FORMYLTETRAHYDROFOLATE SYNTHETASE

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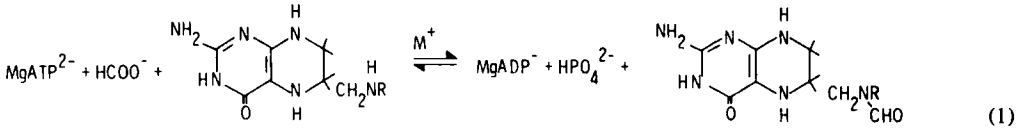
INTRODUCTION

Formyltetrahydrofolate synthetase (EC 6.3.4.3.), designated tetrahydrofolic acid formylase and formate activating enzyme in the earlier literature, catalyzes the formylation of tetrahydrofolate at the N^{10} position using ATP as an energy source (Equation 1).

R = *p*-aminobenzoyl-L-glutamate
M^+ = a monovalent cation (best satisfied by NH_4^+)



(1)

The enzyme is responsible for bringing formate into the one-carbon metabolic pool since the product, N^{10}-formyltetrahydrofolate, is used directly in the biosynthesis of purines and fMet-$tRNA_f^{met}$ or is reduced and used in the biosynthesis of pyrimidines, serine, and methionine. Formyltetrahydrofolate synthetase is widely distributed in nature, having been found in almost every species examined. Table 1 contains a representative list of some of the species from which the enzyme has been isolated as well as the level of activity found in these sources. The level of the enzyme in leucocytes apparently is increased in patients with acute leukemia (Table 1) and infectious mononucleosis.[10] Although there is some variation in the temperature at which the activities were determined, the data in Table 1 emphasize the wide distribution of the enzyme which suggests that it must have an important metabolic role.

In spite of its ubiquitous nature, questions have been raised concerning the metabolic importance of formyltetrahydrofolate synthetase.[11,12] Apparently it has been assumed that the formate concentration in physiological fluids is too low for the enzyme to have much metabolic significance. However, formate is present in physiological fluids as well as in some plants.[13] A study by Annison[14]

TABLE 1

Distribution of Formyltetrahydrofolate Synthetase

Source	Specific activity[a]	Ref.
Animals		
35 species from 12 Phyla[b]	0–800	1
Chicken: liver	30	1
spleen	42	
ovary	21	
heart	36	
muscle	6	
Rabbit: liver	258	1
kidney	108	
spleen	26	
heart	13	
muscle	5	
intestine	15	
testes	42	
Sheep: liver	10	51
Pigeon: liver	2	52
Chick embryo – liver 14 day	6	2
Erythrocytes from 8 species	1–13	3
Leukocytes (human)		4
normal	7	
chronic lymphocytic leukemia	7	
chronic myelocytic leukemia	11	
acute leukemia	16	
acute lymphoblastic leukemia	13	
Plants		
Spinach leaves	9	5
Pea leaves	9	5
Pea roots	3	5
Bean roots	12	5
Bean leaves	2	5
Bean cotyledons	2	5
Germinating		5
tomato seeds	4	
Tobacco leaves	3	5
Tobacco roots	1	5
Germinating		
tobacco seeds	4	5
Tobacco seedlings	4	5
36 species[c]	1–36	6
Bacteria		
Micrococcus aerogenes	1,000–2,800	7
M. lactilyticus, strain 221	50–100	7
M. lactilyticus, strain 416	320–500	7
M. prevotii	100–150	7
M. anaerobius	100–180	7
Clostridium cylindrosporum	15,000–30,000	8
C. acidi-urici	15,000–30,000	8
C. tetanomorphum	40–60	7
C. kluyveri	30–40	7
C. thermoaceticum	11,600	9
Bacillus megatherium	70–100	7
B. brevis	50–70	7
Escherichia coli	10–30	7
Proteus vulgaris	10–20	7
Streptococcus faecalis	10–20	7
Staphylococcus albus	5–20	7
Sarcina lutea	5–10	7
Pseudomonas aeruginosa	3–4	7
Cytophaga succinicans, strain 8	4–6	7
Rhodomicrobium vannielii	30–50	7
Saccharomyces cerevisiae	30–40	7
Neurospora crassa	40–50	7

[a] Specific activities of crude extracts are given in mμmole/min/mg protein. The values in the literature have been rounded off to the nearest whole number.
[b] Several tissues from each species were examined. In only three cases was the enzyme lacking.
[c] Various tissues were examined.

has shown that formate is present in blood at a significant and fairly constant concentration (Table 2). The constant level suggests that formate is being continuously produced by other metabolic pathways and since the utilization of formate by animal tissues in metabolic reactions involving folate has been well documented, it is apparent that formyltetrahydrofolate synthetase does indeed have an important metabolic role. This is substantiated by the finding[14a] that the urinary excretion of formic acid in folic acid deficient rats is markedly increased over that in normal rats.

In some systems the synthesis of formyltetrahydrofolate synthetase is under biological control. For example, purines repress the synthesis of the enzyme in an antifolic-resistant mutant of *Streptococcus faecalis.*[15] Repression is released when the supply of purines is exhausted. Since the enzyme is undetectable in the nonresistant strain of the organism, it is possible that resistance is a result of removal of repression caused by an endogenous repressor.[12] It has also been reported that increased synthesis of the enzyme can be induced by formate.[1]

By far the highest concentration of formyltetrahydrofolate synthetase is found in the bacteria *Micrococcus aerogenes, Clostridium cylindrosporum, C. acidi-urici,* and *C. thermoaceticum.* The first three of these organisms have in common the fact that they utilize purines as carbon and nitrogen sources. The rather unique pathway involves the formation of N^{10}-formyltetrahydrofolate in the penultimate step and the reverse of Equation 1 as the last step.[16] Formyltetrahydrofolate synthetase in these organisms may be responsible for ATP production. It has been suggested that its synthesis may also be depressed in these bacteria explaining the high concentrations found.[15]

TABLE 2

Formate Concentration in Blood[a]

Animal	Total volatile fatty acids (mmol/l)	Percent of total as formic
Horse	0.27–0.96	4–45
Dog	0.18–0.48	31–53
Rabbit	0.89–1.55	6–30
Cat	0.26–0.46	36–54
Goat	0.51–1.61	10–30
Cattle	0.55–1.32	12–28
Human	0.28–0.32	23–34

[a]Data taken from Ref. 14.

MOLECULAR PROPERTIES

Formyltetrahydrofolate synthetase has been purified to homogeneity only from three clostridial species and therefore physical and chemical properties for the enzyme from other sources are not available. It is not known whether the characteristics determined for the clostridial enzymes are generally applicable to formyltetrahydrofolate synthetase from all sources.

Physical Properties

The physical properties of formyltetrahydrofolate synthetase purified from *C. thermoaceticum, C. cylindrosporum,* and *C. acidi-urici* are compiled in Table 3. The enzyme is a tetramer with a molecular weight of 240,000 and is composed of 4 identical subunits as determined by molecular weight, peptide mapping, and isoelectric focusing in 8 *M* urea.[19] Results of gel filtration experiments done at dilute protein concentration and in the presence of substrates established that the tetramer is the catalytically active form of the enzyme.[17] The sedimentation coefficient of the tetramer is between 9S and 10S, while that of the monomer depends on the degree of loss of tertiary structure. Monomer produced under mild conditions (removal of monovalent cations, or lowering the pH to 5 to 6) retains its tertiary structure and sediments as a 3.6 to 4.2S species, while monomer produced with strong denaturants – e.g., extremely high pH or greater than 3 *M* guanidine-HCl – has a sedimentation coefficient of about 1.7S.[17]

Spectral Characteristics

The known spectral properties of the enzyme are summarized in Table 4. The enzyme has a characteristic fluorescence emission due to excitation of the aromatic amino acids. The contribution to fluorescence from tyrosine and tryptophan for the *C. thermoaceticum* enzyme, but not for the enzyme from *C. cylindrosporum,* can apparently be separated since the wavelength of maximum fluorescence shifts as the excitation wavelength is changed from 280 to 295 nm.[24] Moreover, since the emission maximum of the *C. thermoaceticum* enzyme occurs at a shorter wavelength, it appears that the residues in this enzyme

TABLE 3

Physical Properties of Formyltetrahydrofolate Synthetase[a]

Property	*C. cylindrosporum*		*C. acidi-urici*		*C. thermoaceticum*	
	Tetramer	Monomer	Tetramer	Monomer	Tetramer	Monomer
Molecular weight, daltons	$2.38 \pm 0.08 \times 10^5$ (17)[b] 2.30×10^5 (18)[c] 2.40×10^5 (17)[f]	$5.98 \pm 0.01 \times 10^4$ (17)[d] $5.85 + 0.01 \times 10^4$ (19)[e] 5.80×10^4 (20)[b]	2.40×10^5[g]	$6.01 \pm 0.02 \times 10^4$ (19)[e]	$2.44 \pm 0.09 \times 10^5$ (21)[b]	6.10×10^4 (21)[b] $6.00 \pm 0.06 \times 10^4$ (21)[e]
$S_{20,w}$	8.8 (17) 9.25 (18)	3.6–4.2 (17)	9.25 (18)		9.86 ± 0.2 (21)	
$\bar{V}_{cc/g}$	–	–	–	–	0.752 ± 0.011 (21)	–
Stokes radius, A	–	–	–	–	53 ± 4.8 (21)	–
Isoelectric point	–	–	–	5.5 (19)	–	–

[a] Numbers in parentheses refer to references
[b] Determined by sedimentation equilibrium
[c] Determined by sedimentation diffusion
[d] Determined by sedimentation equilibrium in 6 *M* guanidine·hydrochloride
[e] Determined by SDS-gel electrophoresis
[f] Determined by Sephadex G-200 chromatography
[g] J. M. Scott, R. E. MacKenzie, and J. C. Rabinowitz, personal communication

TABLE 4

Spectral Characteristics of Formyltetrahydrofolate Synthetase[a]

Source	ϵ_{280} (ml x mg^{-1} cm^{-1})	ϵ_{280} (M^{-1} cm^{-1})	Fluorescence		$-(M')_{233}$	b_o
			λ_{max}	$\lambda_{excitation}$		
C. thermoaceticum	0.737 ± 0.024 (21)	1.80 x 10^5	316 nm (21)	280 nm		
			328 nm (21)	295 nm	–	–
			355 nm (21)	280 nm[b]		
C. cylindrosporum	0.53 (22)					
	0.528 ± 0.028 (23)	1.27 x 10^5	352 nm[c]	280,292 nm	3656 (17)	−102 (17)
C. acidi-urici	0.370 ± 0.011 (23)	8.88 x 10^4	350 nm[c]	280 nm	–	–

[a] Numbers in parentheses refer to references
[b] Determined in guanidine·HCl
[c] Unpublished work of J. A. K. Harmony and R. H. Himes.

TABLE 5

Amino Acid Content of Formyltetrahydrofolate Synthetase from *Clostridia* Species

Amino acid	*C. cylindrosporum*[a]	*C. acidi-urici*[b]	*C. thermoaceticum*[c]
Serine	76 ± 2	62 ± 3	69
Threonine	123 ± 3	110 ± 2	121
Proline	96	76 ± 3	96 ± 1
Glycine	215 ± 6	204 ± 2	196 ± 4
Alanine	280 ± 8	253 ± 3	239 ± 5
Valine	184 ± 6	152 ± 2	167
Methionine	65 ± 1	45 ± 1	52
Isoleucine	129 ± 4	124 ± 6	148
Leucine	217 ± 6	210 ± 12	206 ± 7
Lysine	181 ± 5	186 ± 3	133 ± 3
Arginine	61 ± 2	66 ± 1	97 ± 2
Phenylalanine	73 ± 2	75	63 ± 1
Tyrosine	36 ± 1	37 ± 1	57 ± 3
Tryptophan	12	9	20
Cysteine	24	24	24
Histidine	32 ± 1	45 ± 1	32 ± 2
Aspartate	260 ± 7	277 ± 3	210 ± 4
Glutamate	197 ± 6	180	179 ± 3

[a] Personal communication, MacKenzie, R. E. and Rabinowitz, J. C.
[b] Data (values have been rounded off to the nearest whole number) from MacKenzie, R. E., D'Ari Straus, L., and Rabinowitz, J. C., *Arch. Biochem. Biophys.*, 150, 421, 1972.[28]
[c] Data (values have been rounded off to the nearest whole number) from Ljungdahl, L. et al., *J. Biol. Chem.*, 245, 4791, 1970.[9]

responsible for the emission spectrum are located in regions of greater hydrophobicity than those in the enzyme from *C. cylindrosporum* or *C. acidi-urici*.[25] The $(M')_{233}$ and b_0 values obtained for the enzyme from *C. cylindrosporum* by optical rotatory dispersion (ORD) spectropolarimetry are consistent with a helical content of 20%. Monomers produced by removing monovalent cations have the same $(M')_{233}$ and b_0 values as the tetramer.[17]

Chemical Composition

The amino acid composition determined for formyltetrahydrofolate synthetase from the three clostridial species is presented in Table 5. The tetrameric protein contains 24 sulfhydryl groups, but no disulfide bonds.[26] Although the enzymes from these sources contain the same number of only two residues, cysteine and leucine, there are no striking differences in the amino acid content. The number of tryptophan residues in each case correlates well with the extinction coefficient determined for the enzyme at 280 nm (Table 4). It has been proposed[27] that thermostable proteins contain more aromatic amino acids and therefore more hydrophobic regions than the corresponding enzymes derived from mesophilic organisms. Formyltetrahydrofolate synthetase from the thermophile *C. thermoaceticum* does contain more tyrosine and tryptophan residues than the protein from the other two sources, but the difference in total number of aromatic amino acids is small. Moreover, the average hydrophobicity for the *C. thermoaceticum* enzyme calculated by Ljungdahl et al.[9] is only slightly greater than the value obtained for the protein from *C. cylindrosporum*.

The purified protein contains little or no carbohydrate, no amino sugars, and less than

0.25% lipid.[9,17] Atomic absorption analyses[17] indicated only trace amounts (< 0.3 atom/mole of enzyme) of nickel, zinc, magnesium, and calcium. No cobalt, iron, or manganese were detected.

