The Monovalent Cation-Induced Association of Formyltetrahydrofolate Synthetase Subunits: A Solvent Isotope Effect†

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ABSTRACT: In the presence of specific monovalent cations (K^+, Cs^+, NH_4^+), inactive monomers of formyltetrahydrofolate synthetase associate to a catalytically active tetramer. The rate and extent of association of enzyme monomers prepared from *C. cylindrosporum* are enhanced 3.3- and about 50-fold, respectively, by the substitution of D_2O for H_2O. Both rate and equilibrium solvent isotope effects are due to a decrease in D_2O of the dissociation constant of the monomer–cation complex. Analysis of rate and equilibria data obtained in solvent mixtures of varying deuterium/protium ratios indicates that the isotope effect may be due to the change in bonding of a single monomer proton during the association process. The data are most consistent with a model in which this proton is in a very weak potential in the cation-free monomer and is converted to a "normal" water-like proton in the monomer–cation complex.

The mechanism of a reaction is normally deduced by observing the effect of perturbants on that reaction. The substitution of deuterium for hydrogen causes a theoretically desirable modest perturbation, affecting only those properties of the system sensitive to mass, and produces easily measurable results known as isotope effects. When the solvent water is replaced by deuterium oxide, the rate and equilibrium constants of a reaction may change. This solvent isotope effect (SIE) may be due to one of or to a combination of the following (Schowen, 1972): (a) water itself may be a reactant, (b) solute protons which are important in the reaction may be replaced by deuterons, or (c) the nature of the solute–solvent interaction may be affected.

This report describes the effect of deuterium oxide on the association of subunits of formyltetrahydrofolate synthetase (EC 6.3.4.3) induced by monovalent cations. The reaction of specific cations (C^+) with the monomer, a catalytically inactive form of the enzyme, produces an enzymatically active tetramer (Scott and Rabinowitz, 1967; MacKenzie and Rabinowitz, 1971; Welch et al., 1971; Harmony et al., 1974).

$$\underset{\text{inactive}}{4\text{monomer}} + 2C^+ \rightleftarrows \underset{\text{active}}{\text{tetramer} \cdot 2C^+} \qquad (1)$$

We have been interested in three aspects of the association process: the mechanism, the nature of the cation–protein reaction, and the nature of the noncovalent forces responsible for subunit–subunit interaction. Previous reports (Harmony et al., 1974; Harmony and Himes, 1975) discussed kinetic and thermodynamic aspects of subunit association in water. Deuterium oxide produces a substantial increase in both the rate and extent of association. We have attempted to identify those steps in the overall reaction sensitive to the isotopic composition of the solvent and to determine the origin of the SIE by a proton inventory analysis (Hopper et al., 1973; Kresge, 1964; Gold, 1969).

Experimental Procedure

Materials

Formyltetrahydrofolate synthetase was isolated from *Clostridium cylindrosporum* and from *Clostridium acidiurici* and was purified by established procedures (Rabinowitz and Pricer, 1962).[1] The methods used to determine the specific activity of the enzyme (Buttlaire et al., 1972; Himes and Cohn, 1967; Curthoys and Rabinowitz, 1971) and to prepare the substrate (+,−)-tetrahydrofolate from folic acid (Samuel et al., 1970) have also been described. Deuterium oxide was purchased from Stohler Isotope Chemicals and was routinely vacuum distilled. The pD of the commercial D_2O was 9.5; that of the distillate, 7.3. One molar stock solutions of Tris and Tris-HCl were prepared in H_2O and in D_2O.

Methods

Dissociation and Reassociation of the Enzyme. Formyltetrahydrofolate synthetase monomers were prepared by dialyzing the enzyme at 4° and at pH 8.1 in the absence of monovalent cations as described previously (Harmony and Himes, 1975). Subunit reassociation was initiated by the addition of 10 μl of the monomer solution to 90 μl of Tris buffer which contained monovalent cation and 4 m*M* dithiothreitol. Reassociation buffers of the desired concentration and pH or pD were obtained by mixing diluted stock solutions of Tris and Tris-HCl in the appropriate ratio. The amount of tetramer formed subsequent to the addition of monovalent cation was determined at various times by catalytic assay. Previous studies (MacKenzie and Rabinowitz,

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[1] A modified purification scheme suggested by J. C. Rabinowitz was employed to obtain formyltetrahydrofolate synthetase from *C. acidiurici*.

1971; Welch et al., 1971) have shown that the tetramer is the active form of the enzyme and that during reassociation only monomer and tetramer are detected. The rate and equilibrium constants of subunit association were calculated by published methods (Harmony et al., 1974; Harmony and Himes, 1975). The units of first- and second-order rate constants are sec^{-1} and liters (moles of tetramer)$^{-1}$ sec^{-1}, respectively.

Proton Inventory Procedure. (a) Theory. In a typical proton inventory study, the rate or extent of a reaction is measured as the amount of deuterated solvent relative to proteated solvent is increased. For reactions exhibiting a solvent isotope effect, it is in principle possible to analyze these rate and equilibrium data obtained in solvent mixtures to determine the number of exchangeable protons involved in the reaction as well as the contribution of each proton to the total isotope effect (Hopper et al., 1973; Kresge, 1964; Gold, 1969). A consideration of equilibria indicates that both reactant and product protons may contribute to the total equilibrium isotope effect and the expression derived to inventory these is:

$$K^n = K^0\left[\frac{\prod_i^{\mu}(1 - n + n\phi_i^{P})}{\prod_j^{\nu}(1 - n + \phi_j^{R})}\right] \quad (2)$$

where there are μ contributing protons in the product state and ν in the reactant state. The other terms are defined as follows: n = atom fraction of deuterium in mixed isotopic solvent; K^n = reaction equilibrium constant in solvent of atom fraction deuterium = n; K^0 = reaction equilibrium constant in pure H_2O; ϕ_i = isotopic fractionation factor of the ith product proton; ϕ_j = isotopic fraction factor of the jth reactant proton. The isotopic fractionation factor, ϕ, is a measure of the preference of the site for deuterium over protium relative to the similar preference of a single site in the solvent molecule (Schowen, 1972). The fractionation factor is therefore a measure of the relative tightness of binding to H or D at the specified site (Hopper et al., 1973).

