Nuclear Magnetic Resonance Studies of Formyltetrahydrofolate Synthetase Interactions with Formate and Methylammonium Ion[†]

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ABSTRACT: Using nuclear magnetic resonance techniques, we have measured the internuclear distances separating the nucleotide-bound metal from the carbon and hydrogen nuclei of formate as well as the carbon of methylammonium cation when bound to formyltetrahydrofolate synthetase. Measurements were made of the paramagnetic effect on the spin–lattice relaxation rates ($1/T_1$) of ^{13}C and ^{1}H nuclei arising from the replacement of Mg^{2+} with Mn^{2+}, which binds to the enzyme in the form of a metal–nucleotide complex. Distances from Mn^{2+} to the formate carbon and proton were found to be 6.3 and 7.4 Å, respectively, in the E·ATP·Mn^{2+}·formate complex and 6.0 and 7.1 Å, respectively, in the E·ADP·Mn^{2+}·formate complex. When tetrahydrofolate was added to the latter complex, the exchange of formate was greatly reduced and became rate limiting for relaxation. These results are consistent with substantial conformational effects produced by the binding of the cofactor. The distance from Mn^{2+} to the methylammonium carbon in the E·ADP·Mn^{2+}·$CH_3NH_3^+$, E·ADP·Mn^{2+}·formate·$CH_3NH_3^+$, and E·ADP·Mn^{2+}·tetrahydrofolate·$CH_3NH_3^+$ complexes was estimated to be in the range of 7.4–12 Å. However, in the E·ADP·Mn^{2+}·formate·tetrahydrofolate·$CH_3NH_3^+$ complex, the data suggest that exchange of cation contributes significantly to relaxation. These results, combined with other known features of the enzyme, suggest that there may be a monovalent cation site within the active site of the enzyme.

Formyltetrahydrofolate synthetase catalyzes the following reaction:

$$HCOO^- + MgATP + H_4folate \overset{K^+}{\rightleftharpoons} 10\text{-}CHO\text{-}H_4folate + MgADP + P_i \quad (1)$$

Enzyme–substrate complexes of formyltetrahydrofolate synthetase from *Clostridium cylindrosporum* have been previously studied by magnetic resonance techniques. Himes & Cohn (1967) used water proton nuclear magnetic resonance (NMR)[1] relaxation rate measurements to establish that divalent metal binds to the enzyme in the form of a metal–nucleotide complex. Free Mn^{2+} did not interact appreciably with the enzyme. Buttlaire et al. (1975a,b) studied the effects of various substrates on water proton relaxation rates and on the Mn^{2+} EPR spectrum. Ternary complexes of enzyme, Mn^{2+}, and ATP or ADP were characterized by greatly enhanced proton relaxation rates and EPR spectra that were very similar to those of binary Mn–nucleotide complexes. It was estimated that three to four exchangeable water molecules were ligated to the metal. The addition of H_4folate greatly reduced the relaxation enhancements to the extent that the observed rate was slower than that observed for the simple aquomanganese(II) solution, and the estimated number of exchangeable water ligands was reduced to a fractional value. EPR spectra for this complex exhibited substantial narrowing, indicating reduced solvent accessibility to the metal. Finally, the further addition of formate led to an additional decrease in proton relaxation rates and greater narrowing of EPR line widths, although the addition of formate to comlexes lacking H_4folate had little or no effect. These results suggest that the binding of H_4folate promotes a conformational change in the active site leading to the catalytically competent structure.

In the present study, we have measured the effect of Mn^{2+} on the spin–lattice relaxation rates of both the hydrogen and carbon nuclei of formate in an effort to compute their distances from the metal, and also to determine the effect of the addition of H_4folate on these values. Further, we have measured the relaxation rate of [^{13}C]methylammonium, an effective monovalent cation activator of the enzyme, first to determine whether there is a cation site in the vicinity of the active site of the enzyme and second to examine the possibility of an interaction between formate and the monovalent cation, as suggested by Welch et al. (1968).

Experimental Procedures

Materials

[^{13}C]Formic acid (90% enriched) and [^{13}C]methylamine (90% enriched) were purchased from Merck Sharp & Dohme, Inc. Tris base (ultrapure) was obtained from Schwarz/Mann. All other reagents were from Sigma Chemical Co. *Clostridium cylindrosporum*, used for the preparation of the enzyme, was provided by the Enzyme Laboratory, University of Kansas.

Methods

Formyltetrahydrofolate synthetase was purified from *C. cylindrosporum* (Rabinowitz & Pricer, 1962) and then chromatographed, first on DEAE-cellulose (Curthoys & Rabinowitz, 1971) and then on Bio-Gel A 0.5 (80 × 4 cm). The last chromatography step was required to systematically remove a very low level contamination of adenylate kinase activity. At the high concentration of protein used in these studies, this contamination presented a problem. The final specific activity was generally in the range of 360–380 μmol min^{-1} mg^{-1}. The crystalline enzyme migrates as a single band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For a more rigorous test of purity with regard to adenylate

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[1] Abbreviations: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; E_{act}, energy of activation; H_4folate, tetrahydrofolic acid; 10-CHO-H_4folate, *N*-10-formyltetrahydrofolic acid; MA, methylammonium; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

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kinase, concentrated enzyme solutions containing ADP, formate, and H_4folate were assayed for the production of 10-CHO-H_4folate after several days of NMR measurements.

H_4folate was prepared by the catalytic hydrogenation of folic acid in aqueous solution and was purified by chromatography on DEAE-cellulose (Samuel et al., 1970). In the purification step, the column was not prewashed with 2 mM EDTA as prescribed; otherwise, a small amount of EDTA eluted with the cofactor and ultimately interfered with NMR measurements. H_4folate concentrations were determined from the absorbance at 298 nm, using an extinction coefficient of 3×10^5 M^{-1} cm^{-1}. The chemical reduction produces a *dl* mixture, of which only the *l* form is a substrate. The *d* form does not inhibit the reaction, and it has been concluded that this isomer does not bind to the enzyme (Himes & Harmony, 1973). Enzyme activity was assayed as previously described (Rabinowitz & Pricer, 1962). Protein concentration was determined from the absorbance at 280 nm by using an extinction coefficient of 1.22×10^5 M^{-1} cm^{-1} (Himes & Cohn, 1967).

