DiSabato, G., & Jencks, W. P. (1961) *J. Am. Chem. Soc. 83*, 4393.
Eckstein, F. (1983) *Angew, Chem., Int. Ed. Engl. 22*, 423.
Gerlt, J. A., & Coderre, J. A. (1980) *J. Am. Chem. Soc. 102*, 4531.
Gerlt, J. A., Mehdi, S., Coderre, J. A., & Rogers, W. O. (1980) *Tetrahedron Lett. 21*, 2385.
Gerlt, J. A., Coderre, J. A., & Mehdi, S. (1983) *Adv. Enzymol. Relat. Areas Mol. Biol. 55*, 291.
Jarvest, R. L., Lowe, G., & Potter, B. V. L. (1981) *J. Chem. Soc., Perkin Trans. 1*, 3186.
Kellerman, G. M. (1958) *J. Biol. Chem. 231*, 427.
Liao, T.-H. (1975) *J. Biol. Chem. 250*, 3721.
Liao, T.-H., Salnikow, J., Moore, S., & Stein, W. H. (1973) *J. Biol. Chem. 248*, 1489.
Lowe, G., Potter, B. V. L., Sproat, B. S., & Hull, W. E. (1979) *J. Chem. Soc., Chem. Commun.*, 733.
Mehdi, S., & Gerlt, J. A. (1981a) *J. Am. Chem. Soc. 103*, 7018.
Mehdi, S., & Gerlt, J. A. (1981b) *J. Biol. Chem. 256*, 12164.
Mehdi, S., & Gerlt, J. A. (1982) *J. Am. Chem. Soc. 104*, 3223.
Moore, S. (1981) *Enzymes, 3rd Ed. 14A*, 281.
Niewarowski, W., Stec, W. J., & Zielinski, W. S. (1980) *J. Chem. Soc., Chem. Commun.*, 524.
Ogilvie, K. K., & Letsinger, R. L. (1967) *J. Org. Chem. 32*, 2365.
Poulos, T. L., & Price, P. A. (1974) *J. Biol. Chem. 249*, 1453.
Schaller, H., Weiman, G., Lerch, B., & Khorana, H. G. (1963) *J. Am. Chem. soc. 85*, 3821.
Stec, W. J. (1983) *Acc. Chem. Res. 16*, 411.
Suck, D. (1982) *J. Mol. Biol. 162*, 511.
Tsai, M.-D. (1979) *Biochemistry 18*, 1468.
Usher, D. A., Richardson, D. I., & Eckstein, F. (1970) *Nature (London) 228*, 663.
Westheimer, F. H. (1980) in *Rearrangements in Ground and Excited States* (deMayo, P., Ed.) Vol. 2, p 229, Academic Press, New York, NY.
Zielinski, W. S., & Lesnikowski, Z. (1976) *Synthesis*, 185.

Solvent Effects on Allosteric Equilibria: Stabilization of T and R Conformations of *Escherichia coli* Aspartate Transcarbamylase by Organic Solvents†

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ABSTRACT: The activity of *Escherichia coli* aspartate transcarbamylase (ATCase) is markedly influenced by the addition of organic solvents to the assay medium. The cosolvents tested, which include simple aliphatic alcohols, amides, and ureas, as well as acetone and dioxane, fall into two different classes: the most polar ones (formamide, acetamide, *N*-methylformamide, and urea) stimulate the enzyme activity for all concentrations tested. In contrast, solvents that are less polar than water inhibit the enzyme at low concentrations but stimulate it at higher concentrations. No comparable effects are observed in the case of the isolated catalytic subunits, a nonregulated form of ATCase. Extensive kinetic studies on ATCase and on two of its Michaelian derivatives, 2-thioU–ATCase and carbamylated ATCase, indicate that solvents modulate the same allosteric transition that is responsible for homotropic interactions between the catalytic sites. The stabilization of the R state of ATCase by comparatively high concentrations of cosolvents is reminiscent of similar findings made on hemoglobin and glycogen phosphorylase, suggesting a common underlying mechanism. Addition of organic cosolvents to water is known to reduce hydrophobic interactions, and we suggest that this effect may preferentially stabilize the more "relaxed" conformations of allosteric proteins, because they have a larger surface exposed to solvent [Chothia, C. (1974) *Nature (London) 248*, 338–339]. On the other hand, we suggest that the stabilization of the T state by low concentrations of all but the most polar cosolvents simply reflects stronger electrostatic interactions in this conformation.

According to the concerted model for allosteric transitions (Monod et al., 1965), allosteric proteins exist in solution as a mixture of conformational states characterized by very different biological activities. Specific ligands can bind preferentially to one conformation or another, thereby shifting the preexisting equilibrium and resulting macroscopically in an increased or decreased activity. Such a conformational equilibrium, like any chemical equilibrium, should be sensitive to physicochemical parameters such as pressure, temperature, or solvent composition: therefore, any model implying an equilibrium between several conformations predicts that a shift in one of these variables eventually results in a change of protein activity, even in the absence of any binding of physiological regulatory ligand. Indeed, early observations made on threonine deaminase (Changeux, 1965) and aspartate transcarbamylase (Weitzman & Wilson, 1966; Chan, 1981) showed that at least some of the effects of positive allosteric effectors could be mimicked by the addition to the assay mixture of molar concentrations of the chaotropic agent urea. More recently, several authors have described the influence of organic solvents on the affinity of human hemoglobin for oxygen (Cordone et al., 1979, 1981; Bulone et al., 1983; Haire & Hedlund, 1983), and the observed effects were interpreted in term of solvent dependence of the T $\rightleftharpoons$ R equilibrium position. Similar but more spectacular solvent effects have been reported in the case of muscle glycogen phosphorylase.

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This enzyme, which is normally inactive in the absence of mononucleotides, is enormously stimulated by the addition of a wide variety of organic compounds to the assay mixture (Dreyfus et al., 1978; Uhing et al., 1979, 1981). Regarding the modifications of the kinetic properties of glycogene phosphorylase, these new activators very much resemble bona fide allosteric activators. However, solvent activation requires unusually large concentrations (i.e., in the molar range) and seems to involve very little structural specificity, since simple aliphatic alcohols, amides, ethers, etc. are all found to activate the enzyme to some degree (the term "cosolvent" will be used as a general name for these compounds throughout the text). Moreover, chemical modification studies showed that neither of the two activator sites known on the phosphorylase molecule was required for cosolvent activation (Dreyfus et al., 1978; M. Dreyfus et al., unpublished results). All these features indicate that the cosolvents do not act through specific binding to a stereochemically defined site but rather that they influence nonspecifically the allosteric equilibrium through modification of the solvent properties.