For rate processes, the reaction rate constant k equals $(kT/h)K^{\ddagger}$ and an equation similar to eq 2 inventories those exchangeable protons in the transition state (T) and the ground state (R) which determine the total kinetic solvent isotope effect:

$$k^n = k^0\left[\frac{\prod_i^{\rho}(1 - n + n\phi_i^{T})}{\prod_j^{\nu}(1 - n + n\phi_j^{R})}\right] \quad (3)$$

In this treatment k^n and k^0 are the reaction rate constants in the mixed solvent and in pure water, respectively; ϕ_i is the isotopic fractionation factor of the ith transition state proton.

Proteins have numerous exchangeable protons, each existing in a distinct environment. Without suitable models it is difficult to predict the isotopic contribution of each proton in the reactant, product, or transition states; exact solutions of eq 2 and 3 are therefore prohibited at this time. However, limiting assumptions may be introduced and the data analyzed for each resulting, simplified situation. For example, in treating equilibria data, if the product protons all have $\phi_i = 1$ and therefore do not contribute to the isotope effect, the total SIE is due to "unusual" reactant protons and eq 2 simplifies to

$$K^n = K^0\left[\frac{1}{\prod_j^{\nu}(1 - n + n\phi_j^{R})}\right] \quad (4)$$

The function $(K^n)^{-1}$ vs. n is then a polynomial in n, the order of which gives the number of contributing motions ν and the coefficients of which can be related to the ϕ_j^{R}. Or if the reactant proton fractionation factors are all unity:

$$K^n = K^0\left[\prod_i^{\mu}(1 - n + n\phi_i^{P})\right] \quad (5)$$

and the isotope effect is entirely due to "unusual" product protons. In this case the relationship K^n vs. n can be analyzed to find μ and the ϕ_i^{P}.

Similar limiting assumptions may be applied when analyzing the kinetic SIE and eq 6 and 7 result from eq 3.

$$k^n = k^0\left[\frac{1}{\prod_j^{\nu}(1 - n + n\phi_j^{R})}\right] \quad (6)$$

$$k^n = k^0\left[\prod_i^{\rho}(1 - n + n\phi_i^{T})\right] \quad (7)$$

(b) Determination of the Number of Contributing Protons and Their Fractionation Factors. Situations described in eq 4–7 can be generalized such that

$$y^n = y^0\prod_k^{x}(1 - n + n\phi_k) \quad (8)$$

where x is the number of contributing protons (k = 1,2,3 . . . x). If the effect is due to the transition state or product state, $y^n = k^n$ or K^n and $y^0 = k^0$ or K^0, respectively. If the isotope effect is due entirely to the reactant state, $y^n = (k^n)^{-1}$ or $(K^n)^{-1}$ and $y^0 = (k^0)^{-1}$ or $(K^0)^{-1}$. Expansion of eq 8 gives

$$y^n = y^0 + c_1n + c_2n^2 + \ldots c_xn^x \quad (9)$$

and it is obvious that y is a polynomial in n of degree x. A polynomial regression computer program, BMDO5R (Dixon, 1968), is employed to determine x. The value of x giving the "best fit" of the data is determined by the F test (Fisher and Yates, 1957). The ϕ_k values could, in principle, be determined directly from the regression coefficients, c_k. However, this is a laborious procedure at best and with data of accessible precision may lead to physically unrealistic values. We have tentatively determined ϕ_k in the following manner. For situations in which the isotopic contributions of the x contributing protons are equal, eq 8 simplifies to

$$\frac{y^n}{y^0} = (1 - n + n\phi)^x \quad (10)$$

or

$$\left(\frac{y^n}{y^0}\right)^{1/x} - 1 = n(\phi - 1) \quad (11)$$

Thus a plot of $[(y^n/y^0)^{1/x} - 1]$ vs. n is a straight line with the slope equal to $(\phi - 1)$. If this relationship is nonlinear, the important isotopic fractionation factors are assumed to be nonequivalent. At this time we have elected to consider in some detail only those cases for which the contributing protons appear to have equal ϕ values. Once suitable models are found to describe the subunit association reaction, nonidentical ϕ values may be determined by fitting the data to equations based on these models.

Reliability of Proton-Inventory Results. The application

of mixed-solvent isotope effects to enzyme systems is a recent phenomenon and there has been some concern that the structural complexity of proteins may lead to difficulty in the interpretation of the results. For example, the linear proton inventory for deacetylation of acetyl-α-chymotrypsin was originally interpreted (Pollack et al., 1973) by assigning the entire solvent isotope effect to a single transition-state proton. In a commentary (Kresge, 1973) on this conclusion, several "alternative models" were adduced. In all of these, at least 75% of the solvent isotope effect was again assigned to a single transition-state proton, while the remainder was imagined to arise from a near-cancellation of effects distributed among from 7 to 21 different reactant-state and transition-state sites, each of which had to be assigned a fractionation factor in a specific range. These mathematical exercises exemplify the "highly fortuitous cancellation of n dependencies", noted as unlikely interpretations in the original report (Pollock et al., 1973), and indeed leave the original conclusion that "one proton in the catalytic transition state produces the solvent isotope effect" (Pollock et al., 1973) substantially unchanged. The "alternative models" are thus not serious mechanistic proposals but merely illustrate the generalization that, for all experiments, there exists an infinity of interpretations of great complexity which may be used to fit data which are also in agreement with simple models. It is, of course, customary to proceed on the basis of the simpler models until more complexity is *required* by the data.[2] In point of fact, the linear proton-inventory behavior observed for deacetylation of chymotrypsin has now been found for its acylation by *N*-acetyltryptophanamide, and for the acylation and deacylation of other serine proteases by various substrates, with a wide range of magnitudes of the solvent isotope effect (Elrod et al., 1975). To pursue the "alternative models" for each of these examples would require the unreasonable postulation in each case of a large number of different, but quite specific, fractionation factors leading to near-cancellation and generating a minor part of the solvent isotope effect. The complexity of proteins therefore does not generate any basic difficulty in the interpretation of proton-inventory results, although it may lend a certain verisimilitude to unnecessarily complicated interpretations.