Preparation of Samples for NMR Measurements. Purified formyltetrahydrofolate synthetase, stored as a crystalline suspension in 50% ammonium sulfate, was collected by centrifugation, redissolved in buffer (25 mM Tris, pH 8.0, 50 mM 2-mercaptoethanol, and 25 mM KCl), dialyzed extensively, and then concentrated to greater than 100 mg/mL by centrifugation in membrane cones (Amicon CF-25). For experiments in which [^{13}C]methylammonium relaxation rates were measured, the enzyme was desalted by chromatography on Sephadex G-25 (30 × 2.5 cm), which dissociated the enzyme to inactive monomers. After concentration, the addition of [^{13}C]methylammonium yielded the active tetrameric enzyme with a specific activity greater than 90% of its initial value. All ^{13}C measurements were performed on solutions containing 20% D_2O to permit internal field–frequency locking. Solutions used in ^{1}H measurements were prepared in D_2O (99.7%), and the enzyme was deuterated by repeated concentration and dilution.

The pH meter reading for all solutions was 8.0. The concentration of Mn^{2+} was determined by atomic absorption spectroscopy. Glassware was soaked overnight in 2 mM EDTA, rinsed thoroughly with deionized distilled water, and dried. All samples were transferred to cylindrical inserts (Wilmad) fitted to NMR sample tubes. Volume capacities of the insert chambers were 0.35 mL for 10-mm sample tubes (^{13}C) and 0.1 mL for 5-mm sample tubes (^{1}H). Inserts were centered within the dimensions of the radio-frequency coils of the spectrometers to minimize field inhomogeneities.

Nuclear Magnetic Resonance Measurements. Spin–lattice relaxation rates ($1/T_1$) were measured on Bruker WP-80 and WP-200 spectrometers by using the fast inversion recovery (FIRFT) method developed by Canet et al. (1975). The pulse sequence was represented by $(T\text{–}180°\text{–}\tau\text{–}90°)_n$ where $T < 5T_1$ were allowed for partial equilibration between scans. The first scan was discarded. $1/T_1$ values and standard errors were calculated by using a nonlinear least-squares computer program which was kindly provided by Dr. R. G. Khalifah of the Veterans Administration Hospital, Kansas City, KS, and modified for the FIRFT sequence. A total of 4–32 scans were accumulated for each τ value, and an exponential multiplication factor corresponding to 5-Hz line broadening was applied to the accumulated free induction decays (FID's) for T_1 measurements. Spin–spin relaxation rates ($1/T_2$) were determined from half-height line widths of fully relaxed spectra.

Nuclear Magnetic Resonance Theory and Calculations. The calculations employed in this study are based upon the theory of Solomon and Bloembergen (Solomon, 1955; Solomon & Bloembergen, 1956; Bloembergen, 1957) which describes the effect of a proximate paramagnetic species on the nuclear relaxation rates of a bound magnetic nucleus. Accordingly, when Mn^{2+} is the paramagnetic species, the equation which describes the spin–lattice relaxation rate of a nucleus bound to a macromolecule close to bound Mn^{2+} is given by eq 2:

$$\frac{1}{T_{1M}} = \frac{B}{r^6}\frac{3\tau_c}{1 + \omega_I^2\tau_c^2} \tag{2}$$

In this equation, T_{1M} is the contribution to T_1 from bound nuclei, r is the distance between the nucleus and Mn^{2+} in the complex, ω_I is the nuclear Larmor frequency, τ_c is the correlation time for the dipolar interaction, and $B = (2/15)\gamma_I^2 g^2 S(S+1)\beta^2$, where γ_I is the magnetogyric ratio of the nucleus, S is the electron spin quantum number, and β is the Bohr magneton. τ_c contains contributions from several rate processes (eq 3):

$$\tau_c^{-1} = \tau_M^{-1} + \tau_r^{-1} + \tau_s^{-1} \tag{3}$$

where τ_M is the lifetime of the nucleus in the bound site, τ_r is the rotational correlation time, and τ_s is the electron spin relaxation time. An equation similar to eq 2 describes the spin–spin relaxation rate ($1/T_2$). This equation contains a second term which describes a scalar contribution that may be important if the paramagnetic ion is connected to the relaxing nucleus via chemical bonds.

The value of r was computed from eq 2 after values for T_{1M} and τ_c were established. The value of T_{1M} was estimated from the following equation, in which it has been assumed that outer-sphere contributions can be neglected (Luz & Meiboom, 1964):

$$\frac{1}{pT_{1p}} = \frac{1}{T_{1M} + \tau_M} \tag{4}$$

In this equation, p is the concentration of the nucleus bound to enzyme sites containing Mn^{2+} divided by the total concentration of the nucleus. T_{1p}^{-1} was obtained by subtracting the observed relaxation rates for the diamagnetic complexes containing Mg^{2+} from the observed rates for the corresponding paramagnetic complexes containing Mn^{2+}. A similar equation can be written for $(pT_{2p})^{-1}$ when Mn^{2+} is the paramagnetic ion. Equation 4 has two limiting conditions, $T_{1M} \gg \tau_M$ and $\tau_M \gg T_{1M}$. The latter condition must not be present in order for distance calculations to be made. Three lines of experimental evidence were used to determine whether τ_M contributed significantly to $(pT_{1p})^{-1}$. First, the effect of temperature on $(pT_{1p})^{-1}$ values was measured. If $(pT_{1p})^{-1} = \tau_M^{-1}$, then the observed rate will increase with increasing temperature, and the magnitude of the temperature effect will be large; E_{act} values will be generally in the range of 6–25 kcal/mol (James, 1975). On the other hand, if $(pT_{1p})^{-1} = T_{1M}^{-1}$, the observed rate may either increase or decrease with increasing temperature. The magnitude of the temperature effect will generally be substantially smaller; $E_{act} < 6$ kcal/mol. Second, the effect of magnetic field strength on pT_{1p} was measured. Since τ_M is not altered by the magnetic field strength, a significant change in the measured rate as a function of magnetic field strength indicates a significant contribution of T_{1M} to pT_{1p} (a lack of field strength effect does not necessarily imply that $pT_{1p} = \tau_M$ because T_{1M} displays field strength dependence only when $\tau_c \gtrsim 1/\omega_I$). Third, values of the ratio of T_{1p} to T_{2p} were computed. Large values for this ratio imply that pT_{1p} does not equal τ_M. This follows from the fact that T_{1M} values are equal to or larger than T_{2M} values, and therefore if $pT_{1p} =$

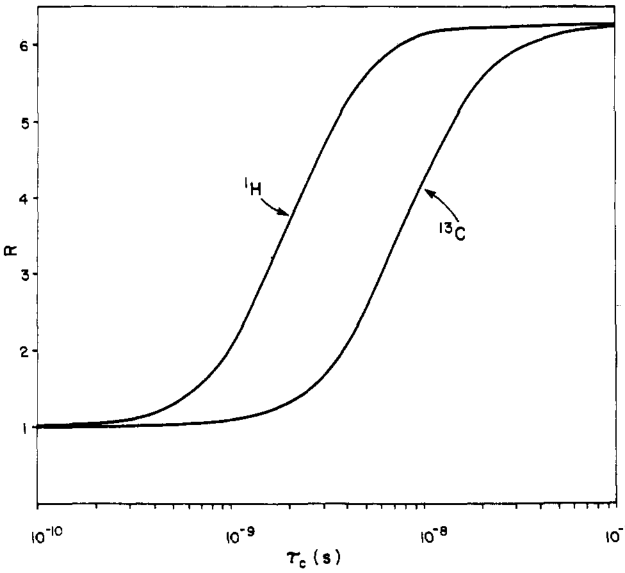

FIGURE 1: Theoretical plot of the value of $R = T_{1p}(47.0\text{ kG})/[T_{1p}(18.8\text{ kG})]$ vs. τ_c for both ^{1}H and ^{13}C relaxation.