Effect of Monovalent Cations on the Quaternary Structure

Formyltetrahydrofolate synthetase requires the presence of ammonium ion or specific alkali metal cations (such as potassium or rubidium) for maximum catalytic efficiency. The requirement for monovalent cations was first demonstrated by Whiteley and Huennekens[29] for the enzyme purified from *M. aerogenes* and has since been demonstrated for the enzyme isolated from human erythrocytes,[3] spinach,[30] pea seedlings,[31] *L. arabinosus* and *L. casei*,[32] *C. cylindrosporum*,[33] and *C. acidi-urici*.[19] Monovalent cations stabilize the enzyme from the thermophilic organism *C. thermoaceticum* against heat inactivation, but are apparently not required for activity.[9]

Using the pure protein from *C. cylindrosporum*, it was shown that the effective monovalent cations have two separable functions. They prevent the catalytically active tetramer from dissociating into inactive monomer subunits;[19,20,34] and they increase by two- to threefold the apparent catalytic activity of the enzyme stabilized as the tetramer in the absence of monovalent cations.[34] Catalytic activation of the enzyme by monovalent cations will be discussed in a later section. Formyltetrahydrofolate synthetase from *C. acidi-urici* has also been shown to dissociate into monomers in the absence of specific monovalent cations[19] and subunit dissociation most likely accounts for the inactivation of the *C. thermoaceticum* enzyme at 55 to 60° in the absence of ammonium or potassium ions.[9]

The Dissociation Process

Studies on the stabilization of the quaternary structure of the protein have been limited to formyltetrahydrofolate synthetase from *C. cylindrosporum* and *C. acidi-urici*. The cations investigated differ in their ability to prevent dissociation of the tetramer. Ultracentrifugal analysis[20] showed that the enzyme exists as a single component of molecular weight approximately 230,000 in the presence of 50 m*M* NH_4^+, K^+, or Rb^+. With the same concentration of Na^+, Li^+, Cs^+, or $Tris^+$, a single component was again obtained, but with a molecular weight of 58,000. The tetramer also dissociates into subunits when the concentration of potassium ion is decreased to 5 m*M*. In comparing the relative abilities of the monovalent cations to prevent dissociation, Welch et al.[34] obtained more quantitative data by measuring the rate of loss of activity at 20° in the presence of 1 m*M* cation. The order of effectiveness was determined to be NH_4^+ (0) ~ $CH_3NH_3^+$ (1) > Rb^+ (12) ≳ K^+ (17) > Cs^+ (26) > Na^+ (35) >> Li^+ (84). The numbers in parentheses represent the percent loss of enzymic activity after eight minutes. Two m*M* ammonium ion is sufficient to stabilize the enzyme for several hours at 20° in 0.1 *M* Tris · HCl buffer, pH 7.7.

Procedures employed to promote dissociation of the tetramer are mild (Sephadex® chromatography or dialysis against a buffer containing no alkali metal ion) and the monomers produced apparently retain their secondary and tertiary structure. Using the enzyme from *C. cylindrosporum*, the $(M')_{233}$ and b_0 values obtained from ORD data[17] and the quantum yield of fluorescence (unpublished results of Harmony and Himes) are identical for the monomer and tetramer.

The Reassociation Process

Dissociation can be reversed by the addition of NH_4^+ and certain other monovalent cations. Depending upon the nature and concentration of the cation and on the reaction conditions (pH, protein concentration, ionic strength), up to 100% of the original enzymic activity can be recovered. The percent of reactivation correlates directly with the percent of tetramer in the solution.[19] The ultraviolet difference spectrum comparing the reactivated tetramer with the monomer form of the enzyme is shown in Figure 1.[17] This spectrum is characteristic of the spectra observed for the aromatic amino acids phenylalanine, tyrosine, and tryptophan in organic vs. aqueous solvents[34a] suggesting that these residues are more exposed to the environment on the monomer than on the reactivated enzyme. The monomer is fairly unstable since after prolonged incubation (9 hr) without monovalent cation the extent of reassociation decreases. This implies that subunit interactions in the tetramer stabilize the tertiary structure of the monomers.

The kinetics of reassociation of monomers

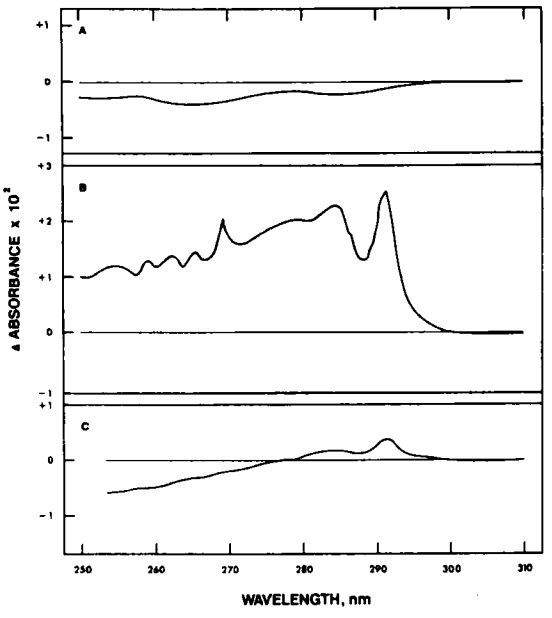


FIGURE 1. Difference spectra between formyltetrahydrofolate synthetase monomers and enzyme reactivated with 100 m*M* NH_4Cl. The enzyme was passed through a Sephadex G-50 column to remove cations, diluted to a concentration of 1.57 mg/ml and incubated at 25° for 30 min to complete dissociation. A. The difference spectrum before addition of NH_4Cl. B. The spectrum obtained 25 min after the addition of NH_4Cl to the sample cuvette. C. The spectrum 13 min after addition of NH_4Cl to the reference cuvette. (Reproduced from Reference 17 by courtesy of *Biochim. Biophys. Acta*)

prepared from *C. cylindrosporum* and *C. acidi-urici* has been investigated extensively with ammonium ion as the reactivating cation. The following interesting difference has been observed: reassociation of subunits from *C. cylindrosporum* is second-order with respect to monomer concentration (unpublished results of Harmony and Himes); reactivation of the *C. acidi-urici* enzyme is first-order[19] with respect to subunit concentration (Figure 2). Since no intermediate dimers or trimers have been detected during the reassociation of either enzyme, it has been concluded that the rate-determining step in reassociation of the monomer from *C. cylindrosporum* involves the dimerization of two monomer subunits followed by rapid association to the tetramer, while the *C. acidi-urici* monomer undergoes a rate-limiting conformational change before actual reassociation is possible. In both cases, Arrhenius plots of the rate of reassociation obtained over the temperature range 4 to 27° are noticeably concave downward, suggesting a complex aspect of reassociation that has not been investigated further. It is possible that there are slight temperature-dependent variations in the conformation of the monomer and/or tetramer.

The extent of reactivation at saturating ammonium ion (200 m*M*) depends on several factors. Maximum recovery of activity (90 to 100%) can be achieved at protein concentrations greater than

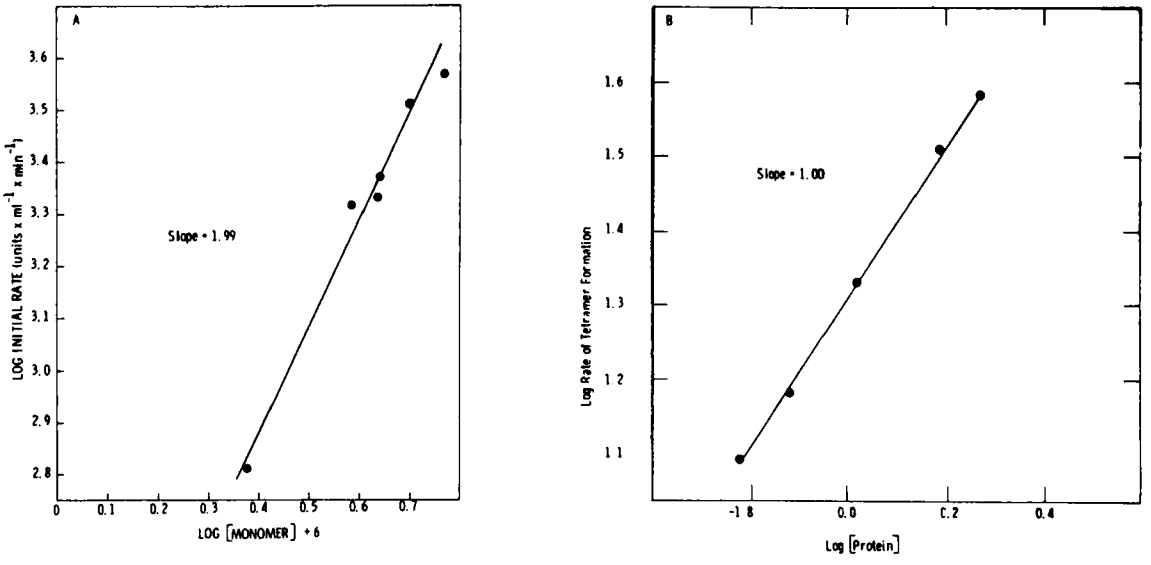


FIGURE 2. Log-log plots of the rate of cation-dependent reactivation at 20° as a function of protein concentration. A. Enzyme from *C. cylindrosporum;* monomer concentration in mol/l; 100 m*M* NH_4Cl. Unpublished results of J. A. K. Harmony and R. H. Himes. B. Enzyme from *C. acidi-urici;* protein concentration in mg/ml; 200 m*M* NH_4Cl. (Reproduced from Reference 19 by courtesy of *J. Biol. Chem.*)

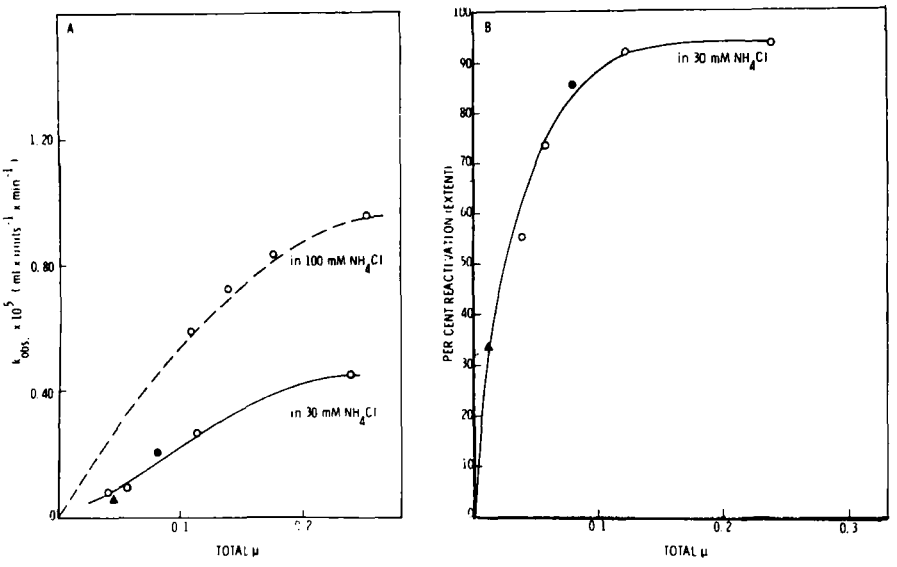


FIGURE 3. Effect of ionic strength on the observed second-order rate constant (A) and the extent (B) of ammonium ion-dependent reactivation of formyltetrahydrofolate synthetase monomers (1 mg/ml) from *C. cylindrosporum*. (▲) 0.05 *M* imidazole · HCl, pH 7.5; (●) 0.1 *M* Tris · HCl, pH 8.0; (○) 0.05 *M* Tris · HCl, pH 7.5 containing varying amounts of NaCl. One unit of enzyme activity refers to the formation of 0.012 μmol of N^{10}-formyltetrahydrofolate/min which corresponds to a ΔA_{350} of 1.0 in 10 min. (Unpublished results of J. A. K. Harmony and R. H. Himes.)

0.5 mg/ml, at temperatures between 20 and 30°, and in the pH range 7.5 to 8.5. In addition to these factors, with nonsaturating cation (10 to 100 m*M* NH_4^+) the ionic strength of the medium significantly influences both the rate and extent of reassociation, as illustrated in Figure 3 for the *C. cylindrosporum* enzyme. The different curves for 100 m*M* and 30 m*M* ammonium ion in Figure 3A suggest that the monovalent cation has a specific role in subunit reassociation which cannot be attributed to ionic strength.

The specificity of the cations is shown in Table 6, which compares the effectiveness of a number of cations in promoting reassociation of the monomers from the two clostridial species. The known hydrated and crystal radius of each cation is also included in the table. Ammonium ion is the most effective cation while the alkali metal ions follow the decreasing order of activity Rb~ K > Cs > Li ~ Na†. A correlation is found between the crystal radius of the cations and their effectiveness (Figure 4). Active monovalent cations have radii around 1.4 Å. It would appear that lithium and sodium are too "small" to cause appreciable reassociation, while cesium is too "large." We have found that thallous ion, ionic radius 1.44 Å, is less effective than NH_4^+ but more effective than K^+ substantiating the importance of the crystal radius. An additional factor in the case of NH_4^+ may be its ability to form hydrogen bonds.[35] At this time, it seems that the hydrated radius cannot play a large role in determining cation specificity, although reassociation takes place in an aqueous environment. The hydrated size of NH_4^+, Rb^+, and Cs^+ are identical, yet the effectiveness of these ions in causing subunit reassociation differs (Table 6). It is possible, however, that factors other than size determine, and must certainly contribute to, the order of cation specificity.

Cesium, sodium, and lithium cations are more

†Our results appear to contradict those reported by MacKenzie and Rabinowitz,[19] who found there was not a significant difference in the amount of reactivation obtained with ammonium, potassium, rubidium, or cesium ions for enzyme from both clostridial species. However, in their experiments the reaction had gone to completion. Our data were obtained under conditions that allow the specificity of the cations to be demonstrated (low cation concentrations and short incubation periods).

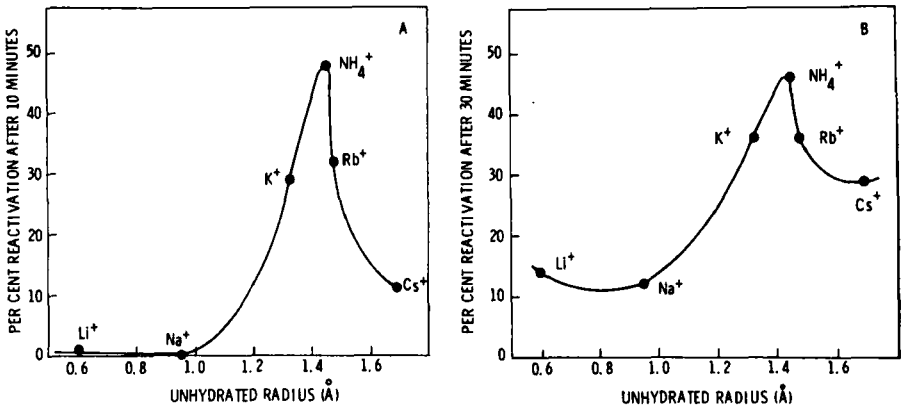


FIGURE 4. Correlation of the crystal radius of the cation with the rate of cation-induced reactivation of monomer (1 mg/ml) from *C. cylindrosporum* (A) and from *C. acidi-urici* (B). The cation (100 m*M*) was present as the chloride salt. (Unpublished results of J. A. K. Harmony and R. H. Himes.)