Results

Kinetic Solvent Isotope Effect

pL Profile for Subunit Association. The pL-rate (L = H,D) curves in H_2O and in 90% D_2O obtained for potassium ion induced association of formyltetrahydrofolate subunits prepared from *C. cylindrosporum* are shown in Figure 1A. To determine an acurate SIE it is essential to compare data constants at the same relative position on the pL profile in each solvent mixture. The curves are similar except that the pL optimum is about 0.4 pL unit more basic in 90% D_2O compared to that in H_2O. This increase is of the magnitude expected if the ionization of a normal weak acid is important in the association process (Glasoe and Long, 1960). Furthermore, the result suggests that the deuterated

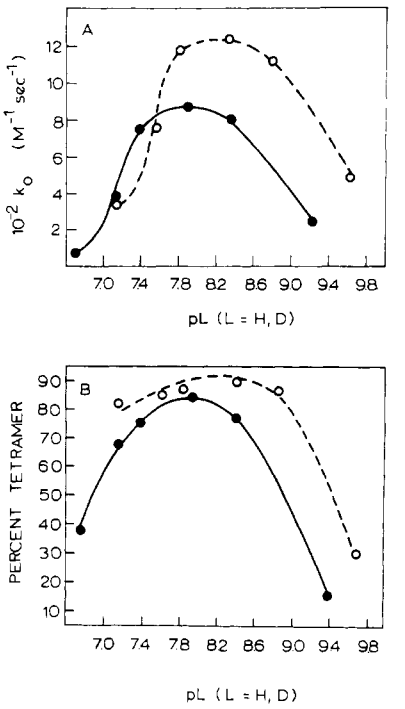

FIGURE 1: The dependence on pL in H_2O (●) and in 90% D_2O (○) of the rate (A) and the extent (B) of potassium ion induced tetramerization of formyltetrahydrofolate synthetase monomers prepared from *C. cylindrosporum*. Reaction solutions at 20° contained 0.7 mg/ml of monomer, 100 m*M* KCl, 4 m*M* dithiothreitol, and 100 m*M* Tris. The total ionic strength at each pH was adjusted to 0.2 by the addition of tetramethylammonium chloride.

solvent does not produce a conformationally altered ground state monomer. In the studies reported herein, rate constants are calculated at the pL optima. The significant enhancement of the association rate in 90% D_2O relative to that in H_2O is evident in Figure 1A.

Effect of D_2O on the Mechanism of Subunit Association. In water the cation-dependent interaction of *C. cylindrosporum* subunits is second order with respect to monomer concentration and first order in monovalent cation (Harmony et al., 1974; Harmony and Himes, 1975). Association of *C. acidi-urici* subunits is first order both in monomer and in cation (MacKenzie and Rabinowitz, 1971; Harmony and Himes, 1975). The proposed kinetically important steps are represented schematically by eq 12 and 13 for *C. cylindrosporum* and by eq 14 and 15 for *C. acidi-urici* (Harmony and Himes, 1975).[3] Cation reassociation of monomers from both clostridial species presumably involves the rapid formation of an initial monomer–cation complex. In the *C. cylindrosporum* system complex formation is followed by a rate-determining dimerization step; in the *C. acidi-urici* system, by a limiting conformational alteration. Reactions which occur after the limiting steps and which result in tetramer are probably rapid since stable intermediates between monomer and tetramer have not been observed

[2] This same principle applies in the examination of proton-inventory data for "pure-reactant" and "pure-product (or transition-state)" fits, as described in the last section. If neither of these approaches yields self-consistent results, more complex fits involving both types of fractionation factors may be examined. If the simple approach yields an apparently valid interpretation, however, the more complicated model is unjustified.

[3] A mechanism for association of *C. cylindrosporum* monomers which has not been considered here or previously (Harmony and Himes, 1975) is the following: $2M \rightleftharpoons D$, $D + C^+ \rightleftharpoons DC^+$, $DC^+ \rightleftharpoons (DC^+)^*$, $2[DC^+]^* \rightleftharpoons D_2C_2^+$ where DC^+ and $(DC^+)^*$ are different conformers. If the equilibrium of the first step lies far in the direction of M and the conformational step is rate limiting, the mechanism is consistent with kinetic and equilibrium data reported previously (Harmony and Himes, 1975). However, the basic conclusion of this work, that the solvent isotope effect is restricted to the cation binding step, is unchanged.