τ_M, then pT_{2p} must also equal τ_M.

The value of τ_c was estimated by measuring pT_{1p} at two magnetic field strengths and from pT_{1p}/pT_{2p} ratios. In the former method, it is important to realize that the value of τ_c itself may be frequency dependent, particularly when Mn^{2+} is used. To compensate for this, we evaluated the dispersion in pT_{1p} values with magnetic field strength changes both with the assumption that τ_c is invariant with frequency and with the assumption that τ_c is maximally frequency dependent. In the first case, the value of τ_c is computed from eq 5 (Fung et al., 1973):

$$\tau_c^2 = \frac{R - 1}{\omega_{I(A)}^2 - R\omega_{I(B)}^2} \tag{5}$$

R is the ratio of the value of pT_{1p} measured at field strength A to that measured at field strength B. The value of R as a function of τ_c is seen in Figure 1 for both ^{1}H and ^{13}C relaxation times at 47 kG (A) and 18.8 kG (B). When τ_c is maximally frequency dependent, then its value varies linearly with the square of the field strength (Fung et al., 1973). In this case, τ_c was computed from eq 6:

$$\tau_{c(B)}^2 = \frac{R(\upsilon_A/\upsilon_B)^2 - 1}{(\upsilon_A/\upsilon_B)^4\omega_{I(A)}^2 - R(\upsilon_A/\upsilon_B)^2\omega_{I(B)}^2} \tag{6}$$

In this equation, υ_A and υ_B are the two field strengths, R has the same definition as in eq 5, and $\tau_{c(B)}$ is the τ_c value at υ_B. The value of τ_c at υ_A is equal to the product of $(\upsilon_A/\upsilon_B)^2\tau_{c(B)}$. In using the T_{1p}/T_{2p} ratio to compute τ_c, it is assumed that exchange is fast for both pT_{1p} and pT_{2p} and that pT_{2p} has no contribution from the scalar term. Under these conditions, τ_c is obtained from eq 7:

$$\tau_c = \frac{(6T_{1p}/T_{2p} - 7)^{1/2}}{2\omega_I} \tag{7}$$

This method for estimating τ_c is the least reliable, as pointed out by Mildvan & Gupta (1978), because of the possibility of scalar contributions to T_{2p}.

Table I: Measured ^{13}C Relaxation Times for Various Solutions[a]

complex[b]	[M^{2+}] (μM)	T_1[c] (s)	T_1[d] (s)	T_{1p} (s)
[^{13}C]Formate				
E·ADP·M^{2+}·formate	20	11.5	1.26	1.4
E·ATP·M^{2+}·formate	20	11.5	1.55	1.8
ADP, M^{2+}, formate	20	20.7	13.5	39.0
E·ADP·M^{2+}·formate·H$_4$folate	80	11.6	6.7	15.9
ADP, M^{2+}, formate, H$_4$folate	80	20.7	4.9	6.4
[^{13}C]Methylammonium				
E·ADP·M^{2+}·[^{13}C]MA	40	10.3	6.6	18.3
E·ADP·M^{2+}·formate·[^{13}C]MA	40	10.8	6.7	17.6
ADP, M^{2+}, [^{13}C]MA	40	12.8	11.5	113.0
E·ADP·M^{2+}·H$_4$folate·[^{13}C]MA	80	11.4	6.1	13.1
E·ADP·M^{2+}·formate·H$_4$folate·[^{13}C]MA	80	11.5	8.9	39.4
ADP, M^{2+}, [^{13}C]MA	80	12.8	10.5	58.4

[a] Data shown represent the results of a single experiment for each complex done at 18.8 kG and 25 °C. Complexes lacking enzyme were measured at ambient temperature (32 °C). [b] Solutions were prepared to contain the following concentrations of components: enzyme sites, 1 mM; nucleotide, 1 mM; formate, 0.1 M; monovalent cation, 0.1 M; *dl*-H$_4$folate, 6 mM; Tris-HCl, 25 mM; 2-mercaptoethanol, 50 mM; and the indicated concentration of M^{2+}Cl$_2$. [c] Relaxation times measured in the presence of MgCl$_2$. [d] Relaxation times measured in the presence of MnCl$_2$.

The value of p in eq 4 for complexes lacking H$_4$folate was determined by calculating the concentrations of the various species containing Mn^{2+} in the enzyme solutions by using a series of simultaneous equations describing the various equilibria. K_d values for Mn·ATP, Mn·ADP, E·ATP·Mn^{2+}, E·ADP·Mn^{2+}, E·ATP, and E·ADP were from Buttlaire et al. (1975a). A K_d of 4×10^{-2} M was used for the formate·Mn^{2+} complex, which is a value measured for the formate·Mg^{2+} complex (Nancollas, 1956). In the calculations, it was assumed that the presence of formate in enzyme complexes does not alter the equilibria between the enzyme, nucleotide, and Mn^{2+}. This is justified by the observation that water proton relaxation enhancements measured for identical complexes were unaffected by the addition of formate (Buttlaire et al., 1975b). The values of p for complexes containing H$_4$folate were taken to equal the ratio of the total concentration of Mn^{2+} to that of formate because the actual K_d values for complexes containing H$_4$folate are not known. Calculation of p in this manner assumes that all of the Mn^{2+} is bound to the enzyme. This is a reasonable approximation since calculations described above show that in a solution of E, Mn^{2+}, and ADP, 75% of the Mn^{2+} is bound, and it is known that H$_4$folate decreases the K_d of MnADP from the enzyme by a factor of 6 (Buttlaire et al., 1975a). Further, H$_4$folate and formate together decrease the K_d by a factor of 20.

