Formyltetrahydrofolate Synthetase

BINDING OF ADENOSINE TRIPHOSPHATE AND RELATED LIGANDS DETERMINED BY PARTITION EQUILIBRIUM

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SUMMARY

The binding of adenine nucleotides and other compounds of related structures to clostridial formyltetrahydrofolate synthetase was examined through the use of the method of partition equilibrium, which utilizes an aqueous biphasic dextran-polyethylene glycol polymer system. The method is rapid and requires minimal amounts of enzyme and ligand. The results of MgATP binding as determined by partition equilibrium agreed well with those obtained by equilibrium dialysis. The enzyme, which has been shown to be tetrameric in structure, possesses four identical, noninteracting nucleotide binding sites per mole of enzyme. MgATP and MgADP bind to the same set of sites. MgATP binding is independent of the presence of the other substrates. The absence of magnesium does not significantly alter the binding of either nucleotide; however, ATP is a less specific ligand than MgATP and is bound to additional sites with a lower affinity. The affinities of other compounds related in structure to ATP were measured by a competitive binding technique. The results of these experiments suggest that the nucleotide site is composed of subsites each of which has a specific binding interaction for a portion of the ATP molecule. Inorganic phosphate can displace MgATP from the enzyme, but it does not affect MgADP binding. The enzyme has a very strong affinity for adenylyl methylenediphosphonate, the β-γ-methylene analogue of ATP, but not for the α-β-methylene analogues of ATP or ADP. Consideration of differences in bond lengths and bond angles between ATP and adenylyl methylenediphosphonate and the specific effect of phosphate on ATP binding suggest that ATP is bound to the enzyme in such a way that the γ-phosphate is strained towards a trigonal-bipyramidal intermediate.

(0)-Tetrahydrofolate + ATP + formate

⇌ (0)-10-formyltetrahydrofolate + ADP + P;

Enzymes catalyzing this reaction have been purified from avian liver (9), from a number of bacteria (3-6), from human erythrocytes (7), and from spinach leaves (8). By far the most active preparations are the crystalline enzymes isolated from the purine-fermenting bacteria, Clostridium cylindrosporum and Clostridium acidi-urici (4).

Formyltetrahydrofolate synthetase belongs to a class of enzymes which utilizes the energy of hydrolysis of the anhydride phosphate bond of ATP to synthesize a carbon-nitrogen bond. The mechanism of this particular reaction is not known, and its relationship to other enzymes of this class remains to be determined (9-11). Because of the large amount of formyltetrahydrofolate synthetase present in purine-fermenting Clostridia, the ease of its purification, and its high degree of purity, it is well suited for mechanistic studies.

Numerous experiments have been performed in order to determine the enzyme mechanism of formyltetrahydrofolate synthetase (12). It has not been possible to demonstrate any partial reactions; the presence of both formate and tetrahydrofolate is required for ADP formation from ATP, and arsenolysis of 10-formyltetrahydrofolate requires catalytic amounts of ADP. Phosphate exchange into ATP requires the presence of all three substrates. A slow exchange of ADP into ATP occurs in the absence of the other two substrates, but addition of formate together with tetrahydrofolate increases the rate considerably. There is some uncertainty as to whether the slow ADP-ATP exchange is catalyzed by formyltetrahydrofolate synthetase or by a minor contaminant of the enzyme preparation. 32P distribution studies showed that an oxygen atom of formate is transferred to the γ-phosphate of ATP in the enzymic reaction. Additional studies indicated that the exchange of formate with formyltetrahydrofolate requires the presence of ADP and phosphate (13). Measurements of equilibrium exchange rates (14) and a detailed kinetic analysis (15) support a mechanism involving the random binding of substrates and the formation of certain dead-end complexes. These results are most consistent with the conclusion that formyltetrahydrofolate synthetase proceeds by a concerted mechanism (12).