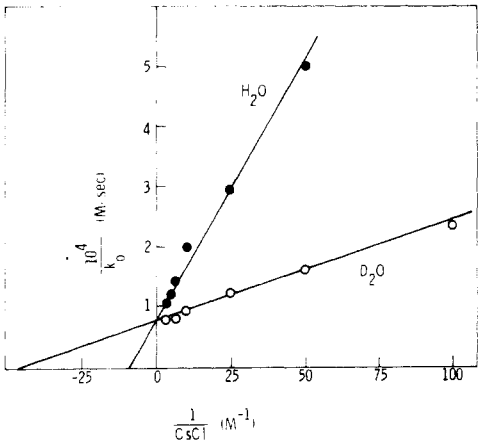

FIGURE 2: The rate in H_2O (●) and in 90% D_2O (○) of cesium ion dependent association of formyltetrahydrofolate synthetase monomers prepared from *C. cylindrosporum*. The monomer concentration was 1 mg/ml; the ionic strength was adjusted to 0.32 by the addition of tetramethylammonium chloride.

(MacKenzie and Rabinowitz, 1971; Welch et al., 1971). Appropriate analysis of the dependence of the association rate on monomer and cation concentration in deuterium oxide indicates that the observed SIE is not due to a change in reaction mechanism. Reaction of the *C. cylindrosporum* monomers in D_2O is second order in monomer, first order in potassium ion; association of *C. acidi-urici* monomers is first order in monomer. In the *C. acidi-urici* case it is not possible to determine the dependence on potassium ion in D_2O. Such experiments require that the total ionic strength be maintained at a constant value with a salt which does not specifically influence the reaction. Cations found to be ineffective in H_2O cause substantial association of *C. acidi-urici* monomers in D_2O. For example, the percent of tetramer formed in 90% D_2O after 3 hr at 20° in the presence of 40 m*M* Tris-HCl, 50 m*M* tetramethylammonium chloride, or 30 m*M* $MgCl_2$ was 60, 70, and 20%, respectively. These salts elicited no subunit association in H_2O. On the other hand, cations with more organic character such as the tri-*n*-butylammonium ion did not cause association in either solvent; they effectively inhibited potassium ion induced tetramer formation, however.

$$M + C^+ \rightleftarrows MC^+ \qquad K_a = K_d^{-1} \qquad (12)$$

$$MC^+ + M \xrightarrow{k_2} DC^+ \qquad (13)$$

$$M' + C^+ \rightleftarrows M'C^+ \qquad K_a' = K_d'^{-1} \qquad (14)$$

$$M'C^+ \xrightarrow{k_1} M^*C^+ \qquad (15)$$

M or M′ = monomer
D = dimer–cation complex
M* = conformationally altered monomer

Influence of D_2O on the Dissociation Constant of the Monomer–Cation Complex. The observed rate constant of association of *C. cylindrosporum* monomers in H_2O and in D_2O is plotted as a function of increasing CsCl concentration in Figure 2. Similar results are obtained with K^+. However, since K^+ is more effective than Cs^+ in promoting association (MacKenzie and Rabinowitz, 1971; Harmony and Himes, 1975), the rates of reaction in D_2O at comparable K^+ concentrations and total ionic strength are extremely rapid and are therefore subject to considerable experimental uncertainty. The important point illustrated in Figure 2 is the decrease in SIE (k_o^n/k_o^0; $n = 0.9$) which occurs with increasing Cs^+ concentrations. At 20 m*M* CsCl k_o^n/k_o^0 is 3.1; at 200 m*M* CsCl the isotope effect is about 1.5.

Since at low [cation] the observed rate constant, k_o, is equal to k_2/K_d (eq 12 and 13) (Harmony and Himes, 1975), D_2O may accelerate the rate by influencing the cation binding process, or the rate-limiting reaction, or both. At low [Cs^+] it is possible to determine $K_d^{Cs^+}$, the dissociation constant of the monomer-Cs^+ complex, and k_2 from data in Figure 2. $K_d^{Cs^+}$, calculated from the intercept on the abscissa, is approximately 100 m*M* in H_2O and 28 m*M* in 90% D_2O. The intercept on the ordinate, k_2^{-1}, is the same in both isotopic solvents. The rate constant k_2 may also be determined experimentally at saturating cation concentration since, under this condition, the reaction is zero order with respect to cation and the observed constant k_o is equal to k_2 (Harmony and Himes, 1975). At 400 m*M* KCl k_2 values did not vary between 0 and 90% D_2O. The average value over this range of D_2O was $7.8 \pm 0.3 \times 10^3$ M^{-1} sec^{-1}. Although obtained at higher ionic strength, these data substantiate the conclusion that D_2O does not influence the rate-determining step. The entire rate acceleration caused by D_2O is therefore accounted for in the initial step of the reaction sequence: formation of the monomer–cation complex. Cs^+ apparently binds about 3.5 times more tightly to the monomer in D_2O than in H_2O.

For the reason cited in the previous section, similar experiments with formyltetrahydrofolate synthetase monomers prepared from *C. acidi-urici* were not feasible. Proposed kinetically important association steps are: (1) formation of the monomer–cation complex and (2) a rate-determining conformational change of this complex (eq 14 and 15). The enhanced activity of cations such as $Tris^+$ and TMA^+ in D_2O nevertheless suggests that the replacement of protium with deuterium likewise decreases the dissociation constant of the *C. acidi-urici* monomer–cation complex. Furthermore, as in the *C. cylindrosporum* case, k_1—the rate constant of the limiting reaction measured at a saturating concentration of KCl (400 m*M*)—was not dependent upon the isotopic composition of the solvent. The average value over the range of D_2O concentration of 0–90% was $7.9 \pm 0.5 \times 10^{-4}$ sec^{-1}.

The Kinetic Proton Inventory. Since proteins contain a number of exchangeable protons, any or all of which may contribute to a SIE, mechanistic conclusions based solely on the magnitude of the isotope effect are unreliable. In addition, the isotope effect in our system is associated with a protein–cation interaction and may therefore be due to changes in hydration of cation and/or protein. It is obviously desirable to inventory those protons in the system responsible for the isotope effect. The proton inventory is accomplished by accounting for the variation in reaction rate or equilibrium constant produced by increasing the mole fraction of D_2O in H_2O (see Experimental Procedure).