Results

Relaxation Rates of [^{1}H]Formate and [^{13}C]Formate in Various Formyltetrahydrofolate Synthetase–Substrate Complexes. Relaxation rates of the two formate nuclei were measured in three enzyme–substrate complexes: E·ATP·M^{2+}·formate, E·ADP·M^{2+}·formate, and E·ADP·M^{2+}·formate·H$_4$folate. Further, [^{13}C]formate relaxation rates were measured in identical solutions lacking enzyme. In the quaternary complexes, spin–lattice relaxation was significantly enhanced by the presence of Mn^{2+}, whereas substantially less enhancement was observed when the enzyme was not present (Table I). It was computed that the concentration of the binary formate·Mn^{2+} complex was about 2–3-fold greater in solutions lacking enzyme than when enzyme was present. If the paramagnetic enhancement measured in the absence of enzyme is dominated by a contribution from formate in the binary complex, then the corresponding contribution in the presence of enzyme would be much less. For this reason, contributions from the binary complex to T_1 relaxation mea-

Table II: Measurements Obtained from [^{13}C]Formate Relaxation Rates in Various Formyltetrahydrofolate Synthetase–Substrate Complexes

complex[a]	pT_{1p}[b] (s) × 10^4	R[c]	$\Delta V_{1/2}$[d] (Hz)	T_{1p}/T_{2p}	E_{act} (kcal/mol)
E·ATP·M^{2+}·formate	3.1 ± 0.4 (4)[e]	3.9 (1)	1.0 ± 0.1 (3)	5.7	2.8 ± 1.2 (4)
E·ADP·M^{2+}·formate	2.2 ± 0.3 (5)	4.3 ± 0.7 (3)	3.2 ± 0.5 (5)	14.6	1.8 ± 0.3 (5)
E·ADP·M^{2+}·formate·H_4folate	102 ± 15 (4)	1.1 ± 0.1 (2)	0.2 ± 0.3 (4)		9.5 ± 0.3 (4)

[a] Experimental solutions were prepared to contain the following: enzyme active sites, 1 mM; nucleotide, 1 mM; [^{13}C]formate, 0.1 M; KCl, 0.1 M; Tris-HCl, 25 mM; 2-mercaptoethanol, 50 mM; *dl*-H_4folate, 6 mM when present; $MgCl_2$ or $MnCl_2$, 20 μM when H_4folate was not included and 80 μM when H_4folate was present. [b] Measured at 18.8 kG and 25 °C. [c] R is the ratio of pT_{1p} measured at 47.0 kG to that measured at 18.8 kG. [d] $\Delta V_{1/2}$ is the difference between the half-height line width at 18.8 kG and 25 °C measured in the presence of Mn^{2+} and that measured in the presence of Mg^{2+}. [e] Number in parentheses indicates the number of measurements performed, each with a different sample.

Table III: Measurements Obtained from [^{1}H]Formate Relaxation Rates in Formyltetrahydrofolate Synthetase–Substrate Complexes[a]

complex	pT_{1p} (s) × 10^4	R	$\Delta V_{1/2}$ (Hz)	T_{1p}/T_{2p}	E_{act} (kcal/mol)
E·ATP·M^{2+}·formate	2.1 ± 0.1 (3)	2.7 ± 0.03 (2)	0.9 ± 0.05 (2)	3.5	0.6 ± 0.05 (2)
E·ADP·M^{2+}·formate	1.65 ± 0.4 (5)	2.6 ± 0.2 (4)	2.9 ± 0.6 (5)	10.0	1.2 ± 0.2 (4)
E·ADP·M^{2+}·H_4folate·formate	147 ± 26 (3)	1.06 (1)	0.3 ± 0.4 (3)		9.0 ± 1.8 (3)

[a] The conditions and definitions are identical with those described in Table II.

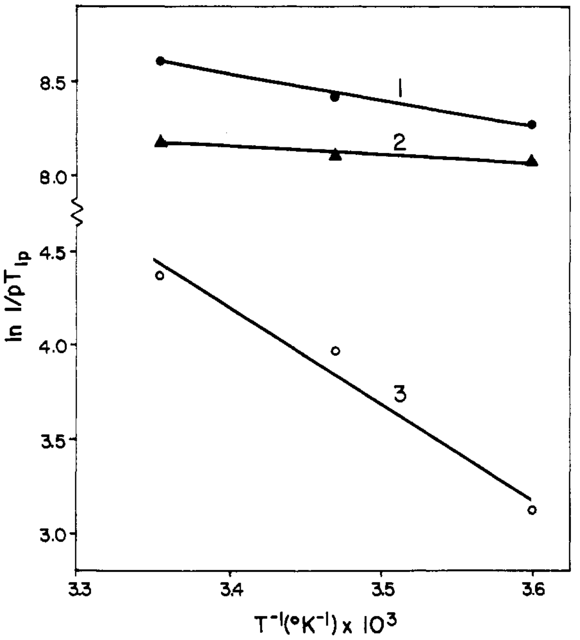

FIGURE 2: Arrhenius plots of the temperature dependence of the relaxation rates, $(pT_{1p})^{-1}$, of [^{13}C]formate·formyltetrahydrofolate synthetase complexes. (1) E·ADP·Mn^{2+}·formate; (2) E·ATP·Mn^{2+}·formate; (3) E·ADP·Mn^{2+}·formate·H_4folate. Solution conditions for these complexes were identical with those described in Table II. All measurements were made at 20.1 MHz. Data shown are the result of a single experiment in each case.

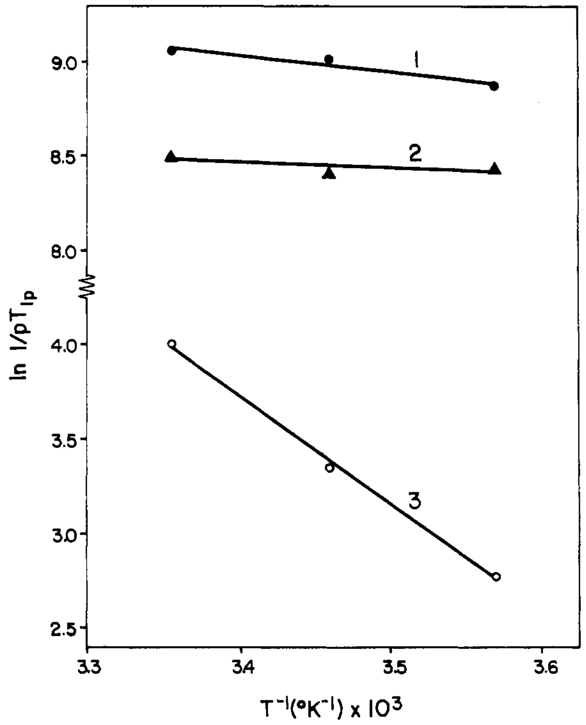

FIGURE 3: Arrhenius plots of the temperature dependence of the relaxation rates of [^{1}H]formate·formyltetrahydrofolate synthetase complexes. (1) E·ADP·Mn^{2+}·formate; (2) E·ATP·Mn^{2+}·formate; (3) E·ADP·Mn^{2+}·formate·H_4folate. Solution conditions for the complexes were as described in Table III. Measurements were made at 80 MHz. Data shown are the result of a single experiment in each case.