However, the experimental results are not inconsistent with some mechanisms that involve covalent intermediates. For example, a mechanism involving an intermediate, but requiring the enzyme to be tetrameric in structure, possesses four identical, noninteracting nucleotide binding sites per mole of enzyme. MgATP and MgADP bind to the same set of sites. MgATP binding is independent of the presence of the other substrates. The absence of magnesium does not significantly alter the binding of either nucleotide; however, ATP is a less specific ligand than MgATP and is bound to additional sites with a lower affinity. The affinities of other compounds related in structure to ATP were measured by a competitive binding technique. The results of these experiments suggest that the nucleotide site is composed of subsites each of which has a specific binding interaction for a portion of the ATP molecule. Inorganic phosphate can displace MgATP from the enzyme, but it does not affect MgADP binding. The enzyme has a very strong affinity for adenylyl methylenediphosphonate, the β-γ-methylene analogue of ATP, but not for the α-β-methylene analogues of ATP or ADP. Consideration of differences in bond lengths and bond angles between ATP and adenylyl methylenediphosphonate and the specific effect of phosphate on ATP binding suggest that ATP is bound to the enzyme in such a way that the γ-phosphate is strained towards a trigonal-bipyramidal intermediate.

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would be consistent with the available experimental observations. A second possibility is an induced fit type of mechanism (16) in which the binding of all three substrates is required to produce the active site, and after the proper catalytic conformation is created, the reaction proceeds through a covalent intermediate.

The binding of adenine nucleotides was, therefore, undertaken as a further probe of the enzyme mechanism of formyltetrahydrofolate synthetase. We chose to use the method of partition equilibrium recently developed by Gray and Chamberlin (17) because it is a more rapid and micro-method than conventional equilibrium dialysis. By comparison of the binding properties of ATP and ADP related ligands, the adenine nucleotide binding site was characterized as consisting of various subsites. The results also suggest that ATP is bound to the enzyme in such a way that the γ-phosphate is strained towards a trigonal-bipyramidal intermediate. A preliminary report of this work has been presented (18).

**EXPERIMENTAL PROCEDURE**

**Materials**

[8-14C]ATP and [PII]ADP were obtained from New England Nuclear with a radioactivity of greater than 97 and 95%, respectively. The radioactivity was confirmed by chromatography on thin layer sheets, impregnated with poly-(ethyleneimine)-cellulose, with 0.25 M LiCl-1 M formic acid as the solvent (19). Adenine nucleotides and the various methylene analogues of ATP and ADP were purchased from Sigma and Miles Labs, Elkhart, Indiana, respectively. The concentrations of solutions of these compounds were determined spectrophotometrically (εmax = 15,400) (20).

(dL)-Tetrahydrofolate was prepared by catalytic hydrogenation of folic acid (Sigma) in neutral aqueous solution (21). It was purified by adsorption on a DEAE-cellulose column equilibrated with 0.25 M 2-mercaptoethanol and eluted with 0.2 M Tris-chloride buffer (pH 7.0). The polymer solutions used in the partition equilibrium experiments were prepared by dissolving Dextran T 500 (Pharmacia) and polyethylene glycol (Carbowax 6000, Union Carbide Corporation, New York) in 50 mM Tris-chloride buffer, pH 8.0.

**Enzyme Purification and Properties**

Formyltetrahydrofolate synthetase from *C. cylindrosporum* was used in the majority of the experiments described in this paper. The organism (ATCC 7905) was grown as previously described (22), except that sodium carbonate and sulfuric acid were omitted from the medium. The original procedure for purification of the enzyme (4) was modified in order to omit the heat step and to increase the scale of the preparation. The enzyme assay and protein determination were performed as previously described (4). In these studies, the international unit of enzyme activity is used and is defined as the amount needed to produce 1 pmole of 10-formyltetrahydrofolate per min. The specific activity is expressed in units per mg of protein. The procedure described below is very reproducible and routinely yields 170 mg of enzyme with a specific activity of approximately 480 units per mg.

The standard buffer used throughout the purification was 0.05 M potassium phosphate-0.05 M 2-mercaptoethanol, pH 7.5, and is referred to simply as "the buffer." All steps were carried out at 30° unless stated otherwise. Ammonium sulfate concentration is expressed as percentage of saturation at 0° (23). After each addition of ammonium sulfate, solutions were readjusted to pH 7.5.

**Extract**—Lyophilized *C. cylindrosporum* cells (21 g) were evenly suspended in 420 ml of buffer. The suspension was stirred slowly at room temperature for 30 min. It was then centrifuged at 105,000 x g for 30 min and the precipitate was discarded.