To test further whether these solvent effects are of general significance in allostery, we have analyzed the effect of cosolvents on another allosteric system, the aspartate transcarbamylase (ATCase) from *Escherichia coli*. This enzyme (EC 2.1.3.2) catalyzes the first reaction of the biosynthetic pathway for pyrimidines, that is, the formation of carbamyl aspartate from carbamyl phosphate and aspartate. The *E. coli* enzyme has a complex oligomeric structure: it consists of two catalytic trimers and three regulatory dimers, held together by noncovalent bonds. This enzyme presents both types of interactions characteristic of an allosteric protein, i.e., homotropic interactions between the catalytic sites for the binding of the substrate, aspartate, and heterotropic interactions between regulatory and catalytic sites, allowing for the retroinhibition of its activity by CTP, the end product of the pathway, and for its activation by ATP. The catalytic and regulatory properties of the enzyme have been extensively reviewed (Jacobson & Stark, 1973; Kantrowitz et al., 1980a,b).

As far as homotropic cooperative interactions are concerned, most observations support the idea that the binding of aspartate (or its analogues) at the catalytic site favors a someway concerted transition of the whole molecule to a conformation having a higher affinity for this substrate. In this respect, ATCase fits the concerted model for allosteric transitions (Monod et al., 1965). However, concerning the heterotropic interactions, the nucleotides CTP and ATP appear not to affect the enzyme activity through preferential binding to one of the two states, as predicted by the concerted model: rather, the fixation of these effectors on their regulatory sites affects the affinity toward aspartate of the nearby catalytic site ("primary" or local "effect"). This phenomenon results in a decreased or increased occupation of this site by aspartate, which in turn causes a shift of the allosteric equilibrium ("secondary effect") (Thiry & Hervé, 1978; Tauc et al., 1982).

The results obtained in the present work show that the allosteric properties of ATCase are very sensitive to the addition of cosolvents, which can stabilize the conformations having either high or low affinity for the substrate, aspartate. Moreover, several aspects of the solvent effect on ATCase, hemoglobin, and phosphorylase are similar, suggesting a common underlying physicochemical mechanism, the nature of which is discussed.

Materials and Methods

Chemicals. Methanol, ethanol, 2-propanol, 1-propanol, 2-methyl-2-propanol, tris(hydroxymethyl)aminomethane (Tris), ammonium persulfate, and dioxane were purchased from Merck; glycine, glycerol, and acetone were from Prolabo; acetamide and formamide were from Carlo-Erba; *N*-methylformamide and aspartate were from Fluka; *n*-butyramide, isobutyramide, propionamide, and urea were from Aldrich. These compounds were of the highest purity available and used as such, except for solids, which were recristallized and then extensively dried before use. Acrylamide, bis(acrylamide), and tetramethylethylenediamine (TEMED) were obtained from Serva; cacodylic acid was from Touzart et Matignon; carbamyl phosphate was from Sigma; [^{14}C]asparate was from Service de Biochimie, C.E.N., Saclay.

Enzyme Preparation and Assay. ATCase was prepared and dissociated into catalytic and regulatory subunits according to Gerhart & Holoubeck (1967), and the enzymatic activity was tested under the conditions previously described (Perbal & Hervé, 1972), with 0.1–0.4 μg of enzyme or as indicated. During the preparation and incubation of the reaction mixture, the tests tubes were kept covered with plastic caps in order to avoid cosolvent evaporation. Double-reciprocal plots and Eadie representations were calculated by using a computerized linear-regression method.

Carbamylated ATCase. This modified enzyme was prepared by reaction with potassium cyanate (Tauc, 1982). The description of this preparation and of the enzymatic properties of this modified enzyme will be published elsewhere.

Electrophoresis through Transverse Gradients of Cosolvents. Acrylamide slab gels containing transverse gradients of cosolvents were run essentially as described by Creighton (1979). The gels (7 cm × 8 cm × 1 mm) were cast with the help of a peristaltic pump (1 mL/mn), care being taken to pour the denser solution first (i.e., the solvent-rich solution in the case of formamide or urea and the water-rich solution in the case of alcohols). Linear polyacrylamide (BDH) was routinely added (0.2%) to all gel solutions to increase the viscosity and stabilize the gradients before polymerization (Douglas et al., 1979). A few crystals of bromophenol blue were added to the denser solution to visualize the gradient. The gels were polymerized chemically with 0.03% TEMED and 0.03% ammonium persulfate. The concentrations of acrylamide and bis(acrylamide) (5 and 0.13%, respectively) were constant throughout the gradient, no effort being made to correct for the variation of protein mobility due to cosolvent (Creighton, 1979).

Electrophoresis was carried out in 10 or 15 mM Tris–glycine buffer, pH 8.3, and lasted 30 min at room temperature. The dissipated power was 2 W. After a short preelectrophoresis, the gel was loaded with the protein sample dissolved (2–20 μg/mL) in the electrophoresis buffer containing 5% glycerol. Carbamyl phosphate (1 mM) was added to the cathodic buffer tank in all experiments. Immediately after electrophoresis, the proteins were visualized by using the rapid silver stain of Ansorge (1982), and the gels were photographed with a polaroid camera.

Results

The effect of cosolvents on ATCase activity was first investigated at an aspartate concentration (5 mM) too low to markedly shift the allosteric equilibrium toward the R state. Simple aliphatic alcohols were assayed because of their well-known effects on other allosteric equilibria (Dreyfus et al., 1978; Uhing et al., 1979, 1981; Cordone et al., 1979, 1981). Amides and ureas were also included because, unlike most other cosolvents, lower members of these series are known to increase the dielectric constant of water. Dioxane and acetone were also assayed as typical apolar and moderately polar

Table I: Difference of Dielectric Constant (ϵ) and Standard Free Energy of Transfer of Model Compounds (F_{tr}) from Water to Aqueous Solutions of the Cosolvents Used in This Work[a]

	ϵ (25 °C)[a]	F_{tr} for *N*-acetyl-L-tryptophan ethyl ester (cal M^{-1})[b]
methanol	−3.5	−256
ethanol	−4.5	−312
1-propanol	−5.4	−383
2-propanol	−5.7	−344
1-butanol		−660
2-methyl-2-propanol	−6.8	−285[d]
formamide	4.0	−366
N-methylformamide	2.0	−568
acetamide	2.0	−337
propionamide	0.2	−395
butyramide		−481
urea	4.0	−212
methylurea		−297
ethylurea		−395
propylurea		−491
butylurea		−624
dioxane	−9.1	−670
acetone	−4.4	