TABLE 6

Effect of Monovalent Cations on Reactivation of Formyltetrahydrofolate Synthetase Monomer[a]

Cation	Crystal radius, Å[34b]	Hydrated radius, Å[34c]	Percent of Original Activity			
			C. cylindrosporum		*C. acidi-urici*	
			After 10 min	After 100 min	After 30 min	After 200 min
NH_4^+	1.45	2.5	48	77	46	75
$CH_3NH_3^+$	–	–	53	74	–	–
Rb^+	1.48	2.5	32	61	36	60
K^+	1.33	3.0	28	59	36	67
Cs^+	1.69	2.5	11	25	29	54
Na^+	0.95	4.0–4.5	0	1	12	31
Li^+	0.60	6.0	1	1	14	37
none	–	–	0	0	1	1

[a]Monomer (1 mg/ml) was reactivated at 20° and pH 7.5 with 100 m*M* monovalent cation as the chloride salt. Unpublished results of J. A. K. Harmony and R. H. Himes.

effective in causing reassociation of the monomer from *C. acidi-urici* than that from *C. cylindrosporum* (See Table 6 and Reference 19). Differences in cation effectiveness between the two systems reflect the differences in the rate determining steps of reassociation mentioned previously. MacKenzie and Rabinowitz[19] reported that they could kinetically distinguish at least two steps in the reassociation of the *C. acidi-urici* monomer: the initial rate-limiting conformational change of the monomer and the actual process of subunit combination. They proposed that the requirement for cations in the first step, the conformational change, is less specific than the cation requirement for subunit reassociation. Since the first step is slow, the overall process of reassociation of monomer from *C. acidi-urici* will appear to be less dependent on the nature of the monovalent cation than reassociation of monomer from *C. cylindrosporum.*

Significantly, ATP or ADP present at concentrations near their binding constants has a pronounced effect on the kinetics of reassociation of the *C. acidi-urici* subunits.[19] It is known that ATP binds to both the monomer and tetramer forms of the enzyme with equal affinity.[36] MacKenzie and Rabinowitz postulated that ATP and ADP cause the conformational alteration of the monomer. They further reasoned that the presence of ADP (to saturate the initial step) would allow the order of specificity of cations in the process of subunit combination to be eluci-

dated. With ADP present, the order of cation effectiveness closely resembles the order found for the *C. cylindrosporum* enzyme for which the rate-determining step is the combination of two monomers. If ATP or ADP causes the necessary conformational change by binding to the monomer, one would predict that after suitable preincubation of the monomer with ATP (which itself does not induce reassociation), addition of NH_4^+ might result in second-order kinetics of reactivation. This experiment has been performed in our laboratory and the results show that, indeed, preincubation with ATP does change the kinetic order of reassociation from first- to second-order. ATP at concentrations near its binding constant does not affect the rate of reassociation of monomer from *C. cylindrosporum* although its presence does increase the extent of reassociation somewhat. This is probably to be expected since the reassociation process is second-order without ATP implying that a conformational change in the monomer is not required.

The active monovalent cations probably act in a specific way to drastically change the association constants for subunit-subunit interaction. The mechanisms by which this is accomplished are not understood. One can visualize that the cation interacts with and neutralizes negatively charged groups at the subunit interfaces which prevent association by electron repulsion. Alternatively, the important interaction might involve the cation with anions or neutral oxygen and/or nitrogen groups causing a requisite conformational alteration. The protein probably has a number of sites which can nonspecifically bind cations, but only a select number of sites to bind specific ions to produce the desired result, namely, reassociation. High ionic strength would result in saturation of nonspecific sites so that low amounts of the active cations can concentrate at sites required for reactivation. This undoubtedly explains the effect of ionic strength on the rate and extent of reassociation at low ammonium ion concentrations observed experimentally (Figure 3). Whether the specific cation effects can be explained by the anionic field strength[37] or the radius-ratio[38] hypothesis remains to be determined.

The Effect of Anions on Reassociation

Since monovalent cations are added to solutions of monomer or tetramer as inorganic salts, a brief discussion of the importance of the counter ion on the structure of formyltetrahydrofolate synthetase is included in this section. Few data have been published regarding the role of the anion in activating the enzyme. Chloride has generally been the counter ion of choice. The effects of several anions (as the ammonium salt) on the activity of the pea seedling enzyme have been compared.[31] Sulfate and carbonate were observed to stimulate the enzymic reaction by a factor of two compared to the chloride salt. Ammonium nitrate appeared to be a good inhibitor. The results were not correlated with any physical properties of the enzyme nor with any possible changes in the K_m values for the substrates.

Studies with the enzyme from *C. cylindrosporum* have revealed that the nature of the anion affects the stability of the tetramer and influences the rate and extent of the cation-dependent reassociation of monomer subunits. The former effect requires relatively high concentrations of anions (~ 100 m*M*) compared to the latter two (~ 5 m*M*). In both cases the effective anions are multivalent. The role of anions in the cation-dependent reassociation process will be considered in more detail.

We have observed that sulfate, when compared to chloride, causes a dramatic increase in the rate and the extent of reactivation induced by ammonium ion. The observed rate constant and the extent of reactivation are plotted as a function of ammonium ion, present as the chloride and sulfate salts, in Figures 5 and 6, respectively. At 70 m*M* ammonium ion the observed second-order rate constant for reactivation with sulfate is 17 times greater than with chloride. At 10 m*M* NH_4^+ only 25% of the original activity is recovered with NH_4Cl compared to 90% recovery with $(NH_4)_2SO_4$. Furthermore, at saturating ammonium ion, 100% reactivation can be achieved with one third less protein if sulfate rather than chloride is the counter ion. Sulfate also increases the amount of reactivation obtained with ineffective cations such as sodium.

These observations led to an investigation of other anions, the partial results of which are summarized in Table 7. Sulfate is twice as effective as chloride in promoting tetramer formation when measured after 10 min of reaction. The difference becomes less pronounced as the time of incubation increases and the reaction approaches equilibrium since the ammonium ion concentration is suffi-

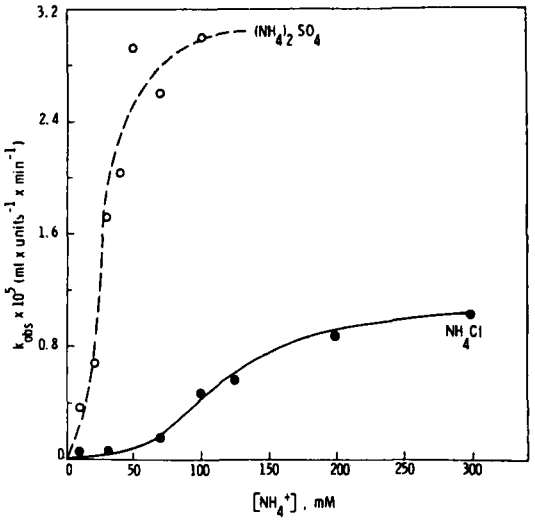


FIGURE 5. Effect of sulfate on the observed rate constant of reactivation at 20° as a function of ammonium ion concentration. One unit of enzyme activity refers to the formation of 0.012 μmol of N^{10}-formyltetrahydrofolate/min which corresponds to a ΔA_{350} of 1.0 in 10 min. (Unpublished results of J. A. K. Harmony and R. H. Himes.)

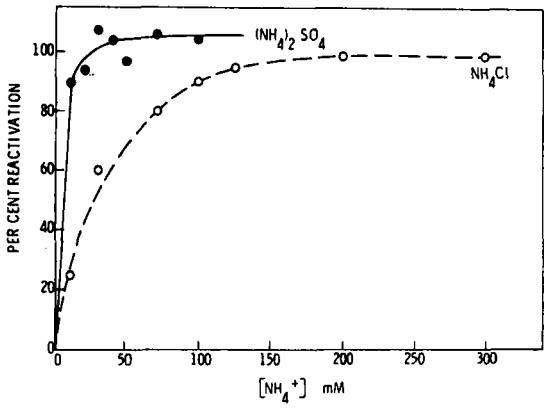


FIGURE 6. Effect of sulfate on the extent of reactivation after 3 hr at 20° as a function of ammonium ion concentration. (Unpublished results of J. A. K. Harmony and R. H. Himes.)

cient to cause 90% reactivation in both cases. Nitrate and bromide ions differ little from chloride in effect. Notably, perchlorate, trichloroacetate, and thiocyanate significantly inhibit reassociation. In order to screen for other effective anions, the experiments were conducted at lower NH_4^+ and anion concentrations. Representative data obtained with the *C. cylindrosporum* enzyme are found in Table 8. Thiosulfate, citrate, phosphate, and ATP are also more effective than chloride, but less effective than sulfate. The concentrations of effective anions required to produce half maximum stimulation of the reassociation rate are between 5 and 10 m*M*; the concentration determined for chloride is an order of magnitude greater. The half-maximum concentration of ATP is 25 times greater than its K_m value, suggesting that it facilitates reassociation through the anionic phosphate groups rather than by binding at the active site. This contrasts with the effect of ATP on reassociation of monomers from *C. acidi-urici* already mentioned. Neither effective nor inhibitory anions cause conformational changes in the

TABLE 7

Effect of Anions on Ammonium Ion-induced Reactivation of Formyltetrahydrofolate Synthetase Monomers[a]

	Percent of Original Activity			
	C. cylindrosporum		*C. acidi-urici*	
Anion	After 10 min	After 100 min	After 10 min	After 100 min
Control (50 m*M* NH_4Cl)	16	52	7	32
Sulfate	100	110	28	65
Chloride	56	96	14	53
Nitrate	52	85	11	47
Bromide	43	84	13	48
Iodide	22	52	5	26
Perchlorate	3	4	1	3
Trichloroacetate	2	4	1	2
Thiocyanate	2	3	2	3

[a]Monomer (1 mg/ml) was reactivated at 20° and pH 7.5 with 50 m*M* NH_4Cl and 70 m*M* of the sodium salt of each anion. Unpublished results of J. A. K. Harmony and R. H. Himes.

TABLE 8

Anion-stimulation of the Rate of Cation-dependent Reactivation of Formyltetrahydrofolate Synthetase Monomer[a]

Anion	Concentration for half-maximum stimulation, m*M*
Sulfate	6
Thiosulfate	8
Citrate	9
Phosphate	10
Chloride	110
MgATP[b]	13
ATP	7

[a]Monomer (1 mg/ml) was reactivated at 20° and pH 7.5 with 10 m*M* NH_4Cl and varying amounts of the sodium salt of the indicated anion. Unpublished results of J. A. K. Harmony and R. H. Himes.

[b]Ratio of Mg: ATP was 2:1.

monomer that could be detected by difference spectroscopy and circular dichroism spectropolarimetry.

We attribute the observed anion effect to the ability of the anion to stabilize or destabilize the structure of the native enzyme. In other words, the anion influences the cation-dependent equilibrium which exists between the monomer and tetramer forms of formyltetrahydrofolate synthetase. From Figure 6 it can be calculated that the equilibrium constant for the reaction $4M \rightleftharpoons M_4$ (M = monomer; M_4 = tetramer) at an ammonium ion concentration of 5 m*M* is increased from 2.6×10^{13} $(mol/l)^3$ with NH_4Cl to 4.6×10^{14} $(mol/l)^3$ with $(NH_4)_2SO_4$.

Anions that facilitate or inhibit subunit reassociation in our study also increase or decrease, respectively, the thermal transition temperature of ribonuclease.[39] In fact, this order of anion effectiveness is well documented [40] and applies to systems in which there is a net transfer of hydrophobic residues from an aqueous to a nonaqueous environment. As mentioned previously, difference spectroscopic studies indicate that aromatic amino acids become "buried" during subunit reassociation in our system (Figure 1). It has been postulated[40] that anions such as sulfate and phosphate stabilize the ordered form of a protein (in our case, the tetramer) by decreasing the free energy of transfer of exposed nonpolar residues to the interior of the protein. Perchlorate and thiocyanate have the opposite effect. As yet, there is no completely satisfactory explanation of the mechanism by which anions decrease or increase the free energy of transfer of hydrophobic residues. It has been suggested[41] that water tends to organize itself into "icebergs" around nonpolar compounds. Anions that increase the extent of iceberg formation about nonpolar groups exposed in the monomer are expected to shift the monomer-tetramer equilibrium toward the tetramer since the thermodynamic consequences of exposing these residues become more unfavorable. It is also possible that certain anions bind directly to one or the other form of the enzyme to affect the equilibrium.

Effect of pH on the Quaternary Structure

Early studies on the stability of formyltetrahydrofolate synthetase[5,18] indicated that the enzyme is not stable at pH values below 7. The only detailed investigation of the effect of pH on the structure of the protein has been done with the enzyme from *C. cylindrosporum.*

Inactivation and Dissociation

The protein dissociates into inactive monomer units at pH values below 7 and above 9.5.[42] Of particular interest has been the dissociation which occurs in acid solution in the pH range 5 to 7. Ultracentrifugal analyses[17,43] showed that complete dissociation at pH 5.3 is not accompanied by unfolding of the subunits. On the other hand, dissociation above pH 10 causes more drastic changes in the enzyme since sedimentation studies[17] indicated the presence of monomers in various stages of unfolding. The Moffitt-Yang parameter, determined from the ORD spectrum of the protein at pH 11.2, has a value of -42[17] indicative of the absence of appreciable helical content at this pH.

The kinetics of inactivation of formyltetrahydrofolate synthetase in basic media has not been determined. Himes and Wilder[42] reported that the substrates MgATP and MgADP increase the rate of dissociation considerably. The concentration of MgATP required to produce 50% of the maximum stimulation is 1.1×10^{-4} *M*, very close to the K_m value determined for this substrate. Other nucleoside triphosphates (with Mg^{2+}), formate, tetrahydrofolate, Mg^{2+}, or combinations of the latter three have no effect. The influence of ATP alone is minimal. Dissociation at high pH values is

probably the result of electrostatic repulsion from the accumulation of negatively charged groups. Amino acid residues expected to ionize in this pH region are those containing sulfhydryl, amino, or phenolic moieties. Since MgATP stimulates dissociation at concentrations close to its K_m value, it must exert its effect while bound at the active site. The presence of this bound substrate may decrease the pKa of a basic group, the protonated form of which is essential for stabilization of the tetramer. Attempts to reactivate the enzyme dissociated at high pH have so far been unsuccessful,[17,42] possibly because low protein concentrations were used.

Inactivation that occurs under acidic conditions is first-order in tetramer concentration.[43] Between pH 5.3 and 7 the dissociation is believed to involve protonation of histidine residues since the imidazole ring is the most likely group which ionizes in this region although other functional groups with unusual pKa values cannot be excluded. The first-order process has been attributed to a rate-limiting conformational change of the protonated tetramer. It could also be due to a one-step dissociation of the protonated enzyme to monomers. Dimers and trimers have not been observed during the dissociation. Difference spectroscopy has revealed that aromatic residues become more exposed to the environment during dissociation (Figure 7). The maximum change at 291 nm, the wavelength of tryptophan absorbance, at pH 5.3 is 40% of the value obtained by denaturing the enzyme in 8 *M* urea indicating that complete unfolding of the polypeptide does not occur. The residues become exposed during or after the rate-determining step since the rate of change at 291 nm equals the rate of inactivation.