The dependence of k_o^n/k_o^0 on n for potassium ion induced association of *C. cylindrosporum* and *C. acidi-urici* monomers is shown in Figure 3. The isotope effects (k_o^n/k_o^0; $n = 0.9$) are 2.9 and 2.3, respectively. The concentration of potassium ion in each case is equal to the dissociation constant of the monomer–cation complex in H_2O (Harmony and Himes, 1975) so that k_o, the observed rate constant, is equal to k'/K_d where k' is the constant of the rate-determining reaction obtained at saturating [KCl]. In

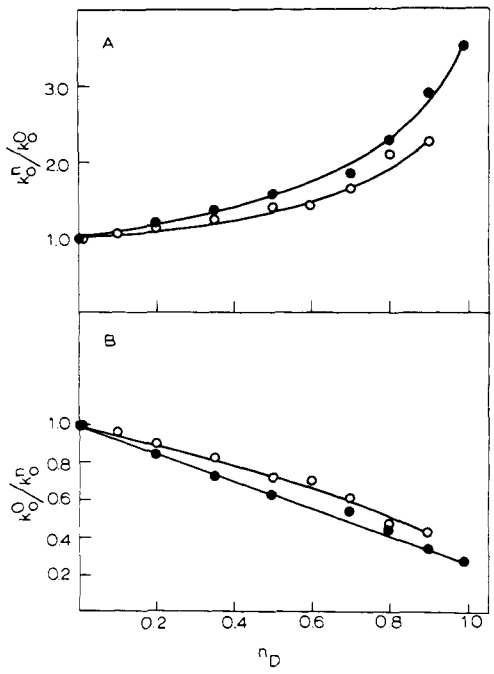

FIGURE 3: Kinetic solvent isotope effect. (A) Dependence of the observed rate of subunit association, relative to the rate obtained in water, on the atom fraction of deuterium. Reaction conditions are described in Figure 2. Monomers of formyltetrahydrofolate synthetase prepared from *C. cylindrosporum* (●) associated to tetramer in the presence of 100 m*M* KCl (k_o, M^{-1} sec^{-1}); monomers of the enzyme from *C. acidi-urici* reactivated in the presence of 20 m*M* KCl (k_o, sec^{-1}). (B) The association rate in water relative to that obtained in mixed solvent of atom fraction deuterium *n* is plotted vs. *n*.

the *C. cylindrosporum* system k' is k_2, the second-order rate constant for dimerization; in the *C. acidi-urici* case k' is k_1, the first-order constant for conformational alteration of the monomer–cation complex. As previously discussed, k_2 and k_1 are independent of the isotopic composition of the solvent and the change in k_o with increasing *n* is therefore directly proportional to the change in $1/K_d$. Thus $k_o^n/k_o^0 \sim K_a^n/K_a^0$, K_a being the association constant for formation of the monomer–cation complex (eq 12). Assuming that only unusual protons in the monomer–cation complex contribute to the isotope effect, a polynomial regression analysis of these data suggests there may be three such protons in the *C. cylindrosporum* case and two in the complex formed from a *C. acidi-urici* monomer and potassium ion. However, the appropriate treatment of the proton inventory data reveals that, in both systems, these potentially "contributing" protons would have dissimilar isotopic fractionation factors. That is, plots of the data according to eq 11 are nonlinear.

At the opposite extreme, assuming that the unusual protons responsible for the SIE are reactant protons, the relationship k_o^0/k_o^n, which is proportional to K_d^n/K_d^0, vs. *n*, graphically represented in Figure 3, is analyzed. The *C. cylindrosporum* data are remarkably linear. This result suggests that a single proton determines the isotope effect. The isotopic fractionation factor of this proton is 0.30. In the *C. acidi-urici* system k_o^0/k_o^n is not a linear function of *n* and the regression analysis indicates that two protons may be responsible for the isotope effect. Since the plot of $[(k_o^0/k_o^n)^{1/2} - 1]$ vs. *n* is also nonlinear, the isotopic fractionation factors of these two protons would not be equal (see Experimental Procedure). In any event, analyses of the SIE data distinguish differences in the interaction with monovalent cations of formyltetrahydrofolate synthetase monomers prepared from *C. cylindrosporum* and those from *C. acidi-urici*.

Thermodynamic Solvent Isotope Effect

Extent of Association–pL Profile. The extent that formyltetrahydrofolate synthetase monomers are associated by monovalent cations depends upon pL. Extent vs. pL curves obtained for potassium ion induced association of *C. cylindrosporum* monomers in H_2O and in 90% D_2O are illustrated in Figure 1B. The profiles are qualitatively similar. The D_2O curve is displaced from the H_2O curve by 0.4 pL unit and the amount of tetramer formed is significantly greater in D_2O than that in H_2O at the same relative position on the pL profile. A comparison of Figures 1A and B shows that the thermodynamic results are consistent with the kinetic data.

Effect of D_2O on the Association and Dissociation Constants for Subunit Association. K_A, the association constant for cation-induced tetramer formation (eq 1), is defined in eq 16 (T = tetramer):

$$K_A = \frac{[T \cdot 2C^+]}{[M]^4[C^+]^2} \tag{16}$$

K_D, the dissociation constant, is the inverse of eq 16. In the *C. cylindrosporum* system,[4] kinetic and stoichiometric evidence (Harmony and Himes, 1975) suggests that the overall reaction represented by eq 1 may be separated into a minimum of three distinct equilibria:

$$4M + 2C^+ \overset{K_1}{\rightleftharpoons} 2MC^+ + 2M \tag{17}$$

$$2MC^+ + 2M \overset{K_2}{\rightleftharpoons} 2DC^+ \tag{18}$$

$$2DC^+ \overset{K_3}{\rightleftharpoons} T \cdot 2C^+ \tag{19}$$

K_A in eq 16 is therefore equal to $K_1K_2K_3$, the product of the association constants of the individual steps. Curve 1 of Figure 4 illustrates the enhancement of K_A^n relative to K_A^0 with increasing D_2O concentration. The equilibrium isotope effect experimentally determined at $n = 0.99$ is approximately 40 (Table I).