**Protease Sulfate**—A protamine sulfate solution (100 ml) containing 10 mg per ml neutralized with KOH, was added in small aliquots, over a 2-min period and with constant stirring, to the supernatant solution (350 ml) obtained in the previous step. The mixture, after standing at room temperature for 10 min, was centrifuged at 30,000 x g for 15 min and the precipitate was discarded.

**Ammonium Sulfate I**—Sufficient solid ammonium sulfate was added to the supernatant solution (470 ml) from the previous step to make the solution 55% saturated (32.6 g/100 ml). The suspension was allowed to stand for 15 min, then centrifuged at 30,000 x g, and the precipitate was discarded.

**Ammonium Sulfate II**—Sufficient solid ammonium sulfate was added to the supernatant solution (530 ml) to bring it to 70% saturation (9.3 g/100 ml). This was allowed to stand for at least 3 hours or overnight and was then centrifuged at 30,000 x g for 15 min. The supernatant solution was discarded, and the precipitate was resuspended with 60 ml of buffer.

**Bio-Gel A-0.5m**—The sample from the second ammonium sulfate precipitation (75 ml) was placed on a Bio-Gel A 0.5m (200 to 400 mesh) column (5 x 180 cm) that had previously been equilibrated by washing with 4 liters of buffer. The flow rate was maintained at 60 ml per hour by use of a 4-liter Mariotte bottle as the reservoir. After 16 hours, the eluate was collected in 10-m1 fractions. The elution pattern is shown in Fig. 1a.

**Ammonium Sulfate III**—Sufficient solid ammonium sulfate was added to the combined fractions from the Bio-Gel column (230 ml) to make the solution 60% saturated (36.1 g/100 ml). It was allowed to stand for 2 to 3 hours and was then centrifuged at 30,000 x g for 15 min. The supernatant solution was discarded, and the pellet was resuspended with 100 ml of buffer.

**First Crystallization**—Solid ammonium sulfate (28.6 g) was added to the solution from the previous step (105 ml). The partially crystalline suspension was allowed to stand overnight. It was then centrifuged at 30,000 x g for 15 min and the supernatant solution was discarded. The pellet was dissolved in 100 ml of buffer and dialyzed for 2 hours against 6 liters of buffer.

**DEAE-Cellulose**—A DEAE-cellulose column (20 x 2.5 cm) was packed and equilibrated by washing with 1.5 liters of buffer. The dialyzed sample was then applied to the column and 10-m1 fractions were collected. The protein was eluted by a linear gradient of KCl in the standard buffer. This was formed by mixing 400 ml of buffer with 400 ml of buffer that was also 0.25 M in KCl. After this, an additional 500 ml of buffer containing 0.25 M KCl were also passed through the column. The elution pattern is shown in Fig. 1b. The combined fractions (440 ml) were concentrated to 80 ml by ultrafiltration in a 400-ml Diaflo apparatus (Amicon Corporation, Cambridge, Massachusetts) with a XM-50 membrane.

**Second Crystallization**—Sufficient solid ammonium sulfate was added to the sample obtained from the DEAE-cellulose column to make the solution 50% saturated (29.1 g/100 ml). The crystalline suspension was allowed to stand overnight, then was cen-
pressed in absorbance units at 280 nm and enzyme activity (O-
-0) in units per ml.

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-0) that it gives a single band weight during polyacrylamide gel electrophoresis in the presence
by isoelectric focusing in the presence of 8
that the enzyme behaves as a single species of 60,000 molecular
on polyacrylamide disc gel electrophoresis.
-0) that the enzyme behaves as a single species of 60,000 molecular
by ultra-
by means of either sedimentation velocity
(c) but is consistent with the difference in the tryptophan con-
when the two polymers are mixed such that the final concentra-
more dense or lower phase consists largely of dextran and con-
the ultracentrifuge (31) was correlated with the absorbance at
for the enzyme from C. cylindrosporum, the extinction
for the enzyme from C. acidi-
zymes is surprising in view of the over-all similarities of the en-
ligand partitions almost equally between the phases under the
less dense or upper phase consists largely of polyethylene glycol. 
The protein concentration measured by a modification of the
method of partition equilibrium as developed by Gray and
Chamberlin (17). The method is based on a dextran-polyethylene
Equilibrium binding of adenine nucleotides and related ligands
to formyltetrahydrofolate synthetase was determined by the
method of partition equilibrium as developed by Gray and Chamberlin (17). The method is based on a dextran-polyethylene glycol two phase system originally described by Albertsson (32). When the two polymers are mixed such that the final concentration of each is approximately 7%, they form two phases. The more dense or lower phase consists largely of dextran and constitutes about one-third of the total volume. Conversely, the less dense or upper phase consists largely of polyethylene glycol. Clostridial formyltetrahydrofolate synthetase was found to partition completely in the dextran phase, whereas the unbound ligand partitions almost equally between the phases under the conditions described here.