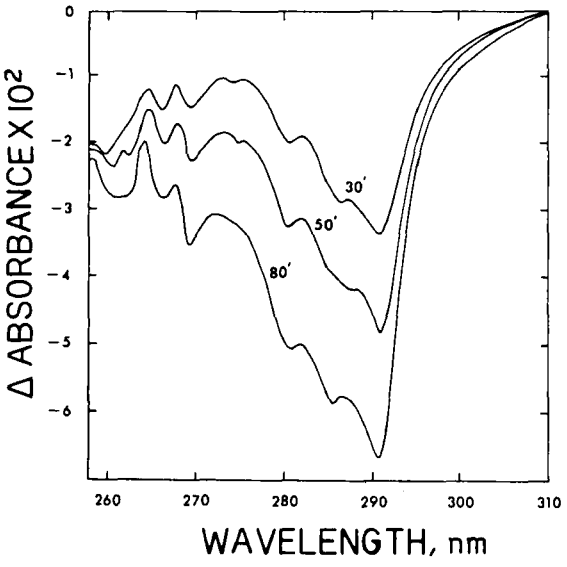


FIGURE 7. Difference spectra between native and acid-inactivated formyltetrahydrofolate synthetase (2.07 mg/ml). The final pH of the solution was 5.3 in the sample cuvette and 7.5 in the reference cuvette. The temperature of both was maintained at 2°. Spectra were recorded as a function of time. (Reproduced from Reference 43 by courtesy of *J. Biol. Chem.*)

Reactivation and Reassociation

Proton-induced inactivation which results from subunit dissociation can be reversed by elevating the pH.[43] Reactivation is influenced by the pH of inactivation and reactivation, the time of incubation under inactivating conditions, the protein concentration, and the presence of sulfate. The effect of pH on the extent of reactivation is shown in Figure 8. The curves in Figure 8 resemble titration curves, suggesting that deprotonation of an ionizable group with a pKa of 6.5 to 7.0, probably the imidazole ring of histidine, is required in order for the enzyme subunits to reassociate. The difference spectrum that resulted from incubation at low pH disappears during reactivation, suggesting that the aromatic residues become reburied. Both the rate of reactivation and of burying of tryptophan are kinetically first-order with respect to the concentration of monomer. In fact, when measured under identical conditions, the rate constants for both processes are the same. Activation parameters obtained for reassociation (at 25°) are $\Delta H\ddagger$, 20.1 kcal/mol, and $\Delta S\ddagger$, -3 eu (entropy unit).

Buttlaire et al.[43] have postulated that the monomer is first deprotonated and then undergoes a rate-determining conformational change. This is followed by a series of rapid steps leading to the tetramer. No intermediate dimers or trimers were detected by ultracentrifuge techniques during reactivation. The aromatic residues probably are transferred to a more hydrophobic environment during the reassociation steps rather than during the conformational change. The situation is similar to that which occurs during reactivation of monomers by specific monovalent cations.

The effect of prolonged incubation under acidic conditions is interesting since it results in a decrease in the extent and rate of reactivation but no decrease in the extent of reassociation. Enzyme incubated for 80 min at 0° and pH 5.2 can be

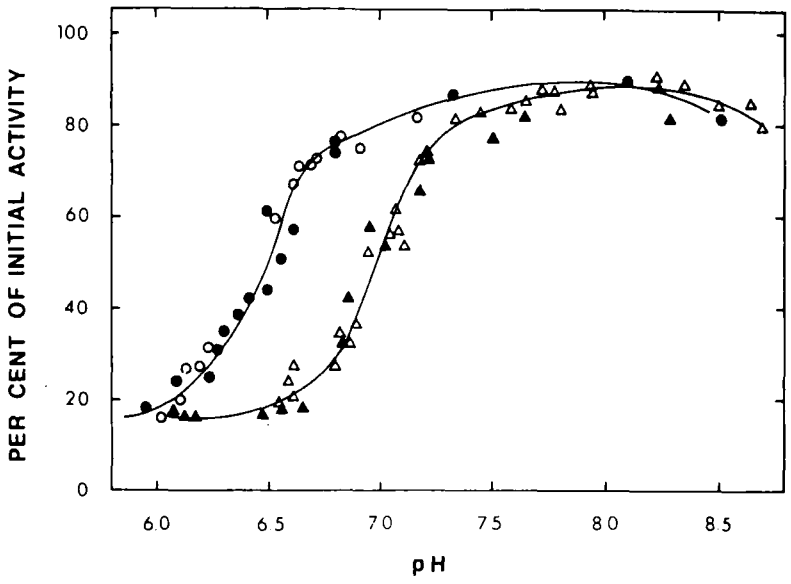


FIGURE 8. Influence of pH on the extent of reactivation. The enzyme (0.1 mg/ml) was inactivated by incubation at pH 5.3 for 80 min at 0°. One-ml aliquots were added to a mixture of varying amounts of 0.1 to 1.0 *N* KOH and 25 μl of 2.0 *M* $(NH_4)_2SO_4$ (•), 50 μl of 1.0 *M* Na_2SO_4 (○), 25 μl of 4.0 *M* NH_4Cl (△), or 40 μl of 3.75 *M* KCl (▲). The enzyme was incubated at 25° until no further increase in activity was observed. The pH values represent the final values. (Reproduced from Reference 43 by courtesy of *J. Biol. Chem.*)

reactivated to 80% of its original activity; that incubated for 9 hr recovers only 35% of its initial activity. The rate constant for reactivation decreases from 13.8 x 10^{-2} min^{-1} after 80 min incubation to 2.3 x 10^{-2} min^{-1} after 4 hr. However, 100% tetramer is obtained in all cases if the pH of reassociation is ≥7.6. The monomers evidently undergo further modification as the time of exposure to the acidic environment increases. This further change does not cause a further change in the difference spectrum from that which resulted from dissociation. The length of incubation in acid solution does determine the magnitude of the ΔA_{291} which occurs during reactivation. Therefore, it seems likely that the tetramer formed after prolonged incubation at pH 5.2 is structurally different from the native enzyme. It was suggested that both fully active and completely inactive tetramers, rather than partially active tetramers, are reconstituted after long periods of incubation.

Sulfate plays an important role in the reactivation process. The pH required to bring about half-maximum reactivation is 7.0 in the absence of sulfate and 6.5 in its presence (Figure 8). Furthermore, sulfate stimulates both the rate and extent of reactivation. At pH 7.8, where the extent of recovery of activity is the same with and without sulfate, the rate constant for reactivation is eight times greater with the anion present. At pH 6.7 the effect of sulfate on the extent of reactivation is dramatic (Figure 9). At low protein concentrations (< 0.1 mg/ml) no reactivation takes place in the absence of sulfate but 100% of the maximum reactivation possible at this pH takes place if 50 m*M* sulfate is present. Five m*M* sulfate is sufficient to produce a half-maximum effect. Of the other anions tested, only thiosulfate is effective, although less so than sulfate. Various cations are also ineffective. The influence of sulfate on reactivation of acid-dissociated enzymes appears to be the same as its effect on cation-induced reassociation of monomers described previously. It is believed that sulfate shifts the equilibrium between monomer and tetramer toward the tetramer by decreasing the free energy of transfer of exposed aromatic residues to hydrophobic regions formed when the subunits reassociate.

CATALYTIC PROPERTIES

Kinetic and Thermodynamic Constants of the Reaction

The equilibrium of the reaction catalyzed by

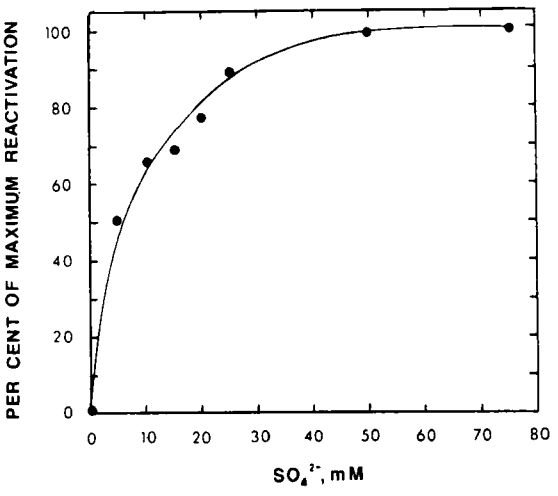


FIGURE 9. Stimulation of reactivation by ammonium sulfate. The enzyme was inactivated as described in Figure 8. The enzyme (0.1 mg/ml) was reactivated at 25° and pH 6.7 in the presence of various concentrations of Na_2SO_4. (Reproduced from Reference 43 by courtesy of *J. Biol. Chem.*)

formyltetrahydrofolate synthetase lies far in the direction of the synthesis of N^{10}-formyltetrahydrofolate. The equilibrium constant was calculated to be 41.[18] In a later publication,[44] using somewhat different reaction conditions, a value of 115 was obtained. It should be pointed out that, in a three substrate-three product reaction for which the equilibrium lies far to one side, small experimental errors in the determination of the concentration of reactants will lead to large errors in the K_{eq} value. Interestingly enough, the equilibrium constant calculated from the Haldane relationship using the V_f, V_b, and K_m values for substrates and products[45] is 76. This is in very close agreement with experimentally determined values particularly considering the number of constants involved. The $\Delta G°$ for the reaction is about 2.1 to 2.8 kcal/mol. Because the equilibrium lies far to the right, the reaction can easily be used as a sensitive assay for formate, tetrahydrofolate, or ATP in the presence of an excess of enzyme.[46] The enzyme is especially useful in the determination of formate in certain carbohydrate structural studies.[47] It can also be used for the preparation of (-)-N^{10}-formyltetrahydrofolate[48] as well as radioactive (-)-tetrahydropteroyltriglutamate and its N^{10}-formyl derivative.[49]

The catalytic center activity of formyltetrahydrofolate synthetase from *C. cylindrosporum* and *C. acidi-urici* is 28,000 moles of substrate transformed/min/active site at 37° (calculated using a specific activity of 450 and an active site molecular weight of 60,000). The corresponding value for the *C. thermoaceticum* enzyme[9] is 15,000 at 50°.

The kinetic constants of the reaction have been determined using the enzyme isolated from many sources. Some reported K_m values are presented in Table 9. The K_m for ATP ranges from 1.5×10^{-5} *M* to 8.84×10^{-4} *M* with most values around 1×10^{-4} *M*. In the majority of cases the K_m for tetrahydrofolate falls between 2×10^{-4} *M* and 1×10^{-3} *M*. A notable exception is the value of 10×10^{-3} *M* found using the enzyme from *L. arabinosus* and *L. casei*. However, as discussed later, in these cases the presence of spermine reduces the K_m value to about $1.5\text{-}1.7 \times 10^{-3}$ *M*.[32] The K_m value for formate is much higher and reported values range between 1.2 and 33 m*M*.

Some of the variation in the reported K_m values is certainly due to the large differences in the purity of the enzyme preparations used. In the case of tetrahydrofolate, instability of the substrate may also help to explain the differences found. Tetrahydrofolate is very susceptible to air oxidation and most methods of preparation lead to a product which is contaminated by colored side products. A recently reported[53] method for the preparation of a highly purified colorless product appears to be the preparative method of choice. Since the K_m of formate has been shown to be affected by the concentration of monovalent cations (see below), the lack of attention to this parameter could lead to some variation in values. All of the enzymes studied show normal Michaelis-Menten kinetics for the three substrates.

Although most workers have reported K_m values for ATP, determined while keeping the total Mg^{2+} concentration constant, kinetic[33] and NMR[22] studies have shown that the true nucleotide substrate for the *C. cylindrosporum* enzyme is MgATP. The K_m value for MgATP was found to be 1.4×10^{-4} *M*.[33] ATP but not Mg^{2+} was found to compete with MgATP for the nucleotide site with the K_i for ATP being about 8×10^{-4} *M*. This competition was also observed in NMR experiments.[22] In the latter case, the enhancement of the proton relaxation rate of the solvent water protons, which is observed when MnATP binds to the enzyme, is decreased when the ratio of

TABLE 9

K_m Values of Substrates

Source	ATP mM	Mg^{2+} mM	(±), THF[a] mM	Formate mM	Ref.
C. cylindrosporum	0.29	2.3	0.52	6.7	18
	0.14 (MgATP)				33
C. thermoaceticum	0.625		1.00	1.67	50
C. acidi-urici	0.12 (MgATP)		0.28	4.8	unpublished
M. aerogenes	0.11		1.1	25	7
S. faecalis A	0.097	0.66	0.54	6.3	12
S. faecalis A_k	0.085	0.71	0.55	6.7	12
L. arabinosus	0.45		10.	8	32
L. casei	0.1		10.	17	32
Pea seedlings			5.6	21	31
Spinach leaves	0.09		0.92	33	5
Sheep liver	0.14	3.0	4.4	1.7	51
Pigeon liver	0.11		1.6	1.2	52
Human erythrocytes	0.015		0.11	2.1	3
Rabbit liver	0.38		0.85	5.1	1
Shizobranchia *insignis* oocytes	0.884		0.23	12.1	1
Pecten caurinus, ovary	0.417		0.35	11.5	1
Balanus nubilis, ovary	0.076		0.32	12.5	1
Cancer magister, ovary	0.013		0.42	31	1
Sebastodes caurinus, liver	0.12		1.87	2	1

[a]Tetrahydrofolate

ATP:MnATP becomes greater than 10, indicating that ATP displaces MnATP from the enzyme.

In the past the reverse reaction was demonstrated either by coupling the reaction to an ATP-utilizing enzyme system, or by using arsenate in place of phosphate, resulting in the arsenolysis of N^{10}-formyltetrahydrofolate. In either case, very large quantities of formyltetrahydrofolate synthetase were required to show the formation of ATP or the disappearance of N^{10}-formyltetrahydrofolate. By using a more sensitive assay, Curthoys and Rabinowitz[45] were able to measure initial velocities of the reverse reaction catalyzed by the *C. cylindrosporum* enzyme and to determine the K_m values of the substrates for this reaction (Table 10). The unexpectedly high K_m value for N^{10}-formyltetrahydrofolate explains why previous investigators had to use high enzyme concentrations to observe the reverse reaction. The amount of this substrate normally used was much below its K_m value. In fact the V_f value is only eight times the V_b value (Table 10).

TABLE 10

Kinetic Constants of the Forward and Reverse Reaction of Formyltetrahydrofolate Synthetase from *C. cylindrosporum*[a]

Substrate	K_m mM
ATP	0.22
ADP	0.13
Phosphate	5[b]
Formate	8.3
(-)-N^{10}-Formyltetrahydrofolate	10
(-)-Tetrahydrofolate	0.37
V Forward reaction[c]	650 μmol/min/mg
V Reverse reaction	80 μmol/min/mg

[a]Taken from Ref. 45.

[b]The K_m of phosphate decreases from 12 mM to 5 mM as the ADP concentration is raised from a value equal to its K_m to a value 3 times greater.

[c]The maximum velocity for the forward reaction was calculated by extrapolating the concentration of all three substrates to infinity.

Effect of pH and Temperature

The enzyme from most sources shows maximum activity between pH 7 and 8.5. The enzyme from *C. cylindrosporum* has been used to examine the effect of pH and temperature on the kinetic constants.[42] Plots of pK_m and log V vs. pH are presented in Figures 10 and 11. Under the conditions used in these studies there was no irreversible denaturation of the enzyme. The maximum velocity data suggest that 2 groups with pK_a values of about 6 and 9.2 are responsible for catalysis. The data concerning the effect of pH on K_m values are more difficult to interpret. When the K_m of one substrate is determined, the other substrates are present at saturating concentrations and since the substrates bind in a random fashion[44,54] the enzyme is present as a ternary complex. The pH effect observed, therefore, is on a different ternary complex for each substrate examined.

The effects of temperature on the kinetic constants are illustrated in Figures 12 and 13. In Figure 12, definite breaks in the curves for plots of pK_m vs. 1/T are seen at about 30°. This suggests that conformational changes in the protein occur in this temperature region which affect the K_m values. As is discussed below, the K_m values are close, but not equal to, the binding constants of the substrates, so it is probably not valid to discuss the results in Figure 12 in terms of enthalpies of binding. If the results are explained by a conformational change, it is one which does not affect the maximum velocity of the reaction since a plot of log V vs. 1/T produces a straight line (Figure 13). The $\Delta H^{\dagger}$ of the reaction is 10.9 kcal/mol and is unaffected by pH in the region 5.5 to 10.

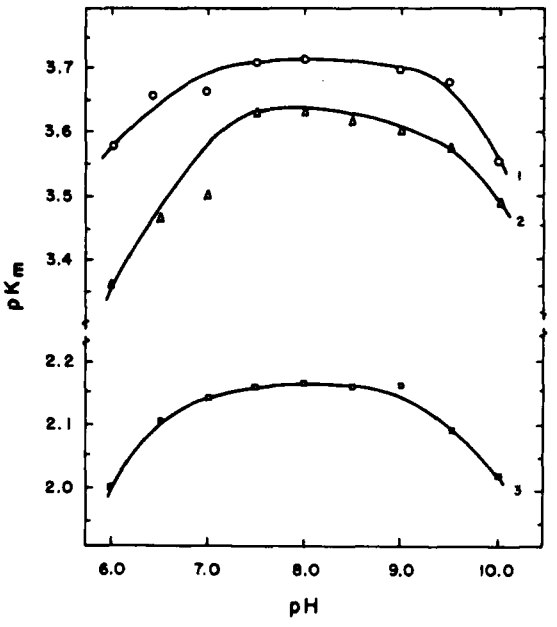


FIGURE 10. The pK_m values as a function of pH at 37°. 1, MgATP; 2, tetrahydrofolate; 3, formate. (Reproduced from Reference 42 by courtesy of *Arch. Biochem. Biophys.*)

Activation by Monovalent Cations

As mentioned earlier, several reports have discussed the requirement of formyltetrahydrofolate synthetase for monovalent cations. Since we now know that the cations are necessary to stabilize the enzyme in its active tetramer conformation, most of these reports cannot be analyzed meaningfully. Under the usual conditions of the reaction (30 to 37°, 10 min, low protein concentrations) the enzyme dissociates into inactive monomers unless a sufficiently high concentration of active monovalent cation is present. However, in one report,[34] attention was given to this problem.

At 20° in the presence of 0.1 *M* sulfate, and

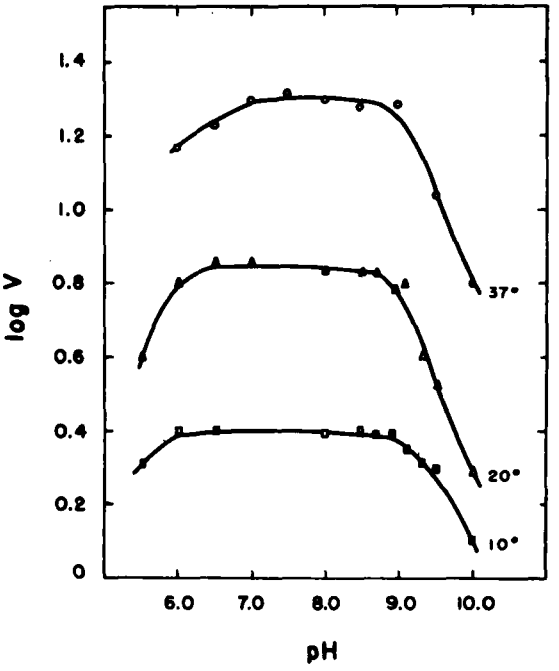


FIGURE 11. Log V as a function of pH and temperature. The maximum velocities were determined with MgATP as the variable substrate. (Reproduced from Reference 42 by courtesy of *Arch. Biochem. Biophys.*)

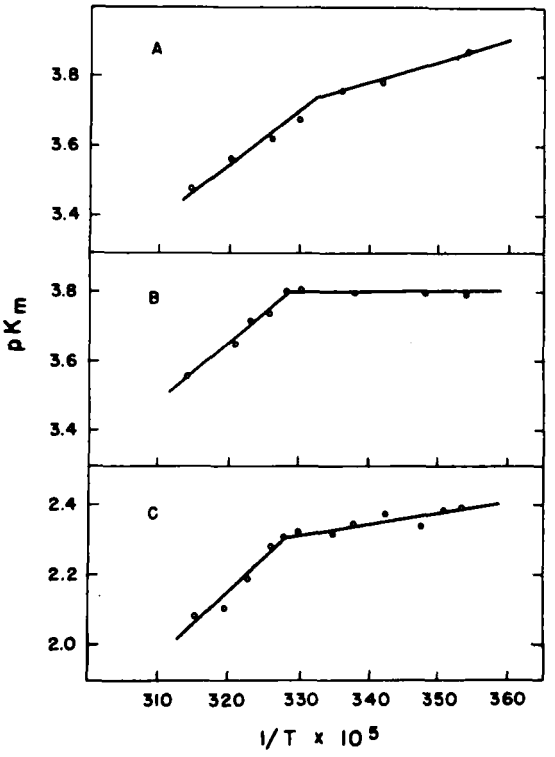


FIGURE 12. The pK_m values as a function of temperature. The K_m values were determined at pH 8.2. A, tetrahydrofolate; B, MgATP; C, formate. (Reproduced from Reference 42 by courtesy of *Arch. Biochem. Biophys.*)

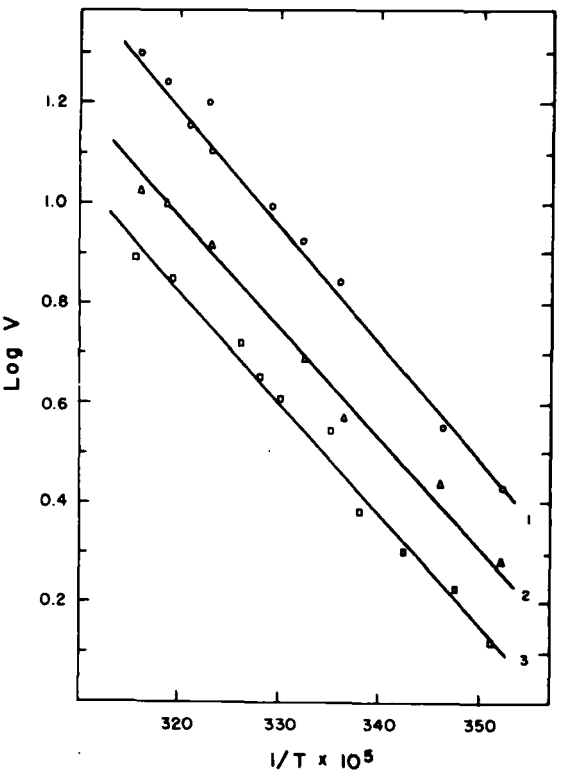


FIGURE 13. Effect of temperature on Log V. The maximum velocities were determined with formate as the variable substrate; 1 = pH 6.5; 2 = pH 9.3; 3 = pH 8.0. (Reproduced from Reference 42 by courtesy of *Arch. Biochem. Biophys.*)

under otherwise normal reaction conditions, the enzyme is maintained as a tetramer.[34] Using these conditions it was shown that the active cations, NH_4^+, K^+, and Rb^+, cause a two- to threefold stimulation in the apparent maximum velocity of the reaction. However, on further examination, it was determined that the cations have a dramatic effect on the K_m of formate. In the absence of an active cation the K_m of formate is 50 m*M* while it is 5 m*M* in the presence of a saturating concentration of NH_4^+. Since the usual concentration of formate present in kinetic studies is 40 m*M*, NH_4^+ appeared to increase the activity of the enzyme. Actually, the cations have no effect on V nor on the K_m values of MgATP and tetrahydrofolate. Apparently, monovalent cations have two functions with formyltetrahydrofolate synthetase – maintaining the protein in the tetramer conformation and decreasing the K_m of formate.

Although activation of enzymes by monovalent cations is well documented, the mechanisms by which activation is accomplished remain to be delineated. The suggestion that cations may allow an enzyme to assume the specific conformation necessary for optimum activity is supported by kinetic studies[55] and by investigations of the effect of monovalent cations on the physical properties of proteins.[56-58] It is also possible for the monovalent cation to act directly at the active site, for example, by forming a bridge between the substrate and protein. Either of these explanations could account for the decreased K_m of formate. Our attempts to observe changes in tetramer conformation caused by the addition of cations using various spectral techniques have not been successful to date.

In only one instance, that involving pyruvate kinase, is there direct evidence for the involvement of monovalent cations at the active site. An NMR study with thallium-205 and the enzyme · Mn complex established that Tl^+ binds to pyruvate kinase 8 Å from the Mn^{2+} binding site.[59] Although the interpretation of this work is subject

to some question,[60] recent NMR experiments of Nowak and Mildvan[61] support the view that the monovalent cation (potassium in this instance) is bound at the active site of pyruvate kinase and it coordinates the carboxyl group of phosphoenolpyruvate analogs, changing the enzyme · Mn · PEP complex to its catalytically active form. An NMR investigation of the enzyme · monovalent cation · MnATP complex of formyltetrahydrofolate synthetase may reveal similar information about the existence of the monovalent cation at the active site of this enzyme.

Effect of Spermine on the Catalytic Activity

Lansford et al.[32] have reported that partially purified formyltetrahydrofolate synthetase from *L. arabinosus* and *L. casei* is stimulated two- to fourfold by 4 m*M* spermine. Other polyamines also produce some stimulation but they are not as effective. Spermine appears to lower the K_m of tetrahydrofolate from 10×10^{-3} *M* to 1.5×10^{-3} *M*. It is tempting to speculate that spermine substitutes for NH_4^+ as a monovalent cation. However, the enzyme from these two organisms requires a very high concentration (200 m*M*) of NH_4^+ for maximum activity. In addition, it was reported that in the presence of 100 m*M* NH_4^+ the addition of 5 m*M* spermine causes some stimulation of activity. Unfortunately, the authors did not indicate how much further stimulation was achieved. These effects are difficult to analyze since, as suggested above, the enzyme may not have been stable in the absence of saturating concentrations of active cations. When similar studies were done with the crystalline enzyme from *C. cylindrosporum*,[33] no effect of spermine was observed when NH_4^+ was present at a concentration of 40 m*M*. Spermine could stabilize the enzyme in the absence of other cations but not as effectively as NH_4^+ and it was concluded that spermine acts simply as a monovalent cation.[33] Why the enzyme from the *Lactobacillus* and *Clostridium* genera should behave differently toward spermine is not clear.

Substrate Specificity and Inhibitors

Formate – The clostridial enzyme appears to have absolute specificity for formate. Using conditions under which there is a complete conversion of formate to N^{10}-formyltetrahydrofolate,[46] the following compounds were completely inactive: methanol, formaldehyde, formamide, acetate, pyruvate, formamidine, glycine, formylglycine, formylaspartate, formylglutamate, formylanthranilate, formiminoaspartate, formiminoglutamate, serine, xanthine, inosinic acid, and phosphite. Specificity for formate was also found with the *M. aerogenes*,[7] pea seedling,[6] and pigeon liver[52] enzyme.

ATP – The specificity for ATP is almost as absolute as for formate. GTP, ITP, CTP, UTP, and the corresponding nucleoside diphosphates cannot substitute for ATP.[6,7,18,51,52] dATP, on the other hand, is an effective substitute[18] having a K_m approximately three times that of ATP. Using dATP, the maximum velocity is 37% of that obtained with ATP.[18] As would be expected, ADP is a competitive inhibitor of ATP with a K_i value of 2.7×10^{-4} *M* when both formate and tetrahydrofolate are present in saturating concentrations.[54] This value is in fairly good agreement with the K_m of MgATP (1.4×10^{-4} *M*)[33] and the K_m of ADP (1.3×10^{-4} *M*) for the reverse reaction.[45] 6-Mercaptopurine, an antileukemia drug, is a competitive inhibitor of ATP[62] having K_i values ranging from 6 to 25×10^{-2} *M*. With such high K_i values it is unlikely that the pharmacologic effect of 6-mercaptopurine is due to the inhibition of formyltetrahydrofolate synthetase as has been suggested.[63]

Perhaps the most interesting inhibitors of formyltetrahydrofolate synthetase are the methylenediphosphonate analogs of the adenine nucleotides. Curthoys and Rabinowitz[23] have determined the K_i and K_d values of several of these compounds. These are presented in Table 11, together with the K_i for adenylyl imidodiphosphate, the β,γ-nitrogen analog of ATP. None of these compounds are active as substrates. The inability to act as substrates is not due to a lack of complex formation with Mg^{2+}.[64] The α,β-methylene analog of ATP has a K_i value close to the K_m of ATP and the K_i of ADP. The dissociation constant for the α,β-methylene analog of ADP is close to that for AMP.[23] When the methylene group is between the β- and γ-phosphates (AMPPCP) the analog is an extremely effective competitor inhibitor of ATP. The K_i value is about 1/20 the K_m of ATP and the K_d, about 1/8 that for ATP. Thus, the specific position of the methylene group, not just its presence, is important.

Curthoys and Rabinowitz[23] explained the tight binding of the β,γ-methylene compound on the basis that it is a transition state analog of ATP.

TABLE 11

K_i and K_d Values for Methylene and Nitrogen Analogs of Adenine Nucleotides[a]

Compound	Abbreviated formula	K_i mM	K_d mM
β-γ-methylene ATP	AMPPCP	0.01	~0.01
α,β-methylene ATP	AMPCPP	0.28[b]	–
α,β-methylene ADP	AMPCP	–	1.9
adenylyl imidodiphosphate	AMPPNP	0.04	–
ATP	–	–	0.076

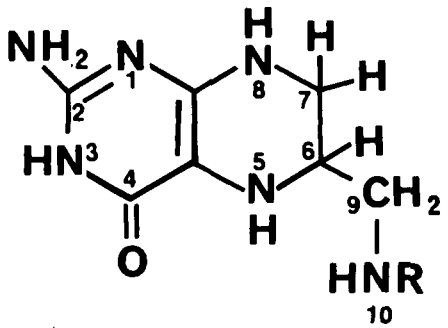
[a]The data for the methylene analogs and ATP are taken from Ref. 23. The data for the nitrogen analog are unpublished work of C. Garrison and R. H. Himes. The AMPPNP was generously provided by R. G. Yount.

[b]In the original article this number was reported to be 0.028 due to a typographical error, N. P. Curthoys and J. C. Rabinowitz, personal communication.

Since there is a transfer of ^{18}O from formate to the γ-phosphate of ATP during the reaction,[65] the mechanism involves an attack of a formate oxygen on this phosphate group. Based on the results of nonenzymic phosphate ester hydrolysis reactions, the authors[23] suggested that attack by formate on the γ-phosphorus may proceed through a trigonal-bipyramidal transition state with the entering and leaving oxygens occupying axial positions. The P-O and P-C bond lengths of pyrophosphoric and methylenediphosphonic acids are 1.63 Å and 1.79 Å, respectively.[64] The P-C distance is closer than the P-O distance to the value (1.76 Å) that the authors cite as being representative of the length of the axial bonds in a trigonal-bipyramidal intermediate. It is possible that ATP is bound to the enzyme in such a way that the γ-phosphate is strained toward the conformation of the transition state. This would require a stretching of the P-O bond. Since the P-C bond is already the proper length, it is suggested that some of the binding energy which is required to stretch the P-O bond in ATP is used to increase the binding affinity of the analog.[23] It may be significant that the K_i for the β,γ-nitrogen analog is higher than that for the methylene analog, but lower than the K_d for ATP (Table 11). The P-N bond length (1.68 Å) is also intermediate between the bond length of the P-C and P-O bonds.[64] The suggestion of Curthoys and Rabinowitz seems reasonable. However, it should be pointed out that only one of the axial bonds of the model trigonal-bipyramidal compound the authors cited (phenanthrenequinone-triisopropyl phosphate 1:1 adduct) has a length of 1.76 Å.[66] This particular axial oxygen is part of a five-membered ring which also includes one of the equatorial oxygens and is therefore in a locked configuration. The free axial bond distance, on the other hand, is 1.63 Å, only slightly longer than the free equatorial P-O bonds. The lengthening of the P-O axial bond in the five-membered ring was attributed to molecular π-bonding with the phenanthrene ring.[67] Whether the compound cited is a good model for the enzymic transition state is questionable.

Tetrahydrofolate – Tetrahydrofolate is a derivative of tetrahydropteridine.

R = benzoyl-L-glutamate



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Since the adduct containing the tetrahydropteridine ring and benzoic acid moieties is sometimes called tetrahydropteroic acid, tetrahydrofolate is tetrahydropteroyl-L-glutamate. Carbon-6 is an asymmetric center and the chemical method of preparation of tetrahydrofolate (usually catalytic hydrogenation of folic acid) leads to a racemic mixture of the (+)- and (-)-isomers. The absolute configuration about C-6 is not known. The specific rotations of the different isomers are listed in Table 12. The rotations of (±)- and (-)-tetrahydrofolate agree with the previously published values,[69] considering that different extinction coefficients were used to calculate concentrations. Asymmetric centers at C-6 and on the L-glutamate moiety contribute to the total rotation of tetrahydrofolate. Since the specific rotation of the (-)-isomer is about -20° and that of the (±)-isomer is +24°, the rotation due to C-6 must be about 44°. The expected rotation of (+)-tetrahydrofolate therefore is +64°. The reported value of +54° is in fair agreement, considering the difficulty in obtaining pure tetrahydrofolate.

The enzyme utilizes only 50%, and therefore only one isomer, of the chemically prepared tetrahydrofolate. This was first shown indirectly to be the (-)-isomer by converting the product of the reaction to N^5-formyltetrahydrofolate with alkali and showing that this compound is fully active in a microbiological assay.[70] (+)-N^5-Formyltetrahydrofolate is inactive in the same assay. The specificity of the enzyme for the (-)-isomer was demonstrated more directly by Mathews and Huennekens[69] who prepared (-)-tetrahydrofolate from folate using dihydrofolate reductase and showed it to be twice as active with formyltetrahydrofolate synthetase as the chemically prepared substrate. We have carried this one step further and isolated the (-)- and (+)-isomers of both tetrahydrofolate and N^{10}-formyltetrahydrofolate. The results reported in Table 12 confirm that the (+)-isomer is not a substrate for the reaction and a comparison of the K_m value of the (-)-isomer with that of the (±)-mixture indicates that (+)-tetrahydrofolate is not an inhibitor. This latter conclusion was substantiated by showing that, at a subsaturating concentration of (-)-tetrahydrofolate, the (+)-isomer present in a 2.5:1 molar excess over the (-)-isomer does not inhibit the reaction. Likewise, (+)-N^{10}-formyltetrahydrofolate prepared by chemical formylation of (+)-tetrahydrofolate does not inhibit the arsenolysis of (-)-N^{10}-formyltetrahydrofolate catalyzed by the enzyme. It is concluded that the (+)-isomers do not bind to the enzyme.

TABLE 12

Properties of Tetrahydrofolate Isomers

Isomer[a]	$[\alpha]_{589}^{25}$[b]	Percent Utilized by Formyltetrahydrofolate Synthetase	K_m m*M*
(-)	-20.4	93	0.25
(+)	+52.8	1	–
(±)	+23.9	50	0.52

[a]The (-) isomer was prepared using dihydrofolate reductase. The (+) isomer was prepared using formyltetrahydrofolate synthetase under conditions where all the (-) isomer is converted to product. The compounds were then purified on DEAE-cellulose using a modified procedure of Scrimgeour and Vitols.[68]

[b]Determined in 0.1 *M* 2-mercaptoethanol, pH 7.

The authors, as well as other researchers, have shown that a variety of potential tetrahydrofolate analogs, some of which are listed in Table 13, are unable to replace tetrahydrofolate as substrates or to act as competitive inhibitors. These compounds therefore are probably incapable of binding to the enzyme. A very low level of activity observed with some compounds can probably be explained by contamination with tetrahydrofolate. Apparently there is an absolute requirement for the 2-amino-4-oxypteridine ring. The binding of the tetrahydro compound is about 10 times better than the oxidized compound.[45] Substitutions on the N^5 position are also not permitted even though this position is not formylated. Interestingly, the dissociation constants for N^{10}-formyltetrahydrofolate and N^{10}-formyltetrahydropteroyltriglutamate are 14 and 20 times higher, respectively, than the dissociation constants of the corresponding unformylated compounds.[45] These results would suggest that the N^5 and N^{10} positions are situated in such close proximity to the enzyme that replacement of the protons on these nitrogen atoms with more bulky groups drastically decreases the binding affinity.

About the only modifications of the tetrahydrofolate molecule that do not lead to a total loss of activity involve the amino acid component. Tetrahydropteroyl-D-glutamate and -L-aspartate are reported to give maximum velocities which are

TABLE 13

Compounds Inactive as Tetrahydrofolate Analogs

Compound	Ref.
2-Amino-4-oxy-6-hydroxymethyl-tetrahydropteridine	51
2-Amino-4-oxy-tetrahydropteroic acid	18,51
2,4-Diamino-tetrahydropteroylglutamate	18,51
N^5-Formyltetrahydrofolate	7,51
N^{10}-Methyltetrahydrofolate	18,51
Dihydrofolate	7,51
2,4-Dioxy-tetrahydropteroylglutamate	18,51
Tetrahydrohomofolate	71
Folate	7
N^5-Methyltetrahydrofolate	unpublished results, R. H. Himes
2-Amino-4-oxy-tetrahydropteridine	unpublished results, R. H. Himes

6 and 20%, respectively, of that obtained with tetrahydrofolate.[18] Tetrahydrofolate should really be considered an analog of the in vivo coenzyme. In *Clostridia* the major form of the coenzyme is tetrahydropteroyltriglutamate,[72] and in rat liver, tetrahydropteroylpentaglutamate.[73,74] Heptaglutamate derivatives have been demonstrated in yeast.[75] The glutamates are linked by amide bonds through the γ-carboxyl groups. An early report[76] of a folate derivative containing other amino acids has never been substantiated. The triglutamate derivatives bind about 130 to 200 times more tightly than the monoglutamate derivatives to clostridial formyltetrahydrofolate synthetase.[45] It would be interesting to know whether the enzyme from a specific source shows maximum specificity for the major polyglutamate derivative found in that source.

Divalent metal ions – Mg^{2+} is the divalent cation which produces the highest maximum velocity. Other metals including Mn^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+} can replace Mg^{2+} with various degrees of effectiveness, depending on the source of the enzyme.[3,6,7,12,18,51,52] As mentioned earlier, the function of the divalent cation is to complex with ATP, forming the true substrate MATP.[33] The enzyme from *C. cylindrosporum* is inhibited by heavy metals. 2-Mercaptoethanol is usually included in reaction mixtures primarily to prevent air oxidation of tetrahydrofolate, but it apparently also complexes heavy metal contaminants. If ascorbate is used as the antioxidant, EDTA must also be included in order for the enzyme to function maximally.[18]

LIGAND BINDING

The binding of nucleotide and folate substrates to formyltetrahydrofolate synthetase has been investigated by Curthoys and Rabinowitz.[23,36,45] Table 14 contains binding parameters determined for substrates and related compounds with enzyme purified from *C. cylindrosporum* and *C. acidi-urici*. Due to the high K_m value for formate, binding of this substrate has not been investigated.

Nucleotides

MgATP binds to formyltetrahydrofolate synthetase from *C. cylindrosporum* at 4 identical, noninteracting sites, each having a dissociation constant of about 0.07 m*M*, and to the enzyme from *C. acidi-urici* at 3.7 sites with K_d = 0.036 m*M*. In the absence of magnesium, ATP binding is less specific, particularly in the case of the *C. cylindrosporum* enzyme, where a binding constant could not be determined due to the nonspecific interaction of ATP with the protein. Kinetic studies[33] which showed that ATP is a competitive inhibitor of MgATP with a K_i of about 0.8 m*M* and NMR experiments[22] mentioned previously, which showed that an ATP:MnATP ratio of 10 is required before MnATP is displaced from the enzyme indicate that ATP does not bind as well as the metal nucleotide to the enzyme from *C. cylindrosporum*. ATP also interacts nonspecifically with the enzyme from *C. acidi-urici*, but only when present at high concentrations. The dissociation constant observed at low ATP concentrations is slightly greater than that determined for the magnesium chelate. The divalent cation prob-

TABLE 14

Binding of Substrates and Related Compounds to Formyltetrahydrofolate Synthetase*

Enzyme	Compound	K_d, m*M*	Number of sites	Method
C. cylindrosporum, tetramer	MgATP	0.076	4	a
		0.073	4.2	b,d
		0.062	4.1	b
	MgADP	0.089	3.3	b
		0.12	3.6	a
	ADP	0.13	3.6	a
	AMP	1.2		c
	Ribose-5′-phosphate	8.6		c
	Adenosine	17		c
	Phosphate	7.1		c
	AMPPCP	~0.01		c
	(-)-N^{10}-Formyltetrahydropteroyltriglutamate	0.034	4	a
	(-)-N^{10}-Formyltetrahydrofolate	4.5		c
	(-)-Tetrahydropteroyltriglutamate	0.0017	3.5	a
		0.0018		b
	(-)-Tetrahydrofolate	0.32		c
	Pteroyltriglutamate	0.015		c
C. cylindrosporum, monomer	MgATP	0.070	0.71	b
	MgADP	0.092	0.62	b
C. acidi-urici, tetramer	MgATP	0.036	3.7	a
	ATP	0.043	3.7	a
	MgADP	0.065	3.6	a

*Taken from Ref. 23, 36, and 45.

[a]Partition equilibrium; 37°; pH 8.

[b]Partition equilibrium; 23–24°.

[c]Partition equilibrium with competitive binding.

[d]Equilibrium dialysis.

ably does not function as a bridge between ATP and the enzyme. This conclusion is based on the fact that ATP does bind to the enzyme and competes with MgATP for the same site, and that enzyme·MnATP but not enzyme·Mn complexes could be demonstrated using NMR techniques.

Dissociation constants for MgADP are 1.5 to 1.8 times greater than for MgATP with enzyme from both sources. Formyltetrahydrofolate synthetase isolated from *C. acidi-urici* binds both nucleotides with twice the affinity observed for the *C. cylindrosporum* enzyme. Competitive binding experiments indicated that MgATP and MgADP bind to the same site.

The nucleotide binding site appears to be intrinsic to the monomer subunit of formyltetrahydrofolate synthetase.[36] Catalytically inactive monomers produced by removing specific monovalent cations bind MgATP and MgADP with an affinity equal to that of the native tetramer. However, the monomer has only 68% of the predicted MgATP site and about 75% of the MgADP site based on the number of 4 and 3.3 sites obtained for the tetramer. In the MgATP

binding study, 69% of the monomers were reactivatable with 200 m*M* ammonium chloride; in the MgADP experiment, 82% reactivation was obtained. This suggests that only monomers capable of reassociating to tetramer can bind nucleotides and, therefore, that there is approximately one binding site per monomer as predicted. The inability of monomers to catalyze the enzymic reaction is not the result of an inability to bind nucleotide substrates.

Competitive binding studies with ATP and a number of related compounds were performed with the tetramer from *C. cylindrosporum* in order to assess the role of various positions of the nucleotide in binding. AMP, ribose-5′-phosphate, and adenosine all bind less well than the complete nucleotide (Table 14). The interaction of the β,γ-methylene analog (AMPPCP) of ATP with the enzyme was also studied. This analog is a strong competitor for the nucleotide binding site, having qualitatively a tenfold greater affinity for the enzyme. It is also a good inhibitor of ATP as mentioned previously. No strong interaction between the α,β-methylene analog of ADP and the enzyme was observed (Table 11).

The free energies of binding of the nucleotides and related compounds to formyltetrahydrofolate synthetase from *C. cylindrosporum* were calculated from the respective dissociation constants. By assuming that the free energy of binding of ATP is equal to the sum of the binding energies of the different components of the molecule, Curthoys and Rabinowitz[23] calculated the free energy contribution of each component. In these calculations, the assumption was also made that the thermodynamic ground state energy of all the compounds is the same. The results in Table 15 imply that there are a number of subsites involved in the ATP binding site, each subsite having specificity for a portion of the ATP molecule. The γ-phosphate contributes little to the overall affinity for ATP in spite of the fact that this is the reactive portion of the ATP molecule. In fact, the γ-phosphate may interact very strongly with the enzyme, but only a fraction of the interaction energy may be available to contribute to the binding affinity. According to Curthoys and Rabinowitz, the remaining energy may be required to distort this group toward a strained, reactive transition-state configuration, as mentioned earlier.

TABLE 15

Calculated Changes in Free Energy Contributed by each of Various Portions of ATP Molecule to its Overall Affinity[a]

Group	Δ(ΔG), kcal
Adenine	1.2
Ribose	1.3
α-P_i	1.6
β-P_i	1.4
γ-P_i of ATP	0.3
γ-P_i of AMPPCP	1.6

[a]Reproduced from Curthoys, N. P. and Rabinowitz, J. C., *J. Biol. Chem.*, 246, 6942, 1971.[23] With permission.

Folate Compounds

The binding parameters of folate substrates to formyltetrahydrofolate synthetase from *C. cylindrosporum* are shown in Table 14. Rabinowitz and Himes[72] reported that the folate coenzymes in this bacterial species exist exclusively as the triglutamyl derivatives. The relative dissociation constants of the mono- and triglutamyl derivatives of N^{10}-formyltetrahydrofolate and tetrahydrofolate show a difference in affinity of approximately 100- to 200-fold in favor of the triglutamate species. Evidence from competitive binding studies suggests that both mono- and triglutamyl substrates bind at the same site. (-)-N^{10}-Formyltetrahydropteroyltriglutamate binds to four independent, noninteracting sites. At high concentrations it binds to additional sites, but with a lower affinity. In the presence of AMPPCP, the affinity for (-)-N^{10}-formyltetrahydropteroyltriglutamate increases by a factor of 2.4. The ATP analog, however, does not prevent the nonspecific binding of (-)-N^{10}-formyltetrahydropteroyltriglutamate to the protein indicating that the folate substrate cannot interact at the ATP site as previously postulated.[54] The presence of AMPPCP has no effect on (-)-tetrahydropteroyltriglutamate binding, nor does the addition of 20 m*M* sodium formate. Pteroyltriglutamate, the oxidized form of the substrate, binds with 10% of the affinity of the reduced substrate. The difference in affinity could be due to differences in pK_a values of various groups on the oxidized and reduced pteridine rings or to the fact that the ring system is planar in the oxidized substrate.

(-)-Tetrahydropteroyltriglutamate does not bind to the monomer form of the enzyme.[36] If

the tetrahydrofolate binding site is intrinsic to the monomer, it may be altered in some manner by subunit dissociation. It is also possible that each tetrahydrofolate site involves more than one subunit. Finally, the binding of tetrahydrofolate to the monomer may require the presence of a specific monovalent cation which was necessarily omitted in these studies. In any case, the inability of the monomer to bind tetrahydrofolate explains its lack of catalytic activity.

Comparison of the dissociation constants (Table 14) with the kinetic constants (Table 10) determined for the substrates in the forward and reverse reactions reveals that the K_d values are generally less than the corresponding Michaelis constants. However, the differences are not great, with the exception of (-)-tetrahydropteroyltriglutamate, indicating that the rate of dissociation of substrates from the enzyme and the rate of interconversion of the quaternary complexes (enzyme · MgATP · formate · tetrahydrofolate and enzyme · MgADP · P_i · N^{10}-formyltetrahydrofolate) are of the same order of magnitude. Joyce and Himes[44] reached a similar conclusion based on a study of equilibrium reaction rates.

AMINO ACID MODIFICATION

Histidine

Studies of the effect of pH on the kinetic constants K_m and V (Figures 10 and 11) have indicated that functional groups on formyltetrahydrofolate synthetase with pK_a values of 6 and 9.2 are involved in catalysis.[42] The group with a pK_a value of about six suggests that histidine may be an active site residue.

Studies by MacKenzie et al.[28] provide much stronger evidence for the involvement of histidine. Using the enzyme from *C. acidi-urici*, these workers found that the loss of activity which occurs upon photooxidation in the presence of methylene blue correlates with the oxidation of histidine residues (Figure 14). In addition, the pseudo first-order rate constants for inactivation increase as the pH is raised from 5 to 7, and a plot of the rate constant as a function of pH shows an inflection point at pH 6.3, implicating histidine as the site of oxidation. Under the conditions of the experiments, tryptophan and tyrosine residues were not oxidized. Destruction of methionine and cysteine residues does not occur until more than 50% of the enzymic activity has been lost.

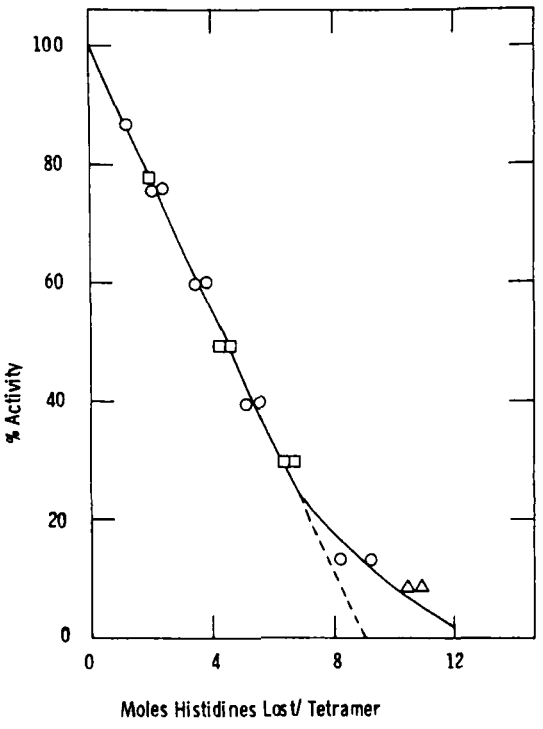


FIGURE 14. Correlation of photoinactivation with loss of histidines. Conditions were 1.25 x 10^{-5} *M* enzyme, 2.5 x 10^{-5} *M* methylene blue, pH 7.8. Histidine was determined after acid hydrolysis. Symbols represent different experiments. (Reproduced from Reference 28 by courtesy of *Arch. Biochem. Biophys.*)

Moreover, the enzyme remains in the tetramer form until it is 80% inactivated.

Enzyme that is 50% inactivated by photooxidation retains the 4 binding sites for ATP and (-)-tetrahydropteroyltriglutamate. The dissociation constants of these substrates are also not affected. The histidine residues that are destroyed are therefore not involved in the binding of these substrates. These results were interpreted by the authors to indicate that at least one histidine residue is part of each catalytic site. The fact that the loss of activity correlates with the destruction of two histidine residues per monomer could mean that there are two histidines at the active site or that the second residue is simply destroyed at the same rate as the active site histidine. These results could probably also be explained by conformational changes produced as a result of the oxidation of histidine residues. Since the authors did not use physical techniques to rule out such conformational changes, it must remain a possibility.

Cysteine

Formyltetrahydrofolate synthetase of different degrees of purity obtained from sheep liver,[51] pea seedlings,[31] *M. aerogenes*,[7] and *Clostridia*[18,26] has been shown to be inhibited by a variety of sulfhydryl reagents. However, there is no strong evidence that an SH group is part of the active site. The role of sulfhydryl groups has been most thoroughly studied with the enzyme from *C. cylindrosporum*.[26] A variety of reagents were used to determine the total number of free SH groups including mercuribenzoate (MB), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide, and mersalyl. An average number of 24 SH groups/240,000 mol wt or 6/monomer were found. Close agreement was obtained with the different methods. This value is in good agreement with the total half-cystine content determined by performic acid oxidation and amino acid analysis. Thus there are no disulfide bonds in the protein. The enzyme from *C. acidi-urici* and *C. thermoaceticum* also contains 24 half-cystine residues (Table 5).

All of the SH groups in the *C. cylindrosporum* enzyme react with MB or DTNB without resorting to the use of denaturing agents. Although all of the sulfhydryl groups are accessible to these reagents, the native enzyme is quite stable to air oxidation. For example, the enzyme can be isolated in the absence of reducing agents or dialyzed extensively against buffer lacking reducing agents without the loss of free SH groups. However, the SH groups in the monomer, prepared by the removal of monovalent cations, are quite susceptible to oxidation.[26]

The reaction of MB and DTNB with the enzyme causes inactivation as well as dissociation into subunits. MB is much more effective in causing dissociation than DTNB. Using MB, monomer was detected after only 5% inactivation had occurred and an average of 1 MB molecule had been bound per tetramer. On the other hand, with DTNB the enzyme remained in the tetramer form until over 75% of the enzymic activity was lost and about 18 SH groups had reacted. This reagent probably produces large changes in the tertiary structure before the subunits dissociate. Slight reactivation of MB- or DTNB-inactivated enzyme could be achieved by the addition of cysteine, but only when the protein was still in the tetrameric form.

The effect of the substrates on the inactivation of the enzyme by MB and DTNB has also been examined.[26] Inactivation by these reagents follows pseudo first-order kindetics. Neither Mg^{2+} nor formate affects the rate. ATP and tetrahydrofolate retard the rate of inactivation while MgATP facilitates the reaction. The concentrations of substrates that produce 50% of the maximal effect are in reasonable agreement with kinetic constants, K_m for MgATP and tetrahydrofolate, and K_i for ATP, suggesting that the effects are produced as a result of the compounds binding at the active site. Since ATP, a competitive inhibitor of MgATP, produces an effect opposite to that of MgATP and since none of the compounds changes the rate of inactivation drastically, it was felt[26] that the substrates produce conformational changes in the protein upon binding, which alter the reactivity of the sulfhydryl groups. The results were not interpreted to mean that there are sulfhydryl groups at the active site.

Surprisingly, DTNB reacts quite differently with formyltetrahydrofolate synthetase from the closely related organisms *C. acidi-urici* and *C. cylindrosporum*, as illustrated in Figure 15A. Within the time period of the experiment there is scarcely any reaction with the *C. acidi-urici* enzyme, whereas 16 of the 24 SH groups in the *C. cylindrosporum* enzyme have reacted. Similar differences in reactivity are also observed when the reaction is carried out in 8 *M* urea, suggesting that this reagent is not very effective in unfolding the *C. acidi-urici* protein. In SDS the enzyme from *C. acidi-urici* reacts with DTNB almost instantaneously. The reaction of enzyme from both species with MB, on the other hand, is almost identical (Figure 15B). The results with DTNB suggest that there are large differences, either in the arrangement of the subunits or in the tertiary structure of the subunits, so that the sulfhydryl groups in the enzyme from *C. acidi-urici* are not exposed enough to react with DTNB.

Hg^{2+} also completely inactivates formyltetrahydrofolate synthetase, but, in contrast to the effect of the other sulfhydryl reagents, it does not result in dissociation.[26] The Hg^{2+}-inactivated enzyme can be completely reactivated by the addition of 2-mercaptoethanol. By using Hg^{203} it was shown that complete loss in activity occurs when 8 Hg^{2+} molecules are bound per mol of enzyme. The binding of Hg^{2+} was found to correlate with the loss of SH groups.

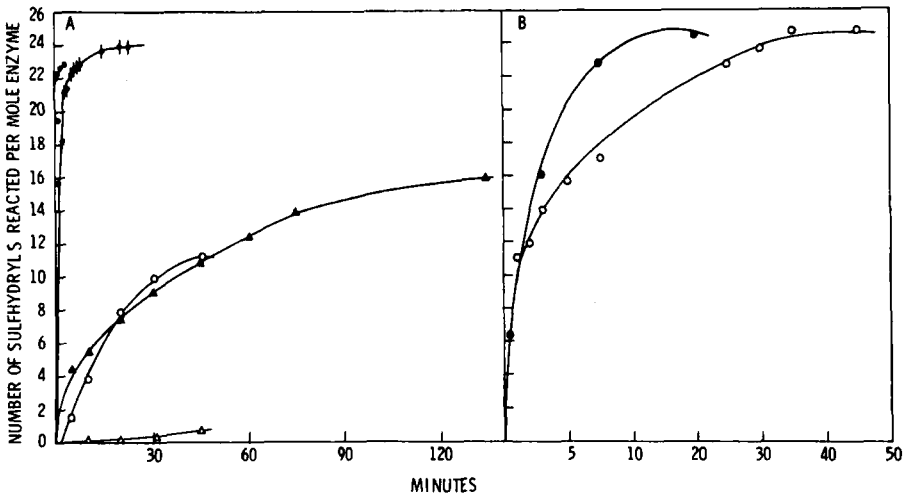


FIGURE 15. Reaction of formyltetrahydrofolate synthetase from *C. acidi-urici* (open symbols) and *C. cylindrosporum* (closed symbols) with (A) DTNB and (B) MB. (A) Enzyme (0.33 mg/ml) was allowed to react with DTNB (3.34×10^{-4} M) and the number of sulfhydryl groups reacted was determined from the change in absorbance at 412 nm. The following conditions were used: (△, ▲) 0.1 *M* phosphate buffer, pH 8.0; (○, ●) 8 *M* urea; and (ϕ) buffer containing 0.1% sodium dodecyl sulfate. (3) Enzyme from *C. acidi-urici* (0.20 mg/ml) and *C. cylindrosporum* (0.64 mg/ml) was allowed to react with MB (4.3×10^{-5} *M*) in 0.05 *M* potassium succinate buffer, pH 7.0. The number of sulfhydryl groups reacted was determined from the change in absorbance at 250 nm. (Unpublished results of C. Garrison and R. H. Himes.)

MECHANISM OF THE REACTION

The detailed mechanism of formyltetrahydrofolate synthetase is not known, in spite of the availability of relatively large quantities of crystalline enzyme and the reasonable amount of effort devoted to the problem. Studies done with the enzyme isolated from various sources have led to a number of proposed mechanisms. Some of these involve phosphorylated tetrahydrofolate, phosphorylated enzyme, and formylphosphate intermediates. For example, investigations of the reaction catalyzed by partially purified enzyme preparations from pigeon and sheep liver led Jaenicke and Brode[52,77] to propose the following mechanism:

$$E + ATP \rightleftharpoons E \cdot P + ADP \quad (2)$$

$$E \cdot P + \text{tetrahydrofolate} \rightleftharpoons E \cdot N^{10}\text{-phosphoryltetrahydrofolate} \quad (3)$$

$$E \cdot N^{10}\text{-phosphoryltetrahydrofolate} + HCOOH \rightleftharpoons E \cdot N^{5}\text{-formyl-}N^{10}\text{-phosphoryltetrahydrofolate} \quad (4)$$

$$E \cdot N^{5}\text{-formyl-}N^{10}\text{-phosphoryltetrahydrofolate} + H_2O \rightleftharpoons E + P_i + N^{10}\text{-formyltetrahydrofolate.} \quad (5)$$

A variation of this scheme[78] involves the conversion of $E \cdot N^5$-formyl-N^{10}-phosphoryltetrahydrofolate (formed in Equation 4) to $E \cdot N^{5,10}$-methenyltetrahydrofolate with a loss of phosphate and the subsequent hydrolysis of the enzyme complex yielding N^{10}-formyltetrahydrofolate.