[a] Features quoted refer to 10% v/v for liquid cosolvents and 10% w/v for solids. [b] Values for alcohols and acetone are extrapolated from Akerlof (1932); dioxane value is from Akerlof & Short (1936); values for amides are estimated graphically from Rohdewald & Möldner (1973); urea value is from Pottel et al. (1975). [c] Extrapolated, assuming linear dependence on solvent concentration, from Herskovits et al. (1970a–c) for alcohols, ureas, and amides, respectively, and from Maurel (1978) for dioxane. [d] The value for 2-methyl-2-propanol seems abnormally small. For instance, it can be extrapolated from Oakenfull & Fenwick (1979) that F_{tr} for one methylene group from water to 10% 2-methyl-2-propanol is much more negative than from water to 10% ethanol.

aprotic solvents, respectively. Table I lists the effects of these compounds on the dielectric constant of water and on the hydrophobic interactions, as expressed by the solubility of the typical apolar compound *N*-acetyl-L-tryptophan ethyl ester.

The cosolvents tested could be separated into two groups according to their effect on ATCase (Figure 1): the four compounds (formamide, acetamide, *N*-methylformamide, and urea) that increase the dielectric constant of the medium were found to stimulate the enzyme for all concentrations tested (data for *N*-methylformamide are not shown). All other cosolvents inhibited ATCase when assayed at low concentrations. However, the activity never decreases to zero, and in some cases, it reaches a well-defined plateau corresponding to about 90% inhibition, while in many other cases increasing the cosolvent concentration results in a sharp reversal of the initial inhibition, followed by a net activation (Figure 1). Since, in this case, the influence of cosolvents on activity is clearly a superposition of two different effects, it was difficult to analyze the influence of the nature of the cosolvents on each of them. In the alcohol series, however, the two effects occurred in distinct concentration ranges, making such analysis possible. It is seen that the concentration needed to reverse the inhibition varies considerably from one alcohol to another: longer chain alcohols are more efficient than short-chain ones, and for a given number of carbon atoms, linear alcohols are more efficient than branched ones (Figure 1).

To ascertain whether the observed effects are due to allosteric shifts or simply to changes in the catalytic properties of the enzyme, we next investigated the effect of cosolvents on the isolated catalytic subunit, a nonregulated, Michaelian form of ATCase (Gerhart & Schachman, 1965). Remarkably, cosolvents generally influenced the activities of ATCase and its catalytic subunit in an opposite way (Figure 1). Most cosolvents, which at low concentrations inhibited ATCase,

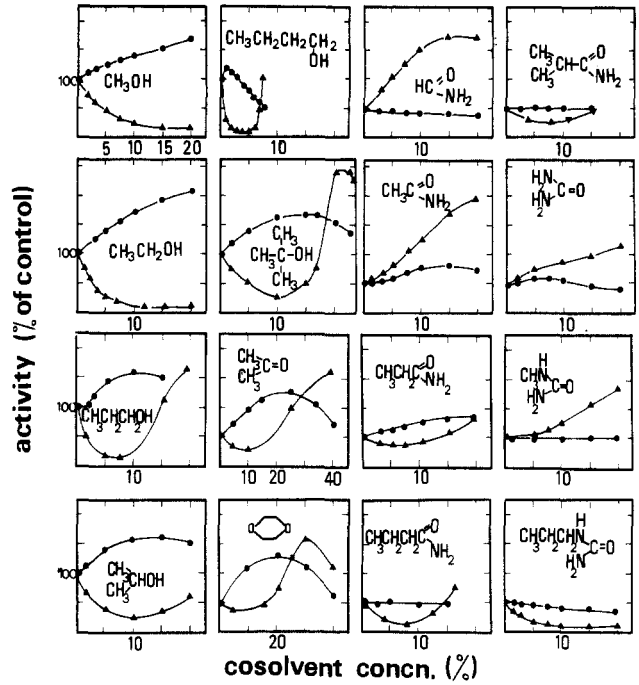

FIGURE 1: Influence of cosolvents on the activity of ATCase and its isolated catalytic subunits. ATCase or its isolated catalytic subunits were assayed as indicated under Materials and Methods in the presence of the different cosolvents, with 5 mM aspartate: (●) catalytic subunits; (▲) ATCase.

activated the catalytic subunits until the activity reaches a maximum and eventually decreases, presumably because of irreversible denaturation (see below). In contrast, the highly polar cosolvents that markedly activate ATCase at any concentration had a much smaller effect on the activity of the isolated catalytic subunit and, in the case of formamide, even inhibited it. It is therefore concluded that the gross changes in ATCase activity seen in Figure 1 are not due to perturbations of the catalytic mechanism by cosolvents but rather are true allosteric effects. This point will be further substantiated below.

For further studies, we decided to select cosolvents that were representative of the various effects seen in Figure 1. The modification of activity seen in the presence of 15% ethanol was considered as typical for the inhibition of ATCase by a solvent less polar than water. On the other hand, the increase of activity seen at 15% formamide and 20% 2-methyl-2-propanol were considered as typical for the ATCase activation by polar and nonpolar solvents, respectively.

Stability of ATCase in the Presence of Cosolvents. Before further analyzing the complex behavior seen in Figure 1, we had to check the stability of the enzyme under these rather harsh conditions. For this purpose, the enzyme (10 μg/mL) was incubated in the presence of a chosen solvent, and aliquots were assayed for enzymatic activity after various incubation times. Since saturating concentrations of carbamyl phosphate were used in all activity tests, this substrate was also included in the preincubation mixture. Under these conditions, both ATCase and its catalytic subunits were stable in 15% ethanol and 15% formamide; however, in the presence of 20% 2-methyl-2-propanol, loss of activity occurred that was more rapid for the more fragile catalytic subunit, while for ATCase is was slow enough not to interfere with a standard 10-min activity assay.