The experimental procedure can be described briefly as follows.

\[^{2}\text{J. M. Scott, and J. C. Rabinowitz, manuscript in preparation.}\]

\[^{3}\text{This large difference in extinction coefficients of the two enzymes is surprising in view of the over-all similarities of the enzymes (J. M. Scott and J. C. Rabinowitz, manuscript in preparation) but is consistent with the difference in the tryptophan content of the two enzymes that has been detected (J. M. Scott and J. C. Rabinowitz, manuscript in preparation).}\]

![Fig. 1. Purification of formyltetrahydrofolate synthetase from C. cylindrosporum. Elution patterns for (a) Bio-Gel A-0.5m column and (b) DEAE-cellulose column. Protein (O--O) is expressed in absorbance units at 280 nm and enzyme activity (O- -0) in units per ml.](http://www.jbc.org/)
A solution (20 µl) containing enzyme (2 to 20 mg per ml) and radioligand were added to 0.5-ml tubes containing 20 µl of solutions of various concentrations of nonradioactive ligand. Then 35 µl of 20% dextran solution and 25 µl of 30% polyethylene glycol were added by using 50-µl syringes (Hamilton 705-LTC) fixed with Cheney adapters. The tubes were then incubated at the desired temperature (usually 37°C), and all subsequent steps were also performed at this temperature. The sample was mixed on a Vortex mixer for 5 to 10 sec, and while still mixing, 70 µl of the sample were withdrawn in a 100-µl capillary tube (Microcaps, Drummond Scientific Company, Broomall, Pennsylvania). The sample was then drawn further into the capillary, and the end was carefully sealed in a flame. The phases were separated by centrifuging the capillary at 1000 × g for 5 to 10 min. The relative volume of each phase was determined by measuring the length of each phase in the capillary. The relative volumes obtained for all the samples generally agreed within 2 to 3%, and the average value was used in the calculations. The phases were separated by scratching the capillary above and below the interface with an ampule file, breaking, and then discarding the small section containing the interface. Each of the two phases was then sampled with a 10-µl microcap and transferred to 0.5 ml of water contained in a scintillation vial. The 10-µl microcap was rinsed with 0.5 ml of water a sufficient number of times to remove all the polymer from the inside wall of the microcap. Then, 10 ml of scintillation solution were added, and the radioactivity of each phase was determined.

Enzyme was prepared by resuspending an aliquot of the crystalline suspension in the appropriate volume of 0.1 M NH₄Cl-0.1 mM EDTA-0.05 M Tris-chloride buffer, pH 8.0, and then dialyzing it for 4 hours against two 1-liter volumes of this buffer. An aliquot of the dialyzed enzyme was diluted with distilled water, and the absorbance at 280 nm was measured. The protein concentration was calculated by using the extinction coefficient given above. The high concentration of NH₄Cl was necessary to prevent inactivation of enzyme during the partition equilibrium experiments. By this procedure, the total enzymic activity could be accounted for within 2 to 3%, and greater than 99% of the enzyme partitioned into the dextran phase. Complete recovery of enzymic activity indicates that the formyltetralhydrofuran synthetase is present in the dextran phase as the active tetrameric enzyme (28).

### Scintillation Counting Procedures and Controls

The radioactivity of ¹⁴C-ligands was determined by use of Bray's scintillation solution (33). The radioactivity added to each sample could be accounted for within 2%, and the counts obtained remained constant with time. Redetermination of the radioactivity of the sample vials up to 18 hours later showed no significant variation in counts. However, when Bray's solution was used to determine the radioactivity of ³¹H-ligands, there was a significant decrease in counts with time. It was also apparent that the rate of decrease of counts was more rapid for samples from the lower phase than for those from the upper phase. Therefore, different scintillation solutions were tried.

The best results were obtained with a toluene, Omnifluor, and Biosolv scintillation solution. It was prepared by dissolving 4 g of Omnifluor (New England Nuclear) and 50 ml of Biosolv-BBS-3 (Beckman) with sufficient toluene (Eastman, reagent grade) to make 1 liter. This toluene base scintillation solution is not miscible with large volumes of water. Therefore, when ³¹H-ligands were used, the 10-µl samples of each phase were transferred to 0.2 ml of water contained in a scintillation vial, and 10 ml of the toluene scintillation solution were added. With this procedure, there was no significant variation in counts, even when test vials were recounted up to 18 hours later.

Determination of radioactivity was performed with a Nuclear-Chicago Mark I scintillation counter. All samples contained at least 500 cpm and were counted for a minimum of 40,000 counts and for a minimum of 2 min. There was no significant quenching of radioactivity by any of the solutions.

### Calculations

From the experimentally determined variables, the concentration of ligand in each phase can readily be calculated. Since the enzyme partitions greater than 99% in the lower phase, the concentration of ligand in this phase is equal to the sum of that which is bound to the enzyme, Aₜ, and that which is unbound, A. The concentration of ligand in the upper phase is related to A by means of a partition coefficient, which is measured directly from control samples minus enzyme. Use of this relationship permits the calculation of Aₜ and A.

For an enzyme which possesses n identical, noninteracting sites, each capable of binding a particular ligand with a dissociation constant, Kₐₜ, the following expression is valid.

\[ K_{diss} = \frac{A(\alpha - r)}{r} \]

where \( r \) is equal to \( Aₜ \) per mole of enzyme. Scatchard (34) rearranged this equation to the form

\[ \frac{r}{A} = \frac{-1}{K_{diss}} + \frac{n}{K_{diss}} \]

so that a plot of \( r/A \) versus \( r \) yields a straight line with a slope of \(-1/K_{diss}\), and an intercept on the abscissa of \( n \).

All of the data for direct binding experiments were calculated in this manner and then plotted in the form of Scatchard plots. The values for \( K_{diss} \) and for \( n \) were obtained by an unweighted least-squares analysis of the experimental data.

The dissociation constant for a ligand can also be measured by a competitive binding technique (35, 36). If ligands A and B bind to the same set of sites on the enzyme and the binding of one ligand excludes the binding of the second, then the following relationship is valid.

\[ A \cdot (\alpha - r) = K_{diss-A} + B \cdot \frac{K_{diss-A}}{K_{diss-B}} \]

where \( r \) is again equal to \( Aₜ \) per mole of enzyme and \( K_{diss-A} \) and \( K_{diss-B} \) are the dissociation constants of A and B, respectively.

Concentrations of radioactive ligand A and enzyme were chosen to give approximately half-maximal saturation. Then, increasing amounts of ligand B were added to identical samples and its effect on the binding of A was measured. The apparent dissociation constant \( (\alpha (n - r)/r) \) for ligand A can be calculated directly from the experimental data. A plot of this value versus B yields a straight line with an intercept on the ordinate of \( K_{diss-A} \) and a slope of \( K_{diss-A}/K_{diss-B} \).

For experiments in which \( K_{diss-B} \) was at least 10 times greater than \( K_{diss-A} \), the amount of B bound to the enzyme is not significant and one can assume that \( B = Bₜ \). This assumption was valid in all the competition experiments.
except in describing the effect of ATP on [H]ADP binding. Because ADP and ATP bind with nearly equivalent affinities, it was necessary to calculate the concentration of unbound ATP. This was done by means of the following equation.

\[
[\text{ATP}] = \frac{Q_{\text{ATP}}}{U + Q_{\text{ATP}}} \cdot \frac{[\text{ADP}] [\text{ATP}]_0 - E_0 [\text{ADP}] - [\text{ADP}] [\text{ADP}] + K_{\text{dis}})}{[\text{ADP}]}
\]

(3)

where \(Q_{\text{ATP}}\) is the partition coefficient for unbound ATP, \(U\) and \(L\) are the relative volumes of the upper and the lower phases, respectively, \([\text{ATP}]_0\) is the amount of ATP added per total volume (100 \mu), \(E_0\) is the concentration of enzyme in the lower phase, and \(K_{\text{dis}}\) is the dissociation constant for ADP, as determined by direct binding experiment. All of the data from competitive binding experiments were plotted in the form of Equation 2, and the dissociation constant of the variable ligand was determined by least squares analysis.