This surprisingly large SIE underscores the necessity to determine its origin. D_2O may affect any of or all of the individual equilibria represented by eq 7–9. Equation 17 is related to the initial kinetic step, formation of the monomer–cation complex (represented by eq 12), and therefore $K_1 = (K_a)^2$. Since K_a^n/K_a^0 is about 3.3 ($1/\phi$; $\phi = 0.30$), K_1^n/K_1^0 is $(3.3)^2$ or approximately 11. Thus, a large part of the equilibrium SIE can be accounted for in the initial reaction of the monovalent cation with a formyltetrahydrofolate synthetase monomer. At present, since the dimer–cation complex is a transient intermediate, we are unable to distinguish the thermodynamics of dimer formation from those of tet-

[4] The solvent isotope effect on cation-induced association of monomers of the enzyme prepared from *C. acidi-urici* was not investigated in detail. In this system, the dissociation constant of the monomer–cation complex in H_2O is one-fifth the value obtained in the *C. cylindrosporum* case (Harmony and Himes, 1975). In order to observe the isotope effect on K_A, the concentration of monovalent cation must be very low which introduces considerable error in the value of K_A obtained in H_2O and in mixed solvents of low deuterium content.

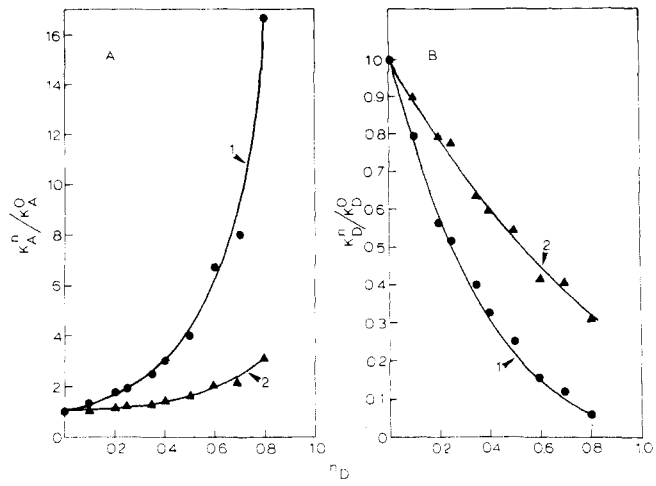

FIGURE 4: Thermodynamic solvent isotope effect. (A) (1) The dependence of the association constant, relative to the value determined in water, on the atom fraction of deuterium. K_A values were calculated according to eq 16. The monomer source was *C. cylindrosporum* and the experimental conditions are described in Figures 2 and 4. (2) K_4^n/K_4^0 vs n. K_4 is the equilibrium constant for the reaction represented by eq 20. (B) (1) K_D^n/K_D^0 vs n. (2) K_4^0/K_4^n vs. n.

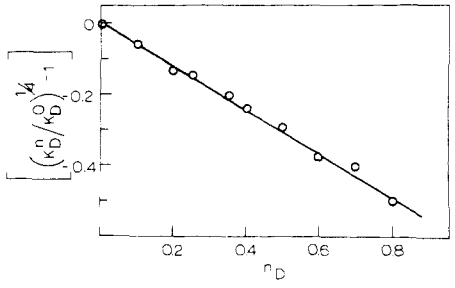

FIGURE 5: Thermodynamic solvent isotope data plotted according to eq 11 [$y^n = (K_A^n)^{-1}$; $y^0 = (K_A^0)^{-1}$; $x = 4$]. K_A values are taken from Table II.

ramer formation. Thus, eq 18 and 19 may pragmatically be combined to facilitate interpretation of the isotope effect:

$$2MC^+ + 2M \overset{K_4}{\rightleftharpoons} T \cdot 2C^+ \qquad (20)$$

K_4 may be estimated by dividing K_A by K_1, and when n = 0.99 K_4^n/K_4^0 is about 3.6 (40/11). Curve 2 in Figure 4A shows the dependence of K_4^n/K_4^0 on the atom fraction of deuterium. A proton inventory indicates that two protons contribute to this SIE, provided that they are both situated on the tetramer–cation complex. If such is indeed the case, their isotopic fractionation factors are unequal. In contrast, assuming that the isotope effect is due to the influence of isotopic solvent on the reactant monomer, analysis of the inverse relationship K_4^0/K_4^n vs. n (curve 2 of Figure 4B) also resulted in a two-proton inventory. In this case, however, the relationship $[(K_4^0/K_4^n)^{1/2} - 1]$ vs. n was linear suggesting that both protons have equal ϕ values. ϕ was calculated to be 0.40.

Considering the array of isotopic fractionation factors that it is possible to calculate from our data for protons which may be important in cation-induced subunit association, the values of 0.30 and 0.40 determined kinetically and thermodynamically in the *C. cylindrosporum* system agree remarkably well. The key postulate relating the two treatments is that only exchangeable monomer protons contribute to the observed isotope effect. The data indicate one contributing proton (of $\phi \sim 0.35$) per monomer. This conclusion is substantiated by analysis of the variation in K_D, the dissociation constant for the overall reaction (the reverse of eq 1), with increasing amounts of D_2O in H_2O (curve 1 in Figure 4B). $[(K_D^n/K_D^0)^{1/4} - 1]$ vs. n is expected to be linear if the isotope effect is due to one proton per monomer, each having the same ϕ value. The linearity of this relationship is evident in Figure 5. Moreover, the isotopic fractionation factor of each proton is 0.37 consistent with the expected value. The isotopic fractionation factor, averaged over three determinations, is therefore 0.36 ± 0.04 (average deviation). The error of 11% seems acceptable at this time in view of the experimental uncertainties associated with the values of k_o and K_A (refer to Table I).