A more sensitive way of detecting protein denaturation and/or dissociation is native gel electrophoresis through a concentration gradient of cosolvent perpendicular to the di-

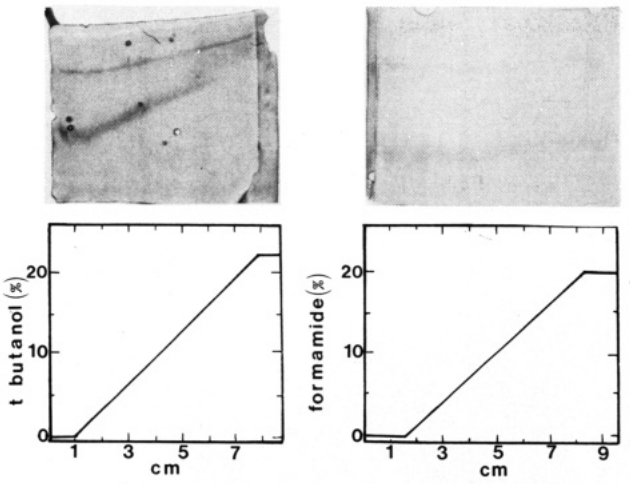

FIGURE 2: Electrophoresis of ATCase and its catalytic subunits through transverse gradients of ethanol and formamide. ATCase and catalytic subunits samples were analyzed by electrophoresis in transverse gradients of cosolvents as described under Materials and Methods, in the presence of 1 mM carbamyl phosphate. Solvent concentration increases from left to right, as schematically indicated in the lower part of the figure. Migration is from the top. The slow-moving and fast-moving bands correspond respectively to ATCase and its isolated catalytic subunit.

rection of migration (Creighton, 1979). In this technique each molecule in the sample migrates through a cosolvent–buffer mixture of uniform composition, but this composition varies from one side of the gel to the other (Figure 2). The use of very small polyacrylamide gels run at high voltages and stained with the very sensitive silver method of Ansorge (1982) allowed experimental conditions to approach those used in the activity assay, with respect to both the duration of the experiment (25–30 min) and the protein concentration used (2–20 μg/mL). In these experiments, we found no evidence for the dissociation of ATCase into its isolated catalytic and regulatory subunits under the influence of cosolvents. Typical gels containing transversal gradients of either 2-methyl-2-propanol (0–23% v/v) or formamide (0–20% v/v) are shown in Figure 2. It is seen that ATCase withstands a 20% concentration of either sovent without dissociation or denaturation, while the isolated catalytic subunit becomes denaturated above 15% 2-methyl-2-propanol. Thus, no conditions were found for which ATCase would dissociate into its isolated catalytic and regulatory subunits in the presence of cosolvents. It is concluded that the effects seen on the enzyme activity (Figure 1) are not due to dissociation.

Influence of the Cosolvents on the Activity of Isolated Catalytic Subunit. Since the presence of either 15% ethanol or 15% formamide was the conditions chosen for further studies on native ATCase activity, the kinetic properties of the isolated catalytic subunits were investigated under the same conditions. Saturation curves for aspartate showed that the stimulation of this subunit by ethanol (Figure 1) is entirely due to an increased enzyme affinity for aspartate. In the case of the slight inhibition provoked by formamide, a small decrease of V_{max} cannot be excluded (not shown).

Stabilization of the T State by 15% Ethanol. We next studied the kinetic properties of ATCase in the presence of 15% ethanol. Under these conditions, the saturation curve for aspartate is concave upward even for the highest aspartate concentrations used (Figure 3), suggesting that unusually large concentrations of this substrate are required to shift the allosteric equilibrium toward the R form. This is confirmed by the pH dependence of the activity at different aspartate concentrations: it is known that the T and R states have distinctly

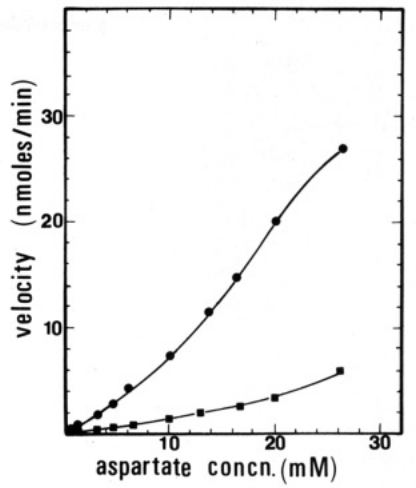

FIGURE 3: ATCase saturation curve for aspartate in the presence of 15% ethanol. ATCase saturation curve for aspartate was determined under the conditions reported under Materials and Methods in the absence (●) and presence (■) of 15% ethanol.

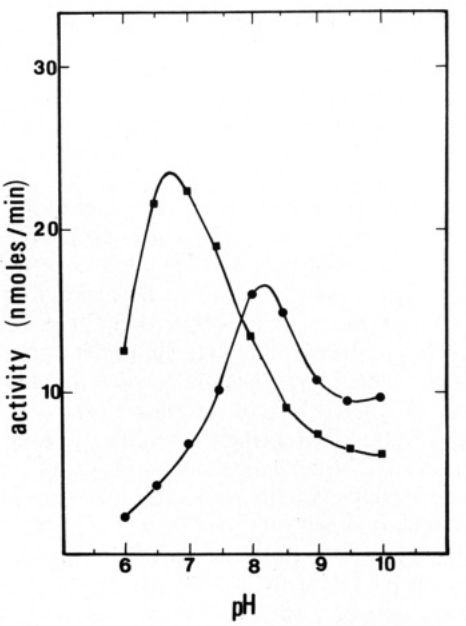

FIGURE 4: pH dependence of ATCase activity in the presence of 15% ethanol. The pH dependence of the activity of ATCase was determined under the conditions described under Materials and Methods in the presence of 20 mM aspartate and in the absence (●) or presence (■) of 15% ethanol with 0.1 and 0.4 μg of ATCase, respectively.

different pH vs. activity profiles (Gerhart & Pardee, 1964; Thiry & Hervé, 1978). At low aspartate concentation where ATCase is essentially in the T state, the activity vs. pH profile shows a maximum around pH 7. In contrast, for aspartate concentrations high enough to shift the enzyme into the R state, the profile shows a maximum around pH 8.2, which is also observed, irrespective of the aspartate concentration, for the isolated catalytic subunit, a Michaelian "R-like" form of ATCase. Figure 4 shows that, in the presence of 15% ethanol, the inhibited enzyme still exhibits the pH dependence characteristic of the T state even at 20 mM aspartate, a concentration normally high enough to shift the enzyme toward the R form. As a control, we checked that ethanol, and the other cosolvents used here, does not influence the pH dependence of the activity of the isolated catalytic subunits (Figure 5) whatever the aspartate concentration. These features indicate that the inhibition of ATCase by 15% ethanol is an allosteric effect.

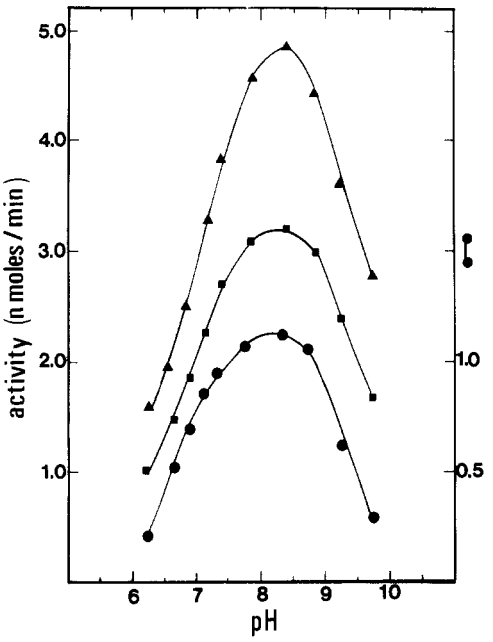

FIGURE 5: Lack of influence of ethanol and 2-methyl-2-propanol on the pH dependence of the isolated catalytic subunits. The pH dependance of the isolated catalytic subunits (0.12 μg) was determined as indicated under Materials and Methods in absence (●) or in the presence of either 15% ethanol (■) or 20% 2-methyl-2-propanol (▲) with 1 mM aspartate.

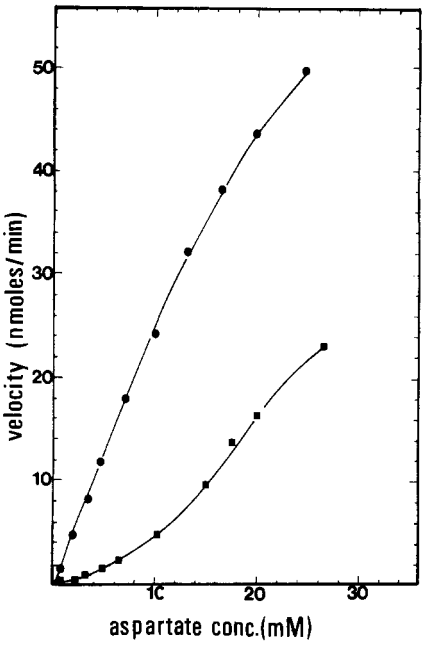

FIGURE 6: Reappearance of cooperativity in carbamylated ATCase under the influence of ethanol. The saturation curve for aspartate of carbamylated ATCase was determined as indicated under Materials and Methods in the the absence (●) and presence (■) of 15% ethanol.

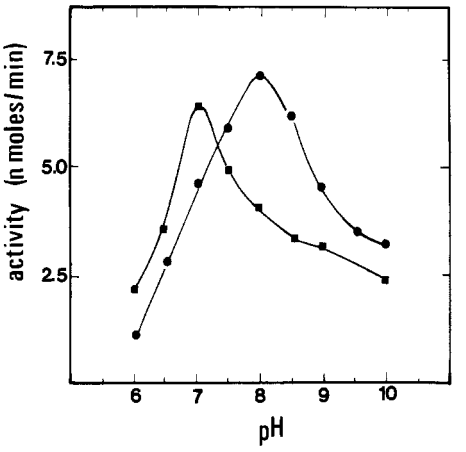

FIGURE 7: pH dependence of the carbamylated ATCase activity in the presence of 15% ethanol. The pH dependence of the activity of the carbamylated ATCase was determined under the conditions described under Materials and Methods in the presence of 15% ethanol and either 1 mM (■) or 20 mM (●) aspartate with 2.1 and 0.21 μg of enzyme, respectively.

Two extreme hypotheses can be proposed to explain this effect: in the first one, alcohol would simply stabilize the T form. This effect on the allosteric equilibrium would prevent, up to high substrate concentrations, the aspartate-promoted T to R conversion. Alternatively, alcohol may not directly influence the allosteric equilibrium, but rather it may decrease the affinity of the T state, or of both the T and R states, for aspartate. Higher substrate concentrations would then be necessary to reach the threshold saturation necessary for the T to R transition. Such an indirect mechanism is known to account for the effect of the nucleotides ATP and CTP on the allosteric equilibrium of ATCase (Tauc et al., 1982). In order to distinguish between these two possibilities, we studied the effect of ethanol on modified ATCases that are "frozen" in the R state, such as 2-thioU–ATCase (Kerbiriou & Hervé, 1972, 1973; Kerbiriou et al., 1977; Kantrowitz et al., 1977) or carbamylated ATCase (Tauc, 1982). These enzymes show a Michaelian response with respect to aspartate: therefore, the eventual restoration of a cooperative behavior in the presence of ethanol would clearly indicate a direct effect on the allosteric equilibrium.

Preliminary experiments showed that both modified enzymes are inhibited by ethanol, albeit less than normal ATCase. Examination of the kinetic response with respect to aspartate indicates that, in the case of the carbamylated ATCase, ethanol restores a sigmoid response (Figure 6). Moreover, while the carbamylated ATCase normally shows an activity vs. pH profile characteristic of the R state at all aspartate concentrations, in the presence of ethanol the pH vs. activity profile varies with the aspartate concentration. Patterns characteristic of the T and R states are observed at 1 mM and 20 mM aspartate, respectively (Figure 7). These findings confirm that ethanol acts directly on the allosteric equilibrium by stabilizing the T state. On the other hand, in the case of 2-thioU–ATCase, alcohol inhibition resulted in an increased K_m for aspartate, but the kinetic response of the inhibited enzyme vs. aspartate remained apparently Michaelian. These features can be explained if the shift to the T form is not efficient enough for its reversion by aspartate to be detected (Hensley et al., 1981).

Stabilization of the R State by 15% Formamide or 20% 2-Methyl-2-propanol. The nature of the activating effects seen in Figure 1 was next investigated. As seen in Figure 8, the kinetic response of ATCase vs. aspartate in the presence of 20% 2-methyl-2-propanol or 15% formamide is Michaelian, with K_m values of 4 mM and 30 mM, respectively.[1] Moreover, Figure 9 shows that in the presence of 20% 2-methyl-2-propanol, ATCase exhibits a maximal activity at pH 8 even for a low (5 mM) aspartate concentration. In this respect, it resembles the two R-like forms of ATCase, 2-thioU–ATCase and carbamylated ATCase. Similarly, in the presence of formamide, the activity vs. pH profile was independent of the

[1] However, it should be pointed out that part of the K_m decrease seen in the presence of 20% 2-methyl-2-propanol may be attributed to the increased aspartate affinity already reported for the isolated catalytic subunit in the presence of alcohols.