All calculations were performed with the aid of programs written for an Olivetti Programma 101.

**Ligand Binding by Equilibrium Dialysis**

Equilibrium dialysis was performed by using the 30-\mu cell dialysis cells and the procedure described by Englund et al. (36). Control experiments showed that equilibration required between 2 and 24 hours. Therefore, the cells were allowed to equilibrate by rotating at 24° for 3 hours.

**RESULTS**

**Direct Binding Experiments with ATP**—Previous kinetic (37) and magnetic resonance (38) studies have shown that a magnesium chelate of ATP is the true substrate for formyltetrahydrofolate synthetase. In order to perform binding experiments with MgATP, sufficient MgCl₂ was added to insure that ATP was present largely in the chelated form. Fig. 2 is a Scatchard plot of MgATP binding to the formyltetrahydrofolate synthetase isolated from *C. cylindrosporum* and *C. acidi-urici*. For the enzyme from *C. cylindrosporum*, there are clearly four identical, noninteracting binding sites per tetramer. There is no indication of binding at additional sites having a lower affinity. Each site binds MgATP with a dissociation constant of 0.076 mM. The formyltetrahydrofolate synthetase from *C. acidi-urici* has 3.7 MgATP binding sites per tetramer. It binds with approximately twice the affinity of the enzyme from *C. cylindrosporum* and has a dissociation constant of 0.036 mM.

The dissociation constants for MgATP obtained for both enzymes are approximately one-third the value of their respective \(K_m\) kinetic constants. Both the kinetic experiments and the partition equilibrium measurements were carried out at 37° and at pH 8.0. The difference between the kinetic constants and the dissociation constants may result from the fact that the kinetic experiments were performed in aqueous solution, whereas, in the partition equilibrium experiments, the enzyme is present in an approximately 20% dextran solution. The presence of such a high concentration of polymer could effect the hydration of MgATP or the conformation of the enzyme. Either of these effects could alter the equilibrium dissociation constant.

In order to determine whether the presence of polymers alters the dissociation constant, MgATP binding to formyltetrahydrofolate synthetase isolated from *C. cylindrosporum* was redetermined at 24° by partition equilibrium, and the results were compared with those obtained by equilibrium dialysis (Fig. 3). Both sets of data fit the same line. Least squares analysis indicated that there are 4.2 binding sites per tetramer, and each has a dis

**Fig. 2.** Binding of MgATP to formyltetrahydrofolate synthetase. Data are given as a Scatchard plot with \(r\) representing the moles of MgATP bound per mole of enzyme and \(r/[\text{MgATP}]\) representing \(10^{-8} \times r\) divided by the molar concentration of unbound MgATP. The data obtained with enzyme isolated from *C. acidi-urici* and from *C. cylindrosporum* are represented by the open and solid figures, respectively. The protein concentrations were \(\bigtriangleup\), \(2.9 \times 10^{-5} \mu\); \(\bigcirc\), \(6.5 \times 10^{-5} \mu\); \(\bigcirc\), \(8.2 \times 10^{-5} \mu\); \(\bigstar\), \(3.9 \times 10^{-5} \mu\); \(\blacktriangle\), \(5.1 \times 10^{-5} \mu\); \(\blacksquare\), \(5.5 \times 10^{-5} \mu\). Partitioning was performed at 37° and pH 8.0.

**Fig. 3.** Comparison of MgATP binding by equilibrium dialysis and by partition equilibrium. Data obtained by equilibrium dialysis (○) and by partition equilibrium (▲) are given as a Scatchard plot. \(r\), moles of MgATP bound per mole of formyltetrahydrofolate synthetase isolated from *C. cylindrosporum*: \(r/[\text{MgATP}], 