Table I: Dependence of the Subunit Association Rate and Equilibrium Constants on the Atom Fraction of Deuterium.[a]

Atom Fraction Deuterium	Monomer Source: *C. cylindrosporum* k_o (M^{-1} sec^{-1}) × 10^{-3}	*C. cylindrosporum* K_A (M^{-5}) × 10^{-19}	*C. acidi-urici* k_o (sec^{-1}) × 10^4
0	2.45	0.04	1.28
0.1		0.05	1.33
0.2	2.90	0.07	1.43
0.25		0.08	
0.35	3.35	0.10	1.56
0.4		0.12	
0.5	3.88	0.16	1.78
0.6		0.27	1.81
0.7	4.51	0.32	2.10
0.8	5.60	0.66	2.69
0.9	7.11	1.25	2.89
0.99	8.60	1.60	

[a] The rate and extent of subunit association were determined at 20° by monitoring the increase in catalytic activity, indicative of tetramer formation, with time. Association was initiated by the addition of KCl. Final KCl concentrations were: *C. cylindrosporum* system, 100 mM (k_o) and 50 mM (K_A); *C. acidi-urici* system, 20 mM (k_o). The observed rate constants are defined in eq 12–15 and in the text; K_A values were calculated from eq 16. The variation in the rate constant is ±2% and in association constant, ±5% except at high deuterium concentration (>90%).

Discussion

The substitution of D_2O for H_2O effectively enhances the stability of numerous proteins as evidenced by their increased ability to "survive" in extreme environments (Hermans and Scheraga, 1959; Maybury and Katz, 1956; Guild and van Tubergen, 1957). In the case of oligomeric proteins, increased stability is often due to the capacity of D_2O to prevent the native, multisubunit form from dissociating to subunits (Henderson and Henderson, 1969). Conversely, D_2O may facilitate the return of the monomeric species, once formed, to its aggregated state (Aune et al., 1971; Timasheff, 1973). The stabilizing influence of D_2O is generally attributed to the prediction that hydrophobic interactions between amino acid side chains in the associated or native configuration will be stronger in D_2O than in H_2O (Timasheff, 1973; Kresheck et al., 1965; Berns et al., 1968). Indeed, the critical micelle concentration of detergents is reduced in D_2O (Kresheck et al., 1965). However, resolution of the molecular basis for a solvent isotope effect requires knowledge of the number of protons which are responsible for that isotope effect, necessitating careful investigation of each individual system.

The mechanism of monovalent cation-dependent tetramerization of formyltetrahydrofolate synthetase monomers

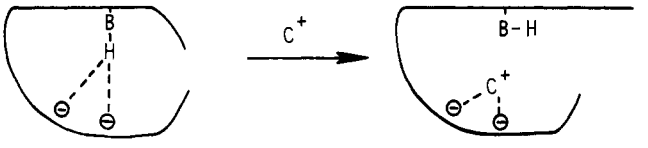

FIGURE 6: Schematic representation of the proposed cation-binding site on the monomer form of formyltetrahydrofolate synthetase prepared from *C. cylindrosporum*.

prepared from two closely related clostridial species is well characterized (Scott and Rabinowitz, 1967; MacKenzie and Rabinowitz, 1971; Welch et al., 1971; Harmony et al., 1974; Harmony and Himes, 1975). In each case the initial step is proposed to be the formation of a reactive monomer-cation complex (eq 12 and 14). When subunit association takes place in D_2O the dissociation constant of this complex is approximately one-third the value obtained in H_2O. In the *C. cylindrosporum* system the rate-determining step is the reaction of a monomer-cation complex with another monomer (eq 13). An alteration in the conformation of the monomer-cation complex (eq 15) limits the rate of subunit association in the *C. acidi-urici* system. In neither case does D_2O influence the rate-determining step. Since the equilibrium between monomer, cation, and the monomer-cation complex is established on a much shorter time scale than that required for the limiting steps (Harmony and Himes, 1975), the influence of D_2O on the dissociation constant of the initial, obligatory complex is reflected as an increase in the rate of subunit association.

The observation that D_2O substantially reduces dissociation of a monovalent cation from a protein-cation complex is extremely interesting. That such a phenomenon may occur in other systems is suggested by the ability of D_2O to prevent dissociation of oligomeric glutamate dehydrogenase into inactive subunits in buffers of very low ionic strength (Henderson and Henderson, 1969). If D_2O also influences the interaction of monovalent cations such as potassium and sodium ions with organic ligands, it is a subject that has received little attention in the literature. We are presently investigating the role of the isotopic composition of the solvent in determining the degree of association of monovalent cations with organic ligands and with model ionophores such as polycyclic crown ethers and antibiotics.