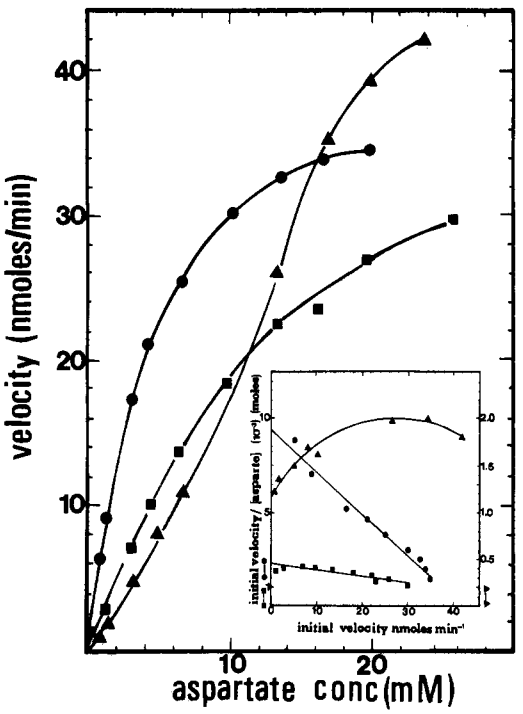

FIGURE 8: Saturation curve for aspartate of ATCase in the presence of 20% 2-methyl-2-propanol and 15% formamide. The saturation curves for aspartate of ATCase were determined as indicated under Materials and Methods in the absence (▲) or presence of either 20% 2-methyl-2-propanol (●) or 15% formamide (■), with 0.4 μg of enzyme in the absence and presence of 2-methyl-2-propanol and 0.15 μg in the presence of formamide. (Insert) Corresponding Eadie plots.

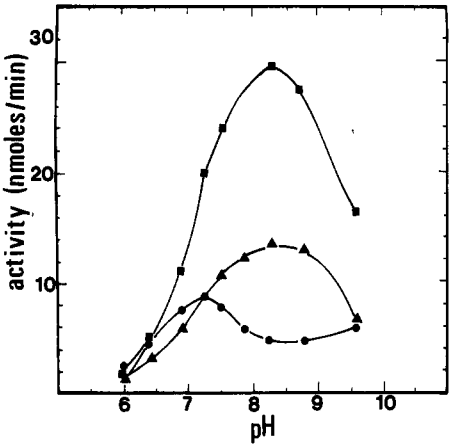

FIGURE 9: pH dependence of ATCase in the presence of 20% 2-methyl-2-propanol. The pH dependence of the activity of ATCase was determined as indicated under Materials and Methods with 5 mM aspartate in the absence (●) or presence (■) of 20% 2-methyl-2-propanol. Catalytic subunits in absence of cosolvent (▲).

aspartate concentration (not shown). Thus, on the basis of these three criteria—Michaelian response with respect to aspartate, high affinity for this substrate, and characteristic pH vs. activity profile—ATCase in the presence of 20% 2-methyl-2-propanol or 15% formamide appears to exist in the R state even at low concentrations of aspartate.

Discussion

Allosteric Equilibrium of ATCase Can Be Perturbed by the Addition of Cosolvents. The results reported here show that the activity of ATCase can be perturbed by the addition of organic cosolvents to its assay medium. We have presented evidence that these activity changes do not reflect modifications of the catalytic turnover of the enzyme. Rather, cosolvents show positive or negative heterotropic effects on the binding of the substrate aspartate and therefore behave as allosteric effectors. Highly polar cosolvents such as urea and the lower amides show positive effects on aspartate binding at all concentrations tested, whereas less polar compounds such as aliphatic alcohols, alkyl-substituted amides or ureas, acetone, and dioxane interact negatively with aspartate binding when assayed at low concentrations and positively at higher ones (Figure 1).

We now address to the possible mechanism of these solvent effects. Since a wide variety of organic structures are able to produce similar results (Figure 1), it is very unlikely that cosolvents act through binding to stereospecific sites as ordinary allosteric ligands do. In contrast, if ATCase is viewed as an equilibrium mixture of conformational states having different catalytic activities, we can readily interpret our results by assuming that cosolvents cause preferential solvation of some conformational states over the others and stabilize one or the other of these conformations. The same interpretation has been given for similar solvent effects observed in the case of glycogen phosphorylase (Dreyfus et al., 1978) and hemoglobin (Cordone et al., 1979, 1981; Haire & Hedlund, 1983). The existence of a conformational equilibrium preexisting to the binding of stereospecific ligands is one of the basic postulates of the concerted model for allosteric transitions (Monod et al., 1965). Although our observations clearly support this postulate in the case of ATCase, they do not indicate that only two states are present. In fact, there is increasing evidence that even the homotropic cooperative interactions in ATCase cannot be completely interpreted in terms of a simple two-state equilibrium, which would be shifted by the preferential binding of the substrate aspartate to the R conformation (Thiry & Hervé, 1978; Foote & Lipscomb, 1981). For the sake of simplicity, we shall nevertheless interpret the negative and positive heterotropic effects of cosolvents in term of stabilization of only two states, T and R, keeping in mind that any model implying equilibrium between conformations having different affinities for aspartate would meet the following interpretations.

When trying to give a precise physicochemical explanation of our findings, we are faced with the difficulty that many features of the solvent–protein interaction remain poorly understood. The delicate allosteric balance presumably results from the cancellation of large energetic contributions, all of which may vary upon addition of cosolvents and account for the observed effect. Thus, at this point, the interpretation is tentative. The stabilization of the T state by low concentrations of all cosolvents less polar than water could be an electrostatic effect: conceivably, the T conformation is constrained by salt bridges that should be stabilized by lowering the dielectric constant of the medium. In addition, it has been suggested that low concentrations of ethanol or 2-methyl-2-propanol can stabilize protein conformations having a high α-helix content [Oakenfull & Fenwick (1979) and references cited therein]. Such an effect may also contribute to the preferential stabilization of the T state by low concentrations of cosolvents, since an 8% decrease of the α-helix content of ATCase during the T to R transition has been reported (Windlund-Gray et al., 1973).

R Structures Are Stabilized by Lowering Hydrophobic Interactions. Concerning the stabilization of the R form, the results obtained here allow a more sound, although still speculative, interpretation. When ATCase (this work), glycogen phosphorylase (Dreyfus et al., 1978), and hemoglobin (Cordone et al., 1979, 1981; Haire & Hedlund, 1983) are compared, a common feature appears: at concentrations high

enough, most cosolvents tend to stabilize the R forms of these three proteins,[2] thereby suggesting a common underlying mechanism.