sured in the presence of enzyme were neglected. In the quinary complex containing H_4folate, the enhancement observed was smaller than that observed when the enzyme was not present. It was not possible to compute the concentration of the binary complex in enzyme solutions containing H_4folate because the requisite K_d values are not known. Nonetheless, it is unlikely that this complex contributed significantly to relaxation measured in the quinary complex because significant paramagnetic line broadening was not observed when the enzyme was present, whereas when the enzyme was absent, the ^{13}C resonance was broadened 9–12 Hz by the presence of 80 μM Mn^{2+}. The pT_{1p} value measured for the quinary complex was much larger, by a factor of 46 from the ^{13}C data and by a factor of about 90 from the ^{1}H data, than that measured in the quaternary complex lacking H_4folate (Tables II and III). Values of $(pT_{1p})^{-1}$ for each complex increased with increasing temperatures (Figures 2 and 3), but the magnitude of the temperature effect was much greater for the quinary complex containing H_4folate. Values of E_{act} for these complexes computed from Arrhenius plots are shown in Tables II and III. Further, $(pT_{1p})^{-1}$ values of the quaternary complexes decreased considerably at the higher field strength, a decrease of about 4-fold for ^{13}C and about 3-fold for ^{1}H. However, when H_4folate was present, the frequency dependence of T_{1p} was lost (Tables II and III). In addition, line widths of the resonances in the quaternary complexes were significantly increased by the presence of Mn^{2+}, leading to T_{1p}/T_{2p} values in excess of 3.0 in all cases; however, when H_4folate was present, the presence of Mn^{2+} did not result in significant broadening of line widths. These data indicate that in the complex containing H_4folate, $(pT_{1p})^{-1}$ values are limited by the exchange of formate (i.e., $\tau_M \gg T_{1M}$), but when the cofactor is absent, the

Table IV: Calculated Values of τ_c and r of Formate Nuclei in Formyltetrahydrofolate Synthetase Complexes[a]

method	E·ATP·Mn^{2+}·formate		E·ADP·Mn^{2+}·formate	
	^{13}C	^{1}H	^{13}C	^{1}H
	τ_c (ns)			
1[b]	8.8	1.4	10.3	1.32
2[c]	2.6	0.53	2.7	0.53
3[d]	21.0	3.7	35	7.0
	r (Å)			
1[b]	6.3 (5.9–6.5)[e]	7.4 (7.1–7.7)	6.0 (5.5–6.1)	7.1 (6.9–7.4)
2[c]	5.8 (5.5–6.1)	6.7 (6.4–7.0)	5.5 (5.3–5.8)	6.4 (6.2–6.7)
3[d]	5.9	7.3	5.2	6.6

[a] Values shown are based upon data obtained at 25 °C. [b] τ_c calculated from $T_{1p}(47\text{ kG})/[T_{1p}(18.8\text{ kG})]$, assuming that τ_c is not frequency dependent. [c] τ_c calculated from $T_{1p}(47\text{ kG})/[T_{1p}(18.8\text{ kG})]$, assuming maximum frequency dependence for τ_c. The τ_c value shown is that at 18.8 kG. [d] τ_c calculated from T_{1p}/T_{2p} ratios. [e] Numbers in parentheses are the range of values computed by assuming a 10% error in the initial measurements.

measured rate is not exchange limited.

Values of τ_c, computed both from T_{1p}/T_{2p} ratios and from the ratio of pT_{1p} values measured at the two frequencies, are shown in Table IV for the complexes in which exchange limitation of formate was not present. Values for the internuclear distance were computed by using τ_c values estimated by the various methods and are also shown. As pointed out earlier, the use of T_{1p}/T_{2p} ratios to calculate τ_c values is the least desirable method because of possible scalar contributions to T_{2p}. Such contributions could explain the larger τ_c values obtained by this method. It is apparent from the table that, regardless of the method of computation, the ^{13}C τ_c is significantly different from the ^{1}H τ_c. In addition, in all cases, the formate proton is about 1 Å farther from the metal than is the carbon.

Although it was not possible from this study to compute internuclear distances when H_4folate was present, it is clear that the presence of the cofactor has an effect on the exchange rate of formate. An estimation of the magnitude of this effect is obtained from a comparison of the values of $(pT_{1p})^{-1}$ for the quinary complex (this is a lower limit of τ_M^{-1} in the presence of H_4folate) to that of $(pT_{2p})^{-1}$ measured for the corresponding quaternary complex which lacks H_4folate (which gives a lower limit of τ_M^{-1} in the absence of H_4folate). For the ^{13}C data, formate exchange is reduced from about 5×10^4 to 98 s^{-1} by the addition of the cofactor, a decrease by a factor of about 500, and from the ^{1}H data, formate exchange is reduced from 4.5×10^4 to 68 s^{-1}, a factor of about 700.

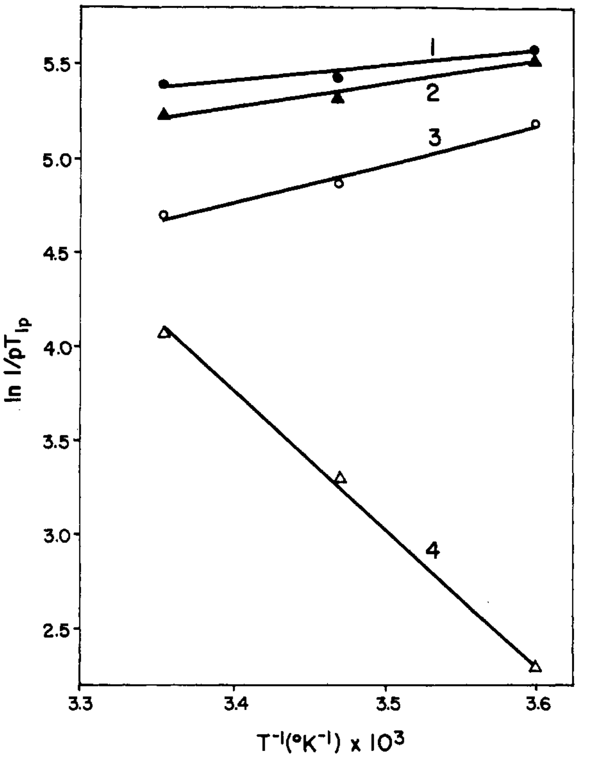

FIGURE 4: Arrhenius plots of the temperature dependence of the relaxation rates of [^{13}C]MA·formyltetrahydrofolate synthetase complexes. (1) E·ADP·Mn^{2+}·[^{13}C]MA; (2) E·ADP·Mn^{2+}·formate·[^{13}C]MA; (3) E·ADP·Mn^{2+}·H_4folate·[^{13}C]MA; (4) E·ADP·Mn^{2+}·formate·H_4folate·[^{13}C]MA. Solution conditions for the above complexes were the same as described in Table V. Measurements were made at 20.1 MHz.