Jaenicke and co-workers observed that their enzyme preparations catalyzed an exchange between labeled ADP and ATP in the absence of the other substrates, and an exchange between labeled formate and N^{10}-formyltetrahydrofolate which required phosphate. The exchange of phosphate and ATP required the presence of all the substrates. These data support the above proposed mechanism. However, considering the state of purity of the enzymes used, the meaning of these exchange reactions is in doubt. It was shown that the sheep liver enzyme contained myokinase, nucleoside diphosphate kinase, and nucleoside triphosphatase activities.[51] In addition, Equation

5 predicts that if the reaction is performed in H_2O^{18}, ^{18}O will be found in the phosphate formed. This experiment has been done recently using the enzyme from *C. cylindrosporum* with the result that no ^{18}O is transferred from the solvent to the phosphate.‡

Another mechanism which involves the formation of a phosphoryltetrahydrofolate intermediate was suggested by Whiteley and Huennekens[29] for the enzyme from *M. aerogenes* and is outlined in Equations 6 through 9.

$$E + ATP \rightleftharpoons E \cdot ATP \quad (6)$$

$$E \cdot ATP + \text{tetrahydrofolate} \rightleftharpoons E \cdot \text{phosphoryltetrahydrofolate} + ADP \quad (7)$$

$$E \cdot \text{phosphoryltetrahydrofolate} + HCOOH \rightleftharpoons E \cdot N^{10}\text{-formyltetrahydrofolate} + P_i \quad (8)$$

$$E \cdot N^{10}\text{-formyltetrahydrofolate} \rightleftharpoons E + N^{10}\text{-formyltetrahydrofolate} \quad (9)$$

However, not all of the evidence presented supports the mechanism. It was found, for example, that to achieve the maximal rate of exchange between formate and N^{10}-formyltetrahydrofolate, ADP was required. According to the proposed mechanism, this exchange occurs in Equations 8 and 9 and ADP should not be required. Moreover, ADP was required for the arsenolysis of N^{10}-formyltetrahydrofolate, a finding which can only be explained if the E·arsenyltetrahydrofolate complex is not hydrolyzed, an unlikely possibility. Evidence was presented to support the first two steps (Equations 6 and 7) of the mechanism. When the enzyme, tetrahydrofolate, and ATP are incubated together in the absence of added formate, the absorbance at 298 nm due to tetrahydrofolate decreases slowly and the λ_{max} shifts from 298 to about 292 to 295 nm. The change in the absorption spectrum was attributed to the formation of free phosphoryltetrahydrofolate which dissociates from the enzyme. ADP is formed as predicted by Equation 7. The amount of enzyme needed to demonstrate this reaction is about 100 times that required for the overall reaction. The results reported by Whiteley and Huennekens in support of Equations 6 and 7 are better explained by the fact that low levels of formate commonly contaminate the reagents used in these experiments.[65] Most of the contaminating formate was traced to solutions of KOH stored in polyethylene bottles and used to neutralize the reagents. The usual concentration of contaminating formate present in the reaction mixture is 1 to 2 x 10^{-5} *M*. This amount is enough to cause the spectral changes observed with the *M. aerogenes* enzyme since tetrahydrofolate has an absorption maximum at 298 nm ($\epsilon^{M}_{298} \sim 32{,}000$[79]) and N^{10}-formyltetrahydrofolate has a maximum at 258 nm.[80] This low concentration of formate also explains the large amount of enzyme required to detect the reaction since the K_m of formate is about 5 x 10^{-3} *M*. An early report of the isolation of phosphoryltetrahydrofolate[81] has not been verified.

A concerted mechanism (Figure 16) has been proposed for the reaction catalyzed by the enzyme from *C. cylindrosporum*.[65] It was found[65,82] that 1. the exchange of $^{32}P_i$ and ATP requires both tetrahydrofolate and formate; 2. the exchange of ^{14}C-formate and N^{10}-formyltetrahydrofolate requires P_i and ADP; 3. the arsenolysis of N^{10}-formyltetrahydrofolate requires ADP; 4. formation of ADP from ATP requires both formate and tetrahydrofolate; 5. during the reaction one oxygen atom from formate is transferred to the P_i formed as shown by using ^{18}O-formate; and 6. the maximal rate of exchange of ADP and ATP requires the presence of formate and tetrahydrofolate. A very slow ADP ↔ ATP exchange was observed in the absence of the other two substrates[65] but this activity can be removed by DEAE-cellulose chromatography of the enzyme.‡ The proposed mechanism is shown in Figure 16. It is suggested that an imidazole ring aids in the removal of the N^{10} proton.[28]

The mechanism depicted in Figure 16 does not involve the formation of freely dissociable intermediates. Such a mechanism is supported by kinetic and equilibrium exchange studies done with the clostridial enzyme.[44,54] The results of a

‡Personal communication, S. J. Benkovic and W. P. Bullard.

‡Unpublished results of R. H. Himes.

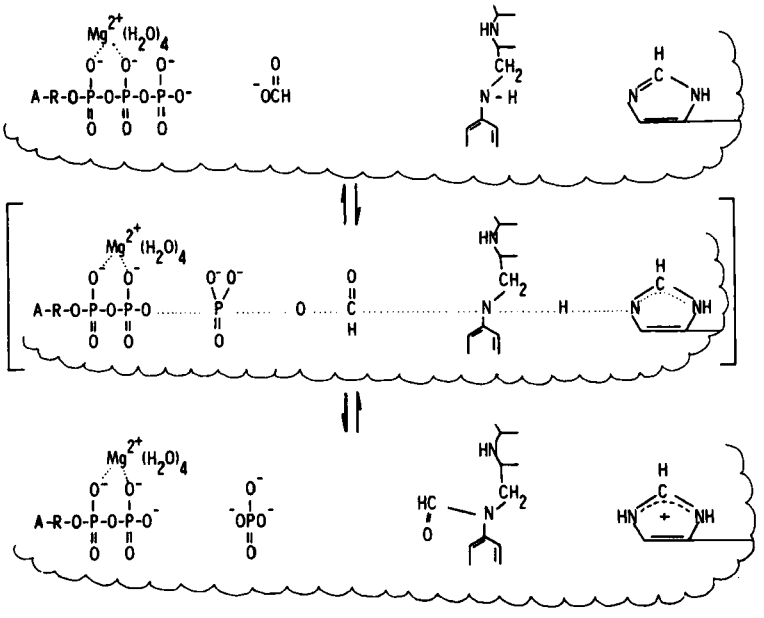


FIGURE 16. The proposed concerted mechanism for formyltetrahydrofolate synthetase.

detailed study[54] of the dependence of initial velocity on the concentrations of the variable and fixed substrates support a sequential mechanism, i.e., one in which all substrates must be present before any products are released from the enzyme. The K_m values of the variable substrates either decrease slightly, or remain constant when the concentrations of the fixed substrates are increased. These results together with the inhibition patterns found for the products ADP, P_i, and N^{10}-formyltetrahydrofolate[54] are consistent with a random ter ter mechansim. The inhibition patterns also suggest that the deadend complexes enzyme ·MgATP·N^{10}-formyltetrahydrofolate and enzyme ·MgADP·formate·tetrahydrofolate are formed.

A kinetic mechanism with a random addition of substrates is also supported by equilibrium exchange data.[44] The effects of substrate concentrations on the ADP↔ATP, P_i↔ATP, formate↔N^{10}-formyltetrahydrofolate equilibrium exchange rates are inconsistent with an ordered mechanism but in agreement with a random binding of substrates and products. At most concentrations of substrates, the formate ↔N^{10}-formyltetrahydrofolate exchange rate is three to four times the P_i↔ATP and ADP↔ATP exchange rates. This result indicates that the reaction does not proceed by a "rapid equilibrium" random mechanism, i.e., the

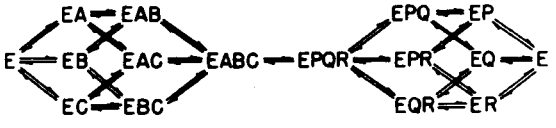


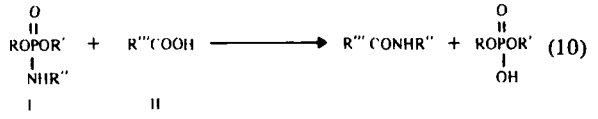
FIGURE 17. The kinetic mechanism of formyltetrahydrofolate synthetase. The letters A,B,C refer to the three substrates; P,Q,R, to the three products.

binding and dissociation of reactants is not fast compared to the interconversion of the enzyme-substrates and enzyme-products quaternary complexes. The relatively small difference between the rates of exchange when all substrates are present at saturating concentrations suggests that the rates of binding and dissociation are the same order of magnitude as the covalent bond-breaking steps. The fact that MgATP and tetrahydrofolate bind to the enzyme independently[23,45] and have dissociation constants only slightly lower than the K_m values (Tables 10 and 14) also supports a random ter ter mechanism. The kinetic mechanism of formyltetrahydrofolate synthetase is depicted in Figure 17.

All of the data taken together definitely rule out the occurrence of partial reactions leading to the formation of intermediates which are freely dissociable from the enzyme. Although a concerted mechanism, as outlined in Figure 16, is

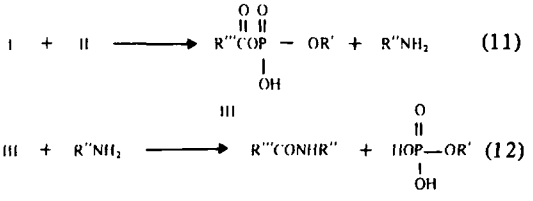
consistent with the available data, mechanisms which involve firmly bound intermediates must also be considered. Two possible bound intermediates are phosphoryltetrahydrofolate and formylphosphate.

Mechanisms involving both N^{10}-phosphoryltetrahydrofolate,[29,52,77] a phosphoramidate, and $N^{5,10}$-phosphoryltetrahydrofolate,[78] a phosphordiamidate, as enzyme-bound intermediates have been postulated, but as already discussed, the supportive evidence has not been unequivocal. If formed, these intermediates would react with formate in some manner to produce N^{10}-formyltetrahydrofolate. Phosphoramidates do react with carboxylic acids to produce amides[83] according to Equation 10.



(10)

Howeyer, the reaction probably occurs in a stepwise fashion with the initial formation of an acylphosphate derivative as shown in Equations 11 and 12.[84-86]

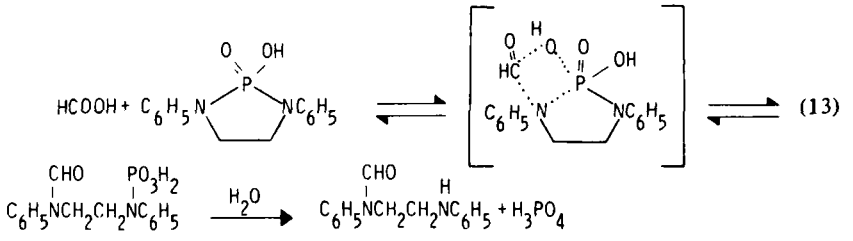


(11)

(12)

A sequence involving the phosphorylation of tetrahydrofolate, followed by the transfer of phosphate to formate, and finally by an attack by tetrahydrofolate on formylphosphate seems excessively complicated for the enzyme reaction.

It has also been found that cyclic phosphordiamidates undergo formolysis to yield *N*-formyl-*N,N'*-diarylethylenediamine.[87] The mechanism described in Equation 13 involving a four-centered



(13)

transition state has been proposed. The reaction could also proceed via the initial formation of a formylphosphoramidate followed by a nucleophilic attack by the secondary nitrogen in a sequence similar to that described by Equations 11 and 12. As pointed out earlier, if such a mechanism were operative in the enzyme reaction, a transfer of an oxygen atom from water to inorganic phosphate would occur and it does not. The involvement of phosphoryltetrahydrofolate as an intermediate does not appear to be likely. The electron-withdrawing tendency of the phosphoryl group would be expected to reduce the nucleophilic character of the nitrogen, making it less reactive.

Formylphosphate is a more likely enzyme-bound intermediate. Its formation from ATP and formate would be followed by a nucleophilic attack by the 10-nitrogen of tetrahydrofolate leading to the final products. A major argument against the participation of formylphosphate has been the finding in several laboratories that neutral hydroxylamine does not inhibit the reaction. Synthetic formylphosphate also fails to act in the reaction.[88] A variety of attempts have been made to demonstrate the existence of enzyme · formyl-

phosphate · MgADP complexes but all have resulted in failure.

The failure to observe the formation of enzyme-bound formylphosphate may be a result of the need for tetrahydrofolate to produce the catalytic center via a conformational change. NMR studies indicate that a conformational change takes place when tetrahydrofolate binds to the enzyme · MnATP complex.[22,89] It was observed that the addition of tetrahydrofolate to solutions of the ternary complexes enzyme · MnATP or enzyme · MnADP causes a significant de-enhancement of the longitudinal relaxation rate ($1/T_1$) of the solvent protons.[22] This effect could be due to one or a combination of three causes: 1. a decrease in the number of water ligands on the manganese; 2. a conformational change of such a nature that the water in the first coordination sphere of the manganese can rotate more freely; and 3. a conformational change so that the rate of exchange of the water in the first coordination sphere, with the water in the solvent, becomes slow compared to the relaxation rate. The magnitude of the decrease in enhancement is too large to be explained by a decrease in the number of water ligands. Moreover, the stability constant of Mn · tetrahydrofolate is much too small[22] for significant complex formation to occur. The other two possibilities can be distinguished by studying the temperature dependence of the enhancement of the quaternary complex.[90] Both involve a conformational change around the Mn · nucleotide binding site. Recent EPR studies[89] indicate that the third possibility is more likely. By examining the effect of tetrahydrofolate on the EPR spectra of the enzyme · MnATP and enzyme · MnADP complexes, it was found that tetrahydrofolate produces a dramatic increase in the electron spin relaxation time of the bound manganese. This, together with the decrease in the water proton relaxation rate, is interpreted to mean that upon formation of the enzyme · Mn · nucleotide · tetrahydrofolate complex, the Mn becomes less accessible to the solvent.[89]

CONCLUDING REMARKS

Formyltetrahydrofolate synthetase, a tetramer with a molecular weight of 240,000 daltons, consists of 4 identical subunits and has 4 active sites. It has been isolated as a pure protein from several species of *Clostridia* where it occurs in large concentrations.

The enzyme is an excellent protein to use in studying the role of monovalent cations in the structure and function of enzymes since specific cations promote the formation of the tetramer from the catalytically inactive monomers and affect the Michaelis constant of formate. The fact that monomers produced by removal of monovalent cations still retain their tertiary structure and bind MgATP but not tetrahydrofolate indicates that subunit interactions produce the binding site for the latter substrate. Moreover, it is obvious from studies of the stability of the monomer that these interactions also stabilize the tertiary structure of the protein. Further investigations of these phenomena would certainly be useful in our attempts to understand the requirement for the polymeric structure of some enzymes.

The mechanism of the reaction catalyzed by formyltetrahydrofolate synthetase still remains in doubt. The proposed concerted mechanism receives some support from NMR and EPR studies which have shown that tetrahydrofolate produces conformational changes in the enzyme · Mn · nucleotide complex. Quite possibly the catalytic center is not formed until all three substrates are present. The use of NMR and EPR techniques with this enzyme appears to be a powerful tool for observing conformational changes. It is hoped these studies will be continued. No information on the nature of the active site residues exists other than the implication of the involvement of histidine by photooxidation studies.

It is unfortunate that only the enzyme from *Clostridia* has been studied in detail. Hopefully, the protein from animal sources will be purified and studied in as much detail to determine whether the properties of the bacterial enzyme are common to the enzyme from higher organisms.

ACKNOWLEDGMENTS

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