Amino acid residues, when transferred from the protein interior to pure water, tend to structurally organize the surrounding solvent, and this phenomenon results in a large, unfavorable entropy contribution to the free energy of transfer. This contribution, named hydrophobic energy, is believed to play a major role in maintaining the native structures of proteins (Kauzmann, 1959; Chotia, 1974, 1975). The addition of organic solvents to the medium tends to weaken the hydrophobic energy, as demonstrated by the increased solubility of model organic compounds (Table I). We suggest that this is the source of the extra stabilization of the R form of ATCase. In the alcohol series, it is clear that short-chain compounds are less efficient than longer ones in stabilizing the R state; for a given number of carbon atoms, the stabilization is lower for branched alcohols than for straight chain ones (Figure 1). The same order is found for the solubilizing power of alcohols on model apolar compounds (Table I). Moreover, again the same order was observed in studies of the stabilization of R states of phosphorylase (Dreyfus et al., 1978) and hemoglobin (Cordone et al., 1979, 1981). These results suggest that, in these three systems, the stabilization of the R state is linked to the decrease of the hydrophobic interactions. A similar interpretation has been given by Cordone et al. (1979, 1981) in the case of hemoglobin.

Chothia (1974) showed that the hydrophobic contribution to the free energy of a protein is proportional to the area of its interface with water. For ATCase, low-resolution structural data indicate a considerable expansion of the molecule during the T to R transition (Kirchner & Schachman, 1971; Moody et al., 1979; Ladner et al., 1982). For hemoglobin, analysis of the high-resolution structure of the T and R forms indicates that the intersubunit contact area is substantially reduced in the R state compared to the T state (Chothia et al., 1976). In the case of phosphorylase, activation results in a small (2-Å) movement of the subunits away from the dimer interface (Sprang & Fletterick, 1980). Therefore, in these three systems, it is plausible that the T state has a reduced water–protein interface area, compared with the R state, and thus is comparatively stabilized by hydrophobic interactions. Any factor—such as the introduction of organic cosolvents—that would decrease these interactions should comparatively stabilize the R state, as observed.

Allosteric proteins are often considered as having evolved from nonregulated subunits that acquired quaternary constraints, which then maintain the protein in a low-affinity state in the absence of ligands (Monod et al., 1965). Indeed, in the case of ATCase and hemoglobin, it is known that the dissociated, Michaelian subunits have higher affinity for their substrates than the oligomeric protein. The binding of the substrates releases these constraints, thus converting the oligomer into a conformation more like that of the isolated protomer. Since the association of proteins usually causes a large decrease of the surface exposed to solvent (Chothia & Janin, 1975; Janin & Chothia, 1976), a partial reversal of this process is expected to result in an increase of this surface. Therefore, the more unfavorable hydrophobic energy in the R state compared with the T state may be a simple and general consequence of the release of quaternary constraints. If so, solvent effects similar to those reported here are expected to hold in other allosteric systems as well. Actually, phosphofructokinase I from *E. coli* (Blangy et al., 1968) is considerably stimulated by ethanol and methanol (M. Dreyfus, unpublished observation). Several other examples of complex allosteric systems that can be activated by cosolvents exist in the literature (Singh & Wang, 1979; Shuster, 1979; Rubio & Lorrente, 1982).

[2] Some exceptions are indeed observed and may, in some cases, be traced back to specific binding of cosolvents to the protein, due to remote structural similarity between them and substrates or allosteric effectors. For example, cyclic ethers such as dioxane and tetrahydrofuran have been found to inhibit, rather than activate, phosphorylase *b* or AMP-saturated phosphorylase *b* (Uhing et al., 1979; B. Vandenbunder and M. Dreyfus, unpublished results). However, kinetic experiments reveal that dioxane is a competitor of AMP, and modification of the enzyme by an AMP analogue, which binds covalently at the AMP site but is hardly an activator, results in an enzyme preparation that is fully activable by dioxane (B. Vandenbunder and M. Dreyfus, unpublished results).

Acknowledgments

We are much indebted to Drs. Henri Buc, Antoine Danchin, and Caris Ghelis for stimulating discussions and to Dr. Beatrice Holton for critical reading of the manuscript.

Registry No. ATCase, 9012-49-1; methanol, 67-56-1; ethanol, 64-17-5; 1-propanol, 71-23-8; 2-propanol, 67-63-0; 1-butanol, 71-36-3; 2-methyl-2-propanol, 75-65-0; formamide, 75-12-7; *N*-methylformamide, 123-39-7; acetamide, 60-35-5; propionamide, 79-05-0; butyramide, 541-35-5; urea, 57-13-6; methylurea, 598-50-5; ethylurea, 625-52-5; propylurea, 627-06-5; butylurea, 592-31-4; dioxane, 123-91-1; acetone, 67-64-1; aspartic acid, 56-84-8.

References

Akerlöf, G. (1932) *J. Am. Chem. Soc. 54*, 4125–4139.
Akerlöf, G., & Short, O. A. (1936) *J. Am. Chem. Soc. 58*, 1241–1243.
Ansorge, W. (1982) *Proceedings of Electrophoresis, 82nd*, Athênes, 1982 (Stathakos, C., Ed.) de Gruyter, Berlin.
Blangy, D., Buc, H., & Monod, J. (1968) *J. Mol. Biol. 31*, 13–35.
Bulone, D., Cupane, A., & Cordone, L. (1983) *Biopolymers 22*, 119–125.
Chan, W. W. (1981) *Can. J. Biochem. 59*, 371–378.
Changeux, J. P. (1965) *Bull. Soc. Chim. Biol. 47*, 267–280.
Chothia, C. (1974) *Nature (London) 248*, 338–339.
Chothia, C. (1975) *Nature (London) 254*, 304–308.
Chothia, C., & Janin, J. (1975) *Nature (London) 256*, 705–708.
Chothia, C., Wodak, S., & Janin, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3793–3797.
Cordone, L., Cupane, A., San Biagio, P. L., & Vitrano, E. (1979) *Biopolymers 18*, 1975–1988.
Cordone, L., Cupane, A., San Biagio, P. L., & Vitrano, E. (1981) *Biopolymers 20*, 39–51.
Creighton, T. E. (1979) *J. Mol. Biol. 129*, 235–264.
Douglas, M., Finkelstein, D., & Butow, R. A. (1979) *Methods Enzymol. 56*, 58–66.
Dreyfus, M., Vandenbunder, B., & Buc, H. (1978) *FEBS Lett. 95*, 185–189.
Foote, J., & Lipscomb, W. N. (1981) *J. Biol. Chem. 256*, 11428–11433.
Gerhart, J. C., & Pardee, A. B. (1964) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 23*, 727–735.
Gerhart, J. C., & Schachman, H. K. (1965) *Biochemistry 4*, 1054–1062.
Gerhart, J. C., & Holoubeck, H. (1967) *J. Biol. Chem. 242*, 2886–2892.
Haire, R. N., & Hedlund, B. E. (1983) *Biochemistry 22*, 327–334.
Hensley, P., Yang, Y. R., & Schachman, H. K. (1981) *J. Mol. Biol. 152*, 131–152.

Herskovits, T. T., Gadegbeku, B., & Jaillet, H. (1970a) *J. Biol. Chem. 245*, 2588–2598.
Herskovits, T. T., Jaillet, H., & Gadegbeku, B. (1970b) *J. Biol. Chem. 245*, 4544–4550.
Herskovits, T. T., Jaillet, H., & Desena, A. T. (1970c) *J. Biol. Chem. 245*, 6511–6517.
Jacobson, G. R., & Stark, G. R. (1973) *Enzymes, 3rd Ed. 9*, 225–308.
Janin, J., & Chothia, C. (1976) *J. Mol. Biol. 100*, 197–211.
Kantrowitz, E. R., & Lipscomb, W. N. (1977) *J. Biol. Chem. 252*, 2873–2880.
Kantrowitz, E. R., Pastra-Landis, S. C., & Lipscomb, W. N. (1980a) *Trends Biochem. Sci. (Pers. Ed.) 5*, 124–128.
Kantrowitz, E. R., Pastra-Landis, S. C., & Lipscomb, W. N. (1980b) *Trends Biochem. Sci. (Pers. Ed.) 6*, 150–153.
Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1–63.
Kerbiriou, D., & Hervé, G. (1972) *J. Mol. Biol. 64*, 379–392.
Kerbiriou, D., & Hervé, G. (1973) *J. Mol. Biol. 78*, 687–702.
Kerbiriou, D., Hervé, G., & Griffin, J. H. (1977) *J. Biol. Chem. 252*, 2881–2890.
Kirchner, M. W., & Schachman, H. K. (1971) *Biochemistry 10*, 1919–1926.
Ladner, J. E., Kitchell, J. P., Honzatko, R. B., Ke, H. M., Volz, K. W., Kalb, A. J., Ladner, R. C., & Lipscomb, W. N. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3125–3128.
Maurel, P. (1978) *J. Biol. Chem. 253*, 1677–1683.
Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118.
Moody, M. F., Vachette, P., & Foote, A. M. (1979) *J. Mol. Biol. 133*, 517–532.
Oakenfull, D., & Fenwick, D. E. (1979) *J. Chem. Soc., Faraday Trans. 1, 75*, 636–645.
Perbal, B., & Hervé, G. (1972) *J. Mol. Biol. 70*, 511–529.
Pottel, R., Adolph, D., & Kaatze, U. (1975) *Ber. Bunsenges. Phys. Chem. 79*, 278–285.
Rohdewald, P., & Möldner, M. (1973) *J. Phys. Chem. 77*, 373–377.
Rubio, V., & Llorente, P. (1982) *Biochem. Biophys. Res. Commun. 107*, 1400–1405.
Schuster, S. M. (1979) *Biochemistry 18*, 1162–1167.
Singh, T. P., & Wang, J. H. (1979) *J. Biol. Chem. 254*, 8466–8472.
Sprang, S., & Fletterick, R. J. (1980) *Biophys. J. 32*, 175–192.
Tauc, P. (1982) Thèse de Doctorat de 3ème Cycle, Paris XI, Orsay.
Tauc, P., Leconte, C., Kerbiriou, D., Thiry, L., & Hervé, G. (1982) *J. Mol. Biol. 155*, 155–168.
Thiry, L., & Hervé, G. (1978) *J. Mol. Biol. 125*, 515–534.
Uhing, R. J., Janski, A. M., & Graves, D. J. (1979) *J. Biol. Chem. 254*, 3166–3169.
Uhing, R. J., Lentz, S. R., & Graves, D. J. (1981) *Biochemistry 20*, 2537–2544.
Weitzman, P. D. J., & Wilson, I. B. (1966) *J. Biol. Chem. 241*, 5481–5488.
Winlund-Gray, C., Chamberlin, M. J., & Gray, D. M. (1973) *J. Biol. Chem. 248*, 6071–6079.

Reinvestigation of the Structure of Oxidized and Reduced Flavin: Carbon-13 and Nitrogen-15 Nuclear Magnetic Resonance Study†

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ABSTRACT: Several chemically substituted flavins are investigated in the oxidized and the reduced state by ^{13}C and ^{15}N NMR techniques. The dependence on the polarity of the solvent and on the concentration is studied. In combination with already published results, a semiempirical theory is developed to interpret the chemical shifts in terms of the solution structure of flavins. Where possible, the results are compared with crystallographic and light absorption data. In contrast to common ideas, the solution structure of the oxidized state is not fully coplanar, but the N(10) atom is situated out of plane to a certain degree. Polarizing the flavin by hydrogen bonds in a high dielectric medium moves the N(10) atom into the molecular plane, and the flavin molecule becomes coplanar. In the coplanar molecule, π electrons are delocalized from the N(10) atom mainly to O(2α) and O(4α). The NMR results show that the solution structure of reduced flavin is mainly governed by sterical hindrance and hydrogen bonds. The findings are in contrast to commonly accepted ideas that reduced flavin is strongly bent. In an apolar solvent, the reduced neutral isoalloxazine is only slightly bent. The formation of hydrogen bonds in a protic solvent of a high dielectric constant decreases the bend. The N(10) atom is now almost fully sp^2 hybridized, and the N(5) atom has an endocyclic angle of 115–117°, indicating its predominant sp^2 character. The results have several important implications for flavin catalysis. Among these, it is shown that the altered redox potential of the semiquinone–fully reduced redox couple of flavodoxin is probably not caused by the planarity of the reduced protein-bound $FMNH^-$.

The flavins are especially remarkable among the various known natural redox coenzymes because of two outstanding features: (i) they can function as one-electron as well as two-electron redox carriers; (ii) they act with considerable efficiency in a wide variety of enzymatic reactions. As a consequence, the flavins are known as very versatile redox coenzymes. This versatility suggests that nature has many possibilities to "tune" the function of the flavin. This has led to the proposal that specific interactions between flavin and apoflavoprotein play a particular role in determining the pathway of flavin catalysis (Müller, 1972; Müller et al., 1970; Hemmerich & Massey, 1982). Other factors such as mobility of the flavin (Moonen & Müller, 1983), microenvironment of the flavin binding site, and the planarity of the reduced

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0006-2960/84/0423-4859$01.50/0