The finding that the dissociation constant of the *C. cylindrosporum* monomer-cation complex decreases linearly with increasing atom fraction of deuterium (Figure 3B), most consistent with a model in which a proton in a very weak potential in the cation-free monomer is converted to a single "normal" water-like proton in the complex, seems more than coincidental and prompts us to propose the cation site illustrated in Figure 6. The site is arbitrarily represented as two negative charges which are stabilized in the absence of monovalent cations by the exchangeable proton, most likely attached to an oxygen base, responsible for the isotope effect. The isotopic fractionation factor of this proton is about 0.36. If the cation site were in a hydrophobic region of the monomer, relatively little water would be available to provide additional stabilization and the interaction of the proton of interest with the "divalent" site in the absence of the monovalent cation could decrease the strength of the protein-H bond sufficiently to account for its low isotopic fractionation factor. The binding of a monovalent cation would free this proton allowing it to assume its normal fractionation factor of about one. It is important to note that the data require the proton to assume a unit fractionation factor, *not* to dissociate from the protein to generate a hydronium ion. If this were the case, three protons of fractionation factor 0.69 (known for the hydronium ion (Schowen, 1972)) would have been required for the product state. The cation binding sites on the monomer forms of formyltetrahydrofolate synthetase isolated from *C. acidi-urici* and from *C. cylindrosporum* are apparently not identical although the dissociation constant of the monomer-cation complex in both systems is similarly decreased by D_2O. Two protons with nonidentical isotopic fractionation factors are apparently responsible for the decrease in the dissociation constant of the *C. acidi-urici* monomer-cation complex which occurs in D_2O. In addition to the difference detected by the proton inventory analysis, the *C. acidi-urici* monomer binds monovalent cations considerably more tightly in H_2O than its *C. cylindrosporum* counterpart (Harmony and Himes, 1975).

In the case of cation-induced association of *C. cylindrosporum* monomers, the thermodynamic solvent isotope effect result is entirely consistent with the kinetic result. It appears that four protons, one per subunit, are responsible for the 40- to 50-fold increase in the amount of tetramer formed in D_2O relative to H_2O. The isotopic fractionation factors of these protons are identical (~0.36) suggesting that the same type of proton on each monomer is involved.

The model indicated by both kinds of data is that: (a) the association of a monovalent cation with a formyltetrahydrofolate synthetase monomer is favored in D_2O over H_2O by a factor of about 2.8 (1/0.36) arising from the change in state of a single proton which is displaced by the cation out of the cation-binding site into a normal environment on the protein; (b) the association of a monomer with a monomer-cation complex is subject to the same thermodynamic solvent isotope effect (2.8), possibly because the single cation joins the two subunits by simultaneously occupying the binding sites of both[5] (thus dimer (DC^+) formation is predicted to be favored thermodynamically in D_2O by a factor of $(2.8)^2$ or 7.8; however, we have, as yet, been unable to devise an experimental method to measure this effect); (c) since the rate of association of MC^+ with M is unaffected by D_2O, the rate-limiting process must be a conformational change or a "reorientation" of the DC^+ complex (unless diffusion in this system is unusually slow; k_2 is only 10^4 M^{-1} sec^{-1}) *preceding* the entry of the cation of MC^+ into the binding site of M; and (d) the dimer DC^+, once formed, rapidly aggregates to form the tetramer $T{\cdot}2C^+$ in a process which has no associated equilibrium solvent isotope effect. According to the proposed model, the overall equilibrium solvent isotope effect K_A^n/K_A^0 is simply the product of the effects for dimer formation, $(7.8)^2$ or $(2.8)^4$, or about 60. The predicted value is in reasonably good agreement with the experimentally determined value of 40 at $n = 0.99$ and with the extrapolated value (from a version of Figure 4A expanded to include all the K_A values reported in Table I) of 50 at $n = 1$. Moreover, at $n = 0.9$ the equilibrium solvent isotope effect is predicted to be 31 $[1/(1 - n + 0.36n)^4]$ which is exactly the experimentally determined value (1.25

[5] This seems to be the simplest way of accounting for the equality of the solvent isotope effects for the process $M + C^+$ and the process $MC^+ + M$. However, this hypothesis necessitates an expanation of why C^+ does not inhibit dimer formation (Harmony and Himes, 1975) (i.e., why the process $MC^+ + M$ and the process $MC^+ + MC^+$ proceed at equal rates). As explained below, this may be true because it is extremely easy to displace *either* the proton (as in M) or the cation (as in MC^+) from the cation-binding site.

$\times 10^{19}/4.0 \times 10^{17}$).

Thus the large stabilization of the tetramer in D_2O is apparently a result of the change in binding state of four protons, one on each subunit, attendant upon entry of a cation into its binding site. None (or no substantial part) of the solvent isotope effect appears to arise from the release of water molecules which occurs as hydrophobic bonds form during the association, although these forces are likely to be involved. Indeed, it is almost certain that large changes in binding of water are associated with tetramerization since the calculated entropy changes are positive and on the order of 100–550 eu (Harmony and Himes, 1975). The present investigation shows that the large solvent isotope effect does not arise from this source. A corollary of these findings is therefore that a large stabilization of oligomer in D_2O cannot be taken as evidence for the importance of hydrophobic bonding. In the present case, the entropy change is strongly indicative of the importance of hydrophobic binding in the oligomer, while the solvent isotope effect results from displacement of four protons out of cation binding sites. The two experiments thus measure independent characteristics of the reaction. The solvent isotope effects from the water molecules displaced by hydrophobic binding are negligible in comparison to those from cation binding.

One aspect of the proposed model—the prediction that a monovalent cation is sandwiched between two enzyme subunits—deserves further comment. This postulate is consistent with the reaction stoichiometry determined at low cation concentration (Harmony and Himes, 1975); i.e., one cation is sufficient to induce dimerization and two are sufficient for formation of the catalytically competent tetramer. Moreover, since the rate of subunit association is not inhibited by high concentrations ($\sim$1 *M*) of monovalent cation (footnote 2 of Harmony and Himes (1975)) and since MC^+ reacts equally well with M and with MC^+ (Harmony and Himes, 1975), the rate-limiting reaction of MC^+ with MC^+ which occurs under conditions of saturating cation must result in the displacement, after the rate-limiting event, of one monomer-bound cation by the cation of the other monomer–cation complex. Proof for this conclusion awaits a detailed determination of the association stoichiometry obtained at saturating concentrations of monovalent cation.

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