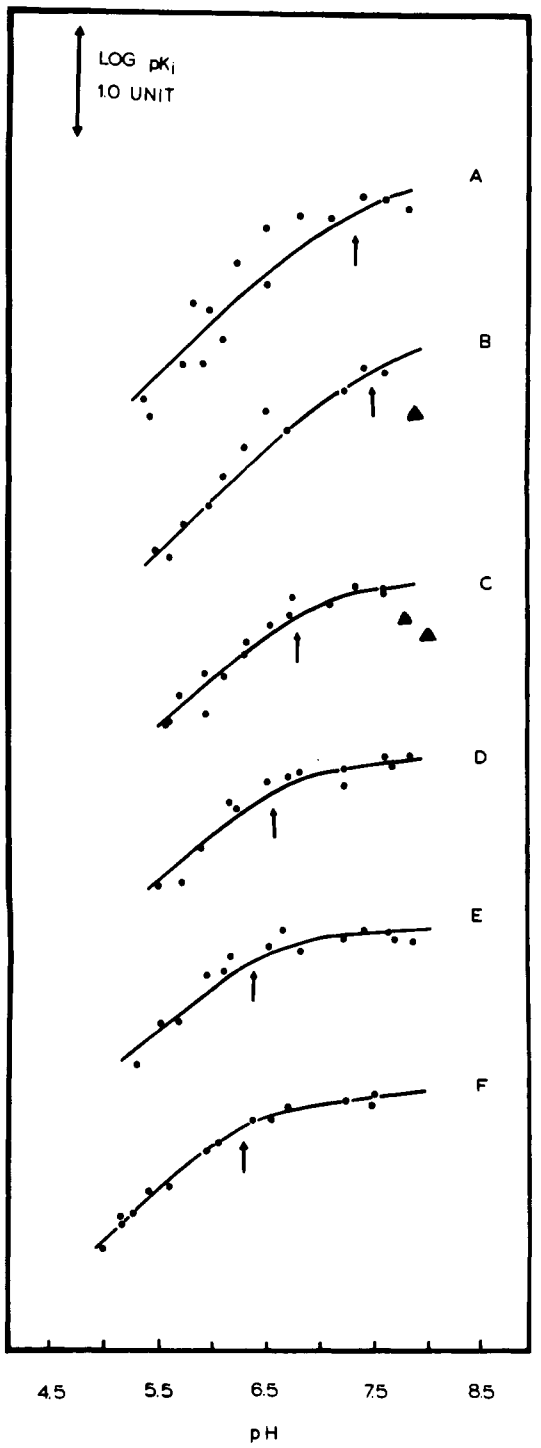

FIGURE 4: Lyxose pK_i profile measured in the presence of 5 mM MgATP and in the presence of (A) 17.5 (v/v) dioxane, (B) 25% (v/v) methanol, (C) 25% (v/v) DMF, (D) 25% (v/v) propylene glycol, (E) 25% (v/v) Me_2SO, and (F) H_2O. The pK_a is indicated by (↑). The curves were computer fitted. The data represented by triangles were not included in the fit. At neutral pH, the K_i for lyxose was determined to be 6.7 mM in H_2O, 0.53 mM in Me_2SO, 1.5 mM in propylene glycol, 2.0 mM in DMF, 1.1 mM in methanol, and 1.2 mM in dioxane.

had been adjusted to different pH values within their buffering range. The results obtained indicate that the pH perturbations are uniform and do correlate well with the perturbation of the Mes pK_a.[3]

[3] It is important to point out that this uniformity of perturbation can be expected only within the buffering range of the salt. Footnote 6 discusses experimental indications of erratic pH changes that occur when solvents are added to improperly buffered solutions.

Solvent Perturbation of Acid pK_a Values. The solvent-induced changes in acid pK_a values shown in Table I generally harmonize with the expectation derived from the preexisting literature on solvent effects: the pK_a values of carboxylic acids and other acids which undergo nonisoionic ionizations are increased by organic solvents, while the pK_a values of cationic acids are unaffected or decreased. The few exceptions that appear in Table I include the polar cationic acids, which show positive perturbations in ethylene glycol and propylene glycol, instead of the expected negative perturbations that are seen in all the other solvents. Boric acid (a neutral acid) shows exceptionally large perturbations, which are negative in the case of the two glycols. Boric acid is atypical, however, since it functions as a Lewis acid, accepting a hydroxyl group, instead of as a proton donor. The glycols accentuate the acidity of $B(OH)_3$ by complexing it (Cotton & Wilkinson, 1966).

Although the data are unsurprising in terms of the direction of the perturbations, there do seem to be differences in the magnitude of the perturbations that depend on the polarity of the neutral form of the solute (see Table II). For the same functional acid group, the change in pK_a is larger when ionization interconverts a nonpolar/polar acid–base pair than when the acid is polar both before and after ionization. It is for this reason that enzyme pK_a perturbation studies which are conducted according to the recommended procedure (Cleland, 1977) must be carried out with "matched" buffers. The use of pH measurements of the fully aqueous reaction mixtures for calculation of the rate data obtained for the mixed solvent systems requires that all of the buffers be affected equally by the solvent in their buffering range. The use of alternating polar and nonpolar buffers would result in a discontinuous shift in pH when organic solvent is added, since the buffers would not be perturbed to the same degree. If the pK_a of the kinetic parameter resided near the discontinuity, error might be introduced into the calculated pK_a. We recommend that the pH of the mixed solvent reaction mixtures be directly measured and the δ factor for the solvent be used to correct the pH reading. This value in turn should be used in the calculation of the rate data rather than the assumed value derived from the corresponding fully aqueous reaction mixture. This procedure would also eliminate the necessity of measuring the pH profiles in both cationic and neutral acid buffers and would reveal any abnormalities in the solvent-induced pH perturbation of the reaction solution.

The solvent-induced pK_a changes of the four classes of acids normalized to 0.1 mole fraction of solvent are shown in Table II. In general, the alcohols give rise to the smallest perturbations while dioxane and Me_2SO give rise to the largest perturbations.

Solvent Perturbation Studies of the Hexokinase Reaction. In proposing the solvent perturbation technique, Findlay et al. (1962) cautioned that a pK_a perturbation cannot be considered an indicator of acid type at the active site of an enzyme unless solvent-induced conformational changes can be excluded. X-ray and solution studies have shown that yeast hexokinase undergoes marked conformational changes when substrates are bound (Anderson et al., 1978; McDonald et al., 1979). Consequently it may seem that yeast hexokinase is a poor choice for a test of the reliability of the solvent perturbation technique. However, from a practical standpoint, it was necessary to work with a well-characterized enzyme. Further, despite the original caution of Findlay et al. (1962), enzymes which are known to undergo conformational changes are being subjected to solvent perturbation studies. It is certainly likely that some degree of conformational change

universally accompanies enzyme catalysis, and solvent perturbation is used principally on enzymes whose sequence and catalytic mechanism are unknown. This series of experiments is, then, a test of the solvent perturbation technique under realistic conditions.

Crystallographic analysis of hexokinase at 2.1-Å resolution has revealed an active-site aspartate which appears to bind to the hydroxyl groups of carbons 4 and 6 of glucose (Anderson et al., 1978). The results from pH studies of the hexokinase-catalyzed reaction have provided evidence for an active-site carboxylate residue which functions in the forward direction as a general base to remove the proton from the glucose 6-hydroxyl group, thereby facilitating nucleophilic attack on the γ-phosphate of ATP (Viola & Cleland, 1978). These studies also showed that the binding of MgATP to the enzyme is pH independent, and therefore, any pH dependence observed in the MgATP V/K must reflect changes in the ionization state of the catalytic residue. Viola & Cleland (1978) carried out a solvent perturbation study in which the V/K for MgATP was measured at a fixed glucose concentration (2 mM) as a function of reaction pH. A perturbation of $+0.47 \pm 0.05$ was obtained by subtracting the pK_a determined in a cationic buffer system in the presence of DMF (25% v/v) from that determined in its absence. The data presented in Table I indicate that this perturbation value is consistent with those exhibited by solvated carboxylic acids. In the present study, this same hexokinase pK_a perturbation was attempted with 25% (v/v) DMF, Me_2SO, methanol, and propylene glycol and with 17.5% (v/v) dioxane. The perturbation observed with DMF agreed reasonably well (see Table III) with that reported by Viola & Cleland (1978). Methanol induced a similar sized perturbation, but the remaining solvents produced perturbations of insignificant size. For this reason, the day to day reproducibility of the pK_a determination was tested. The five pK_a values determined for the fully aqueous system and for the 25% (v/v) DMF system agreed quite well (see Results for the actual pK_a values). In Figure 1, the pH profiles measured for each of the solvents are shown in order that we may point out a subtle feature of the data-fitting procedure. If one examines the curves carefully, it becomes apparent that those profiles which reflect higher pK_a values appear to drop with a slope greater than 1. The computer attempts to fit the data to a curve having a slope of unity, and as a result, the experimental curve appears to be sliced by the computer-fitted curve. This in turn forces a premature break in the curve and, therefore, an inaccurately high pK_a value. Because 2 mM glucose was found not to be sufficiently saturating in the mixed solvent systems, the MgATP V/K vs. pH profiles were repeated in the presence of 50 mM glucose. The data obtained, which are presented in Figure 2, fit well to the computer-drawn curves, and the perturbations calculated from these data are too small to be considered significant (see Table III). Why is the observed pK_a of the aspartate in the E·glucose complex not perturbed by organic solvent? It is possible that glucose partially shields the aspartate residue from solvent (either in a direct manner or via an induced conformational change) or significantly delocalizes the charge on the aspartate via H bonding to it or stabilizes the enzyme against solvent-induced conformational changes (vide infra).

Shown in Figure 3 are the V and K_m profiles of MgATP (50 mM glucose) measured in the presence and absence of 25% (v/v) DMF. The data shown for the DMF system are representative of those obtained by using the other organic solvents. Note that the V and V/K (see Figure 2) for MgATP in water respond in the same way to pH so that the K_m for MgATP is pH independent. In contrast, the V profiles measured for the organic systems do not show a strong pH dependency over the range studied, and thus, MgATP K_m values vary with pH. It is not clear how the organic solvent perturbs the aspartate pK_a to a slightly higher value when only glucose is bound to the enzyme and to a dramatically lower value when both glucose and MgATP are bound.

Ideally, pK_a determinations should be made from pH profiles of the kinetic parameters measured with slow-reacting substrate analogues or, where appropriate, from the pK_i profiles of competitive inhibitors, so that solvent effects on binding steps will not alter the measured pK_a. The hexokinase aspartate residue is thought to function not only in catalysis, by deprotonation of the glucose, but also in glucose binding. Viola & Cleland (1978) had shown that the K_i of 6-deoxyglucose is pH dependent. Thus, in order to study the aspartate perturbation under equilibrium conditions, the pK_i vs. pH profile of lyxose, also a competitive inhibitor[4] vs. glucose, was measured both in the presence and in the absence of organic solvents. The MgATP concentration was held constant at its K_m level of 5 mM (Viola et al., 1982). The pK_i data are presented in Figure 4, and the calculated pK_a values are listed in Table III. The data obtained for the fully aqueous system show a good fit to the computer-drawn curve. The pK_a value obtained is somewhat higher than that determined from the MgATP V/K profile and may be at least in part a reflection of the stabilization of the carboxylate anion via hydrogen-bonding interactions with glucose.

The lyxose pK_i vs. pH profiles measured in the presence of Me_2SO or propylene glycol were similar to those measured in water although they were slightly less defined and when computer fitted yielded pK_a values slightly higher than that obtained for the fully aqueous system. It is important to note that hexokinase showed no sign of denaturation during the velocity measurements over the entire pH range for all three solvent systems. In contrast, DMF, methanol, and dioxane did induce activity loss which was observable during the velocity measurements (the actual velocity was derived from the tangent to an initial velocity curve) and which was more evident for assays which contained glucose at the low end of the glucose concentration range than those that contained glucose at the high end. In short, these three solvents alter the hexokinase conformer population in solution in a time-dependent manner which appears to be slowed down by the presence of glucose. Although the pK_i data measured for these three solvent systems were computer fitted by assuming that a single ionization is responsible for the change in lyxose binding with pH, the quality of the data is actually not sufficient to define an accurate pK_a value. First, the data are quite "noisy" (no doubt due to the dynamics of the enzyme denaturation during the velocity measurement), and second, the plateau of the pH curve could not be defined. As indicated by the triangles in Figure 4 (representing data which were not included in the pK_a calculations), the binding of lyxose drops dramatically at pH values exceeding 7.5. While the pK_a perturbation determined from the lyxose pK_i profile measured in 25% (v/v) DMF (Table III) is consistent with that one would expect for a small solvated carboxylic acid (Table I), those obtained for the dioxane and methanol systems are much too large. It is likely

[4] In our own hands, lyxose was found to be a linear competitive inhibitor at pH 7.7 and 5.5.

that the large perturbations observed with methanol, dioxane, and to some extent with DMF reflect solvent-induced changes in hexokinase other than the protonation state of the active-site aspartate residue.

Conclusions

When the solvent is a mixture of water and a miscible organic liquid, the basicity, dielectric constant, and structure of the mixture will differ from those of either pure solvent. The exact qualities of the mixture vary with its composition, so that predicting the exact pK_a of a solute in a mixed solvent system would seem to be impractical. In the present study, the organic solvent was held at 25% v/v, and therefore, the solutions contained at least 9 times as many water molecules as organic molecules. To a first approximation, the solvents' perturbing effects are a result of their effects on the predominant species, water. The complexity of the pK_a change phenomena can be appreciated by recognizing that preexisting water–water and water–organic interactions are altered by the addition of the acid solutes. Changes in enthalpy and entropy associated with these alterations are combined with those resulting from new interactions between the solvent and the acid, both in its protonated and in its unprotonated forms, to determine the pK_a. As indicated by the data shown in Table II, the organic solvents, when normalized to 0.1 mole fraction, do in fact differ in their perturbing strengths.

The charge type of the acid determines the direction of the pK_a perturbation, while the polarity of the uncharged form of the acid determines the extent to which it is perturbed by a given organic solvent (Table II). Thus, despite the complexity of the underlying changes, one can make a resonable prediction of the pK_a perturbation that will be observed on the basis of the solvent composition and the charge and polarity of the acid.

Enzyme Active-Site Acid Perturbations. It is clear that an acid residue in an enzyme active site is not exposed to the same environment as a fully solvated acid. Further, it is not subject to the same environmental changes that occur when organic solvent is added to the aqueous medium. Five very distinct complications inhibit predictions concerning the effect of an organic solvent on the pK_a of the active-site residue. The first problem arises from the potential solvent effect on the stickiness[5] of the proton, substrate, and product. If the pK_a is determined from the V or V/K profile, an apparent pK_a is measured. Its deviation from the true pK_a is determined by a ratio of rate constants indicative of substrate and/or product stickiness. A second problem derives from the effect of the solvent on the enzyme forms present in solution. The ionization of an active-site residue is sensitive to the presence of substrates and cofactors. The organic solvent may loosen or tighten the binding of these molecules, changing the enzyme form undergoing ionization, and thus the pK_a for that ionization. A third problem is one of solvation. To what extent is the effect of the organic solvent on the structure of water and its ability to solvate ions mediated to the active-site residue? The fourth ambiguity arises from solvent-induced changes in the microscopic environment of the acid group. The pK_a of an active-site residue often greatly deviates from that of the corresponding amino acid in solution. A classic example is the active-site lysine of acetoacetate decarboxylase which has been shown to have a pK_a of 6.0 (Frey et al., 1971; Kokesh & Westheimer, 1971). The variation of pK_a observed for an amino acid in different enzyme active sites may result from its involvement in H bonding, may be a result of electrostatic interactions with neighboring residues, or may result from desolvation effects. In any case, the organic solvent may be expected to alter the environment of these neighboring groups as well as that of the catalytic group and thus may alter the pK_a of the active-site residue secondarily in some significant but unforeseen way. Last, and perhaps most important at least in the case of hexokinase, the presence of the organic solvent may alter the average conformation of the enzyme in solution, and the degree of alteration may be itself pH dependent.

Although our studies of the solvent perturbation of the pK_a of the yeast hexokinase catalytic group were reasonably comprehensive, we were not able to make a distinction between a neutral acid and a cationic acid group on the basis of the results obtained. A careful examination of other reported solvent perturbation studies leads one to further doubts about the reliability of the technique. In some cases, investigators have reached conclusions without acknowledging the restrictions imposed on the technique itself. For example, the perturbation study carried out for soybean β-amylase (Nitta et al., 1979) did not adhere to the logic of the perturbation method. Specifically, while part of the pH range was controlled by neutral buffers and part of it by cationic buffers, the compensation solvent-induced pH changes that would result in the buffers were not taken into consideration in the interpretation of the perturbation data. Other problems arise from deciding what is the acceptable magnitude of a "real" perturbation. For example, mitochondrial F-1 ATPase was reported to show a (MgATP V/K profile, 15% DMF–10% Me_2SO) +0.4 pH unit perturbation in cationic buffer and a +0.2 pH unit perturbation in neutral buffer. On the basis of the net experimental difference of +0.2 pH unit, an assignment of a neutral acid was made. For further complication of the matter, the pK_a measured in the presence, but not in the absence, of the organic solvent in the cationic buffer system is actually a composite of two pK_a values (Godinot & Penin, 1981). The analysis of the benzoyl-L-arginine *p*-nitroanilide V/K profile for arginine esterase yielded pK_a values of 7.54 and 7.68 in cationic and neutral acid buffers, respectively. The pK_a values appeared −0.59 and −0.52 units lower when measured in the presence of 20% DMF, and on this basis, a neutral acid was assigned (Viljoen & Bates, 1981). Sometimes the perturbation observed is not significant as a result of the large standard error associated with the pK_a value itself. This would appear to be the case for the assignment of a neutral acid residue which was made to the catalytic group of bovine brain hexokinase based on an observed perturbation of $+0.29 \pm 0.27$ pH unit (25% Me_2SO–glucose V/K profile) (Solheim & Fromm, 1980).

A more disturbing feature of the published perturbation studies is that they clearly demonstrate that nonspecific solvent and buffer effects not only can contribute to the observed perturbation but also can in many cases dominate it. A particularly dramatic example of solvent effects is observed with rabbit muscle creatine kinase (Cook et al., 1981). The pK_a measured from the V/K profile for phosphocreatine was perturbed −0.82 pH unit in cationic buffer and −1.19 pH units in neutral buffer by 25% DMF. The difference between the two perturbations was used as evidence for a cationic acid group.[6] Similar reasoning was applied in the assignment of a cationic acid to L-alanine dehydrogenase on the basis of a −0.7 and −2.1 pH unit perturbation determined from the pK_i

[5] A sticky substrate, for example, reacts to product faster than it is released from the enzyme.

profile of pyruvate and measured in the presence of 30% Me_2SO in cationic or neutral acid buffer, respectively (Grimshaw et al., 1981). Since the pK_a of this group measured in fully aqueous neutral buffer was not reported, it is difficult to assess how much of the perturbation observed in neutral buffer is a solvent effect and how much is a buffer effect. The buffer effect can actually be quite large. For example, the malate V/K profile for the pigeon liver malic enzyme shows three pK_a values, all of which appear at least 0.6 pH unit higher when measured in fully aqueous neutral buffer than in fully aqueous cationic buffer (Schimerlik & Cleland, 1977). Similarly, the pK_a determined from the dihydrofolate V/K profile for dihydrofolate reductase appears 0.71 pH unit higher when measured with aqueous neutral acid buffer than with aqueous cationic acid buffer. Surprisingly, this difference was acknowledged but not taken into account in interpreting the data. Both cationic and neutral mixed solvent systems were compared to the cationic aqueous system (Williams & Morrison, 1981).

The large differences observed in systems that are expected to be identical for catalytic purposes are disturbing. Nonspecific solvent and buffer effects are probably the rule in enzyme–solvent perturbation experiments rather than the exception. Where they do exist, the size and direction of perturbation predicted by the behavior of small molecules may be irrelevant. While the results reported herein do not support the reliability of the solvent perturbation technique when indiscriminately applied, they of course do not exclude the possibility that it may be applicable to some systems. If it is to be used, however, more care must be taken with the methodology, and the results must be interpreted more conservatively.

[6] The pH was controlled by a mixed neutral buffer system containing acetate, cacodylate, and borate. Borate is a bad choice for a mixed buffer system, as it is perturbed to a much greater extent than are most neutral acid buffers. A discontinuous solvent-induced pH change may result when it is included in a mixed buffer system. If the pH of the mixed solvent system is measured directly and used in the pK_a calculations, this potential problem can be avoided. However, these authors used the standard protocol, measuring only the pH of the aqueous system. A further point of concern with the mixed buffer system used in the creatine kinase study is that over the pH range 7.0–8.2 the reaction mixture is not truly buffered. We prepared the exact buffer solutions used by the authors and measured in duplicate the pH which results upon addition of DMF to a concentration of 25% (v/v). A mixture 25 mM in each acetate and cacodylate was used to buffer the range from pH 5.5 to 7, 25 mM cacodylate and 12.5 mM in each acetate and borate from pH 7 to 8.2, and 25 mM in each cacodylate and borate from pH 8.1 to 9. When DMF was added to the buffer solutions, the following pH changes were observed. At several pH values up to pH 6.6, the first buffer system was perturbed +0.47 unit. At pH 6.94, the pH increase was +0.54, and at pH 7.05, it was +0.81. The change in the pH is not surprising as the buffering capacity of the solution has been exhausted at neutral pH. The pH range covered by the second buffering system is not truly buffered, nor is that at the low end of the pH range covered by the third buffer system. The perturbation of the second buffer solution at pH 7.01 upon addition of DMF was +1.05, and the change at pH 7.58 was +1.46. The third buffering system showed a ΔpH of +0.962 at pH 8.43, which is consistent with what would be expected for borate. For determination of the extent the nonuniform perturbations would influence the pK_a determination, a pH profile was constructed by using kinetic parameter values from a computer-drawn curve which defined a pK_a of 7.7. The new curve, which showed a discontinuity beginning in the pH 7.0–7.2 region, was computer fitted to give a pK_a of 5.9. Thus, independent of any perturbation of the creatine kinase residue, one would expect that the perturbation measured with the neutral buffer system could be as large as −1.8 pH units. The negative perturbation of −1.17 units reported by Cook et al. (1981) could have resulted entirely from the buffer system and therefore cannot be used to draw any conclusion about the nature of the active-site group. The literature contains at least two other examples of dubious results obtained in mixed neutral buffer systems. F-1 ATPase was studied by using a similar borate–acetate–cacodylate system (Godinot & Penin, 1981). Schimerlik & Cleland (1977) reported unexplained negative pK_a changes that could have been caused by undetected pH deviations in the solvent-containing reaction mixtures.

Acknowledgments

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Registry No. MeOH, 67-56-1; EG, 107-21-1; Me_2SO, 67-68-5; DMF, 68-12-2; PG, 57-55-6; THF, 109-99-9; Hepes, 7365-45-9; Mes, 4432-31-9; Taps, 29915-38-6; Bis-Tris, 6976-37-0; Tris, 77-86-1; L-aspartic acid, 56-84-8; hexokinase, 9001-51-8; MgATP, 1476-84-2; lyxose, 65-42-9; ethanol, 64-17-5; dioxane, 123-91-1; glycylglycine, 556-50-3; acetic acid, 64-19-7; anthranilic acid, 118-92-3; succinic acid, 110-15-6; citric acid, 77-92-9; 2,5-dihydroxybenzoic acid, 490-79-9; 3,5-dihydroxybenzoic acid, 99-10-5; *p*-hydroxybenzoic acid, 99-96-7; benzoic acid, 65-85-0; nicotinic acid, 59-67-6; nicotinamide, 98-92-0; 2-methylimidazole, 693-98-1; imidazole, 288-32-4; piperazine, 110-85-0; boric acid, 10043-35-3; cacodylic acid, 75-60-5; KH_2PO_4, 7778-77-0; $Na_3HP_2O_7$, 14691-80-6.

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Resonance Energy Transfer between the Adenosine 5′-Diphosphate Site of Glutamate Dehydrogenase and a Guanosine 5′-Triphosphate Site Containing a Tyrosine Labeled with 5′-[*p*-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine†

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ABSTRACT: The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBεA) reacts irreversibly with bovine liver glutamate dehydrogenase and modifies one of the natural inhibitory guanosine 5′-triphosphate (GTP) sites [Jacobson, M. A., & Colman, R. F. (1982) *Biochemistry 21*, 2177–2186]. Enzyme with 1.28 mol of 5′-(*p*-sulfonylbenzoyl)-1,N^6-ethenoadenosine/mol of subunit incorporated and exhibiting maximum change in sensitivity to GTP inhibition is now shown by amino acid analysis to contain 0.95 mol of *O*-[(4-carboxyphenyl)sulfonyl]tyrosine (CBS-Tyr) and 0.33 mol of N^{ϵ}-[(4-carboxyphenyl)sulfonyl]-lysine (CBS-Lys), quantitatively accounting for the total incorporation prior to acid hydrolysis. As a function of time of incubation with 5′-FSBεA, the amount of CBS-Tyr formed was directly proportional to the change in GTP inhibition. In contrast, an initial formation of CBS-Lys was observed, followed by relatively little additional CBS-Lys although the percent change in GTP inhibition continued to increase. It was concluded that the tyrosine is an essential residue in the GTP binding site of glutamate dehydrogenase, while the lysine modified is not involved in the inhibitory action of GTP. The nucleotide analogue 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate (TNP-ADP) was evaluated for its ability to occupy the adenosine 5′-diphosphate (ADP) activator site and to function as an energy acceptor conjointly with 5′-SBεA covalently bound at the GTP site as the energy donor. TNP-ADP activates native enzyme 2-fold and competes kinetically with ADP. As determined by fluorometric titration, the maximum number of TNP-ADP binding sites on native enzyme was 0.5 mol/mol of subunit in the absence and 1 mol/mol of subunit in the presence of reduced coenzyme. The 5′-SBεA-modified enzyme also binds TNP-ADP: 0.5 mol/mol of subunit in the absence or presence of reduced coenzyme. TNP-ADP competes for binding with ADP to native and 5′-SBεA-modified enzyme, indicating that this nucleotide analogue is a satisfactory fluorescent probe of the ADP site of glutamate dehydrogenase. An energy-transfer efficiency of 0.77 was determined from the decrease in donor fluorescence upon addition of TNP-ADP in the absence of reduced coenzyme to modified enzyme containing 1.23 mol of 5′-SBεA/mol of subunit. A value of 18 Å was calculated as the average distance between the GTP and ADP regulatory sites. This result indicates that the inhibitory GTP and the activatory ADP sites are close but not identical.

The distance between two chromophores on a protein can be determined by fluorescence energy transfer (Förster, 1959) provided that the emission spectrum of the donor chromophore overlaps with the absorption spectrum of the acceptor chromophore and that the two species are sufficiently close to each other (within 80 Å). Studies on a variety of biomolecules and macromolecular assemblies have been carried out by use of energy transfer (Fairclough & Cantor, 1978; Stryer, 1978)

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