Relaxation Rates of [^{13}C]Methylammonium Ions in Various Formyltetrahydrofolate Synthetase–Substrate Complexes. Spin–lattice relaxation rates of [^{13}C]methylammonium ([^{13}C]MA) were measured in four enzyme–substrate complexes: E·ADP·M^{2+}·[^{13}C]MA, E·ADP·M^{2+}·formate·[^{13}C]MA, E·ADP·M^{2+}·H_4folate·[^{13}C]MA, and E·ADP·M^{2+}·formate·H_4folate·[^{13}C]MA. In these studies, it was assumed that the 0.1 M concentration of MA used is sufficient to saturate the enzyme, since the apparent K_d values of active cations are in the range of 2.5–4.0 mM (Welch et al., 1971). For the first three of the above complexes, the addition of Mn^{2+} resulted in modest, yet significant, rate enhancements (Table I), indicating that cation(s) bind(s) near enough to the metal to experience the paramagnetic environment. However, in the complex containing both formate and H_4folate, the addition of Mn^{2+} resulted in an enhancement that was not significantly greater than that observed when the enzyme was not present. Significant line broadening was not observed when Mn^{2+} was present in any of the complexes. Further, it was found that $(pT_{1p})^{-1}$ values of the three complexes which did not contain both formate and H_4folate decreased with increasing temperature. However, when both substrates were present, $(pT_{1p})^{-1}$ values increased with increasing temperature (Figure 4). Computed values of E_{act} for these complexes are shown in Table V. It should be noted that in the case of the complex containing both formate and H_4folate, the diamagnetic rates were so similar to the paramagnetic rates at the lower tem-

Table V: Measurements Obtained from [^{13}C]Methylammonium Relaxation Rates[a]

complex	pT_{1p} (s) × 10^4	R	$\Delta V_{1/2}$ (Hz)	E_{act} (kcal/mol)
E·ADP·M^{2+}·[^{13}C]MA	50 ± 6.5 (2)	2.4 (1)	0.45 ± 0.45 (2)	−2.9 ± 1.4 (2)
E·ADP·M^{2+}·formate·[^{13}C]MA	52 ± 6.9 (3)	1.9 (1)	0 ± 0.6 (3)	−1.4 ± 0.8 (3)
E·ADP·M^{2+}·H_4folate·[^{13}C]MA	106 ± 1.2 (2)	3.3 (1)	−0.12 ± 0.05 (2)	−5.1 ± 1.1 (2)
E·ADP·M^{2+}·formate·H_4folate·[^{13}C]MA	275 ± 58 (3)	2.0 ± 1 (2)	−0.4 ± 0.5 (2)	7.3 ± 3.6 (3)

[a] Conditions and terms are the same as described in Table II except that the solutions were prepared to contain the following: enzyme sites, 1 mM; ADP, 1 mM; [^{13}C]methylammonium, 0.1 M; formate (when present), 0.1 M; *dl*-H_4folate (when present), 6 mM; $MgCl_2$ or $MnCl_2$, either 40 or 80 μM; Tris-HCl, 25 mM; 2-mercaptoethanol, 50 mM. T_{1p}/T_{2p} ratios were not calculated because the diamagnetic line widths were within error of the paramagnetic line widths.

Table VI: Calculated Values of τ_c and r of [^{13}C]Methylammonium in Formyltetrahydrofolate Synthetase Complexes[a]

complex	τ_c (ns)		r (Å)	
	method 1[b]	method 2[c]	method 1[b]	method 2[c]
E·ADP·Mn^{2+}·[^{13}C]MA	4.5	1.95	9.9 (7.4–11)[d]	8.9 (7.4–11)
E·ADP·Mn^{2+}.formate·[^{13}C]MA	3.6	1.71	9.7 (7.5–11.1)	8.8 (7.5–10.7)
E·ADP·Mn^{2+}·H_4folate·[^{13}C]MA	7.0	2.34	11.4 (8.5–12.2)	10.3 (8.5–12.2)
E·ADP·Mn^{2+}·formate·H_4folate·[^{13}C]MA	3.8	1.76	12.9 (9.5–15.8)	11.6 (9.5–15.8)

[a] Values shown are based upon data obtained at 25 °C. [b] τ_c calculated from T_{1p}(47 kG)/[T_{1p}(18.8 kG)], assuming that τ_c is not frequency dependent. [c] τ_c calculated from T_{1p}(47 kG)/[T_{1p}(18.8 kG)] ratios, assuming maximum frequency dependence of τ_c. The τ_c value shown is that calculated at 18.8 kG. [d] Lower and upper limits of r were calculated as described in the text.

peratures that very small errors in either rate would result in large errors in pT_{1p} values. This led to a very large variation of E_{act} values for this complex (range of three experiments was 3–14 kcal/mol). Observation of a positive temperature effect was reproducible, however. Finally, it was found that $(pT_{1p})^{-1}$ values for each complex decreased when the magnetic field strength was increased.

These data indicate that for the three complexes which do not contain both formate and H_4folate, exchange of the cation does not limit relaxation. However when both formate and H_4folate are present, an unambiguous assessment cannot be made. The data suggest that both τ_M and T_{1M} may contribute to pT_{1p}, since the observed frequency dependence of pT_{1p} can only arise from T_{1M} yet the temperature dependence of pT_{1p} suggests that τ_M contribution is significant. However, it must be kept in mind that a change in the temperature effect from negative to positive could also arise from changes in τ_c. Since E_{act} for this complex is not reproducibly large, it is not clear that the observed changes in the temperature dependence are due to the τ_M contribution.

Values of τ_c were estimated from the ratio of pT_{1p} values measured at the two field strengths for each complex. Internuclear distances were computed by assuming that one cation molecule per active site is in the paramagnetic environment (Table VI). When a 10% uncertainty was assumed in the measured rates, upper and lower limits of τ_c could not be estimated from the frequency dispersion in pT_{1p} values [R values less than 1 and greater than 6.25 were within the error range (see Figure 1)]. An upper limit of τ_c, and therefore a lower limit of r, was calculated from Stokes' law by assuming that the upper limit for τ_c is equal to τ_R, the rotational diffusion time for the protein (Dwek, 1975). Upper limits of r were calculated by using $1/\omega_I$ as the value for τ_c. Although it is possible that more than one cation molecule per active site contributes to the measured relaxation, the maximum effect would be to increase the computed distances only by a factor of the sixth root of the number of cations, assuming that the τ_c and r values are the same for each cation.

Discussion

The use of NMR relaxation rate measurements to estimate the internuclear distance between a nucleus and a paramagnetic probe has provided considerable insight into enzyme mechanisms. Often, the information derived from such studies is not accessible from other techniques. In the present system, results have been obtained which add to the current understanding of the mechanism of formyltetrahydrofolate synthetase.

It is clear from the data that the presence of H_4folate in the active site has a considerable effect on the measured relaxation rates of the formate nuclei (Tables II and III). Furthermore, this effect arises from a large decrease in the exchange rate of formate. Upon the addition of H_4folate, the k_{off} (τ_M^{-1}) of formate decreases from (4.5–5) × 10^4 to 68–98 s^{-1}. The catalytic center activity for the reverse reaction calculated from the known V_M (Curthoys & Rabinowitz, 1972) is 80 s^{-1}, suggesting that the complex detected by NMR is kinetically competent. That formate is "trapped" in the active site by the addition of H_4folate is consistent with the idea that binding of H_4folate to formyltetrahydrofolate synthetase induces a structural change in the active site (Buttlaire et al., 1975a,b). It is also possible that the cofactor physically obstructs the exchange of formate. Substantial effects of H_4folate on the enzyme–formate interaction have been observed in other studies as well. Joyce & Himes (1966) found that the K_m value of formate is very sensitive to the concentration of H_4folate. The formate K_m was determined to be about 5 mM at saturating concentrations of H_4folate, and as the concentration of the cofactor was reduced, the formate K_m approached an extrapolated limit of about 50 mM in the absence of H_4folate (Joyce, 1965). Furthermore, Buttlaire et al. (1975a,b) observed that the addition of formate to E·nucleotide·Mn^{2+} complexes had no measurable effect either on water proton relaxation rates or on the Mn^{2+} EPR spectrum. However, in the E·ADP·Mn^{2+}·H_4folate complex, water proton relaxation rates, which were substantially diminished by the presence of cofactor, were diminished further by the addition of formate. The Mn^{2+} EPR resonance, which was substantially narrowed by the presence of cofactor, exhibited greater narrowing upon further addition of formate. When these results are considered together, it becomes apparent that there are at least two distinct modes in which formate is bound to the enzyme. In the absence of H_4folate, formate may be relatively weakly bound to the enzyme, exchanging rapidly with formate in solution, and its presence in the active site has little or no effect, either direct or indirect, on the environment of the nucleotide-bound metal. When H_4folate is present, formate may be more tightly bound to the enzyme, its exchange with unbound formate restricted, and its presence in the active site affecting the environment of the metal, either directly or through conformational effects.

In the quaternary E·nucleotide·Mn^{2+}·formate complexes, distance calculations indicated that formate is quite close to the nucleotide-bound metal. Since it is likely that formate is not tightly bound in the complexes, the computed distances are upper limits for the true distance. If the formate K_d is about 50 mM, then enzyme sites would be about 67% saturated and the computed distances would be 7% greater than the true distance. An internuclear distance of 6.3 Å or less allows very close approach of formate to the γ-phosphoryl of ATP. This result is interesting because previous studies have suggested that an intermediate formyl phosphate is formed in the course of the reaction (Buttlaire et al., 1976, 1979). It was also found that the formate proton is significantly farther from Mn^{2+} than is the formate carbon. This implies that the formate oxygen is pointed toward the metal. The apparent orientation of formate, coupled with its close proximity to the metal, is suggestive of a metal–formate interaction. Clearly, a Mn^{2+}–formate carbon distance greater than 5 Å is too large to permit direct ligation, but a second sphere complex, assuming an intervening water molecule, is allowed. Such an interaction is not inconsistent with the earlier observation that formate addition does not measurably perturb water proton relaxation rates in this complex (Buttlaire et al., 1975a) because a water molecule would not be trapped as long as formate exchanges rapidly.

Finally, it was found that the τ_c value computed from [^{13}C]formate relaxation was significantly different from that computed for the formate proton. This observation is unusual for two nuclei directly bonded to each other. For this reason, the possibility was considered that this discrepancy in τ_c values, rather than being real, was due to some unaccounted process which contributes more to the frequency dispersion observed for one nucleus than that for the other. One possibility is that equilibria between various formate species in solution is sensitive to the concentration of D_2O. To test this, we measured the frequency dispersion of [^{13}C]formate relaxation at the same D_2O concentration as was used in the proton studies. No significant change in the ^{13}C relaxation rates was observed. Other potential explanations considered were the following: (a) τ_M contributes more significantly to pT_{1p} values of ^{1}H than to the ^{13}C pT_{1p} values; (b) the frequency dependence of τ_c gives rise to the observed behavior; and (c) $(pT_{1p})^{-1}$ values contain significant contributions from T_{1M} of the binary formate·Mn^{2+} complex. The first two possibilities can be readily excluded. Since the pT_{1p} values observed for the carbon and hydrogen nuclei are so similar, τ_M contributions, if important, would affect the frequency dispersion of the two nuclei almost equally. The frequency dependence of τ_c, although possible, does not account for the different values of τ_c observed because relaxation rates of the two nuclei were measured at the same field strengths. As seen in Table IV, disparate τ_c values are calculated for the formate carbon and proton, regardless of the extent to which τ_c is frequency dependent. Whether contributions from the binary formate·Mn^{2+} complex can account for the observed differences is difficult to evaluate. However, this alternative suffers from the fact that the magnitude of the contribution from the binary complex is directly related to its concentration. Regardless of the uncertainty in the formate·Mn^{2+} K_d value, the concentration of this species is 2–4 times higher in the E·ADP·Mn·formate solution than when ATP is present, yet the discrepancy in the τ_c value is the same for both of these complexes. A real difference in τ_c for the two nuclei would indicate that bound formate is undergoing some internal motion which gives rise to different τ_r values for the carbon and proton. Clearly, τ_M and τ_s experienced by nuclei in the same molecule must be identical. Such motion would be consistent with the idea that formate is loosely bound in this complex.

The relaxation rates of [^{13}C]methylammonium were modestly enhanced by the addition of Mn^{2+}, and the magnitude of enhancement was significantly greater than that observed in the absence of enzyme, indicating that at least one cation molecule binds close enough to Mn^{2+} to experience the paramagnetic environment. However, in each complex, the paramagnetic effect was sufficiently small that the range of error in all calculated terms is considerable. This led to an uncertainty on the order of ±2 Å for the computed internuclear distances. The situation is clearly present that the Mn^{2+}–cation carbon distance is too large to measure with great accuracy by using this method. Since these values pertain to the cation carbon rather than the ammonium functionality, the uncertainty in the distance between Mn^{2+} and the cation site is the sum of the computed values ± ~1.3 Å, an approximate length of the C–N bond.

Regardless of the uncertainty in calculated distances, several significant results were obtained in this study. It is clear from the data that the presence of the substrates formate and H_4folate affects the spin–lattice relaxation time of the cation carbon. In Tables I and V, it is seen that addition of formate to the E·ADP·Mn^{2+}·[^{13}C]MA complex resulted in no significant change in any of the measurements. On the other hand, addition of H_4folate to the quaternary complex resulted in an approximate 2-fold increase in both pT_{1p} and the magnitude of E_{act}. This suggests that r has increased and/or the magnitude of τ_c has changed. When both formate and H_4folate were added to the quaternary complex, the pT_{1p} value increased 5–6-fold and the temperature effect was inverted from negative to positive. This indicates that either a large change in r and τ_c has occurred or else τ_M has increased to the extent that it contributes significantly to pT_{1p}. Importantly, these results parallel those obtained from the study of water proton relaxation rates in similar complexes (Buttlaire et al., 1975a), where it was found that H_4folate addition diminished relaxation rates substantially, whereas formate addition had a significant effect only in the presence of H_4folate. This similarity indicates that the bound cation is quite close to the active site, such that its environment is measurably influenced by whatever conformational effects occur when these substrates bind. The idea that the exchange rate of the cation is reduced in the higher ordered complex is an attractive one because it would imply that the exchange rate of the monovalent cation is coupled to or limited by that of formate. This is consistent with the observed data because the summation of the pT_{1p} value measured for formate in the quinary E·ADP·Mn^{2+}·formate·H_4folate complex (where $pT_{1p} = \tau_M$ of formate) and the pT_{1p} values of [^{13}C]MA in the E·ADP·Mn^{2+}·H_4folate·[^{13}C]MA complex (where $pT_{1p} = T_{1M}$ of [^{13}C]MA) yields a value that is very similar to that measured for the E·ADP·Mn^{2+}·formate·H_4folate·[^{13}C]MA complex. When this treatment is extended to the measurements made at the different temperatures and the two field strengths, an E_{act} value of 5.8 kcal/mol and a $T_{1p}(47\text{ kG})/[T_{1p}(18.8\text{ kG})]$ value of 2.0 are computed, which are very similar to the values reported for the higher ordered complex. Implications of this model are that a cation molecule is bound within the active site of formyltetrahydrofolate synthetase and that this cation may be involved in the binding of formate. This latter idea, although quite speculative, is supported by the earlier observation that removal of the cation in the presence of H_4folate resulted in a 10-fold increase in the formate K_m. Monovalent cation has also been implicated in the binding of pyruvate to pyruvate kinase (Nowak & Mildvan, 1972). Investigations are currently under way to further probe the idea that a formate–monovalent cation interaction is important in the binding of formate to formyltetrahydrofolate synthetase.

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Registry No. H_4folate, 135-16-0; Mn, 7439-96-5; MA, 17000-00-9; formic acid, 64-18-6; formyltetrahydrofolate synthetase, 9023-66-9.

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Affinity Chromatography of Brain Cyclic Nucleotide Phosphodiesterase Using 3-(2-Pyridyldithio)propionyl-Substituted Calmodulin Linked to Thiol-Sepharose[†]

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ABSTRACT: [3-(2-Pyridylthio)propionyl]calmodulin (PDP-CaM), an activated thiol derivative of calmodulin (CaM), was synthesized. Preparations of this derivative containing an average of 2.8 mol of substituent/mol of protein activated purified cyclic nucleotide phosphodiesterase in a manner indistinguishable from that of native CaM. PDP-CaM was covalently coupled to free thiol-Sepharose 4B through formation of a stable mixed disulfide bond for use in affinity chromatography. The binding capacity of the disulfide-linked CaM–Sepharose for phosphodiesterase activity was proportional to substituent level up to 4 mg of CaM/mL of gel; the total capacity of the gel for binding phosphodiesterase was 4 times that of CNBr-coupled CaM–Sepharose. Quantitative recovery was achieved by desorption of both ligand and bound proteins with a reducing agent. The thiolated CaM derivative was then separated from phosphodiesterase by rapid gel filtration; the overall recovery of phosphodiesterase activity was greater than 70%. Preparations of homogeneous enzyme in good yield were obtained after a second chromatography step on CaM–Sepharose. Binding and recovery of phosphodiesterase activity were entirely reproducible, since each preparation of affinity gel was used only once. As it permits separation of interacting species in free solution, this general method may be useful with other ligands for increasing yields from affinity chromatography, particularly when dissociation of molecules in their matrix-bound conformation may be difficult to achieve.

Affinity chromatography (Cuatrecasas et al., 1968) depends on the interaction of macromolecules with specific ligands that have been covalently bound to a solid matrix such as agarose. The use of CNBr for coupling of amine-containing ligands to inert supports (Axên et al., 1967) has enjoyed widespread use, although a variety of other chemical procedures are also available [for a review, see Lowe & Dean (1979)]. These chromatographic media are extremely valuable for many types of selective separations; however, changes in their properties with use can cause problems. Gradual leakage of matrix-bound ligand has been noted (Parikh et al., 1974), and decreases either in functional capacity of the affinity matrix or in recovery of bound proteins occur in some instances. The reliability and reproducibility of purification procedures that depend upon such steps may thus be compromised with repeated usage of the affinity gel. We have observed such changes in the chromatographic behavior of calmodulin (CaM)[1] coupled to CNBr-activated Sepharose (Kincaid &

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[1] Abbreviations: CaM, calmodulin; QAE-Sephadex, [diethyl(2-hydroxypropyl)amino]ethyl-Sephadex; PDP-CaM, [3-(2-pyridyldithio)-propionyl]calmodulin; NaDodSO$_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl chloride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DEAE, diethylaminoethyl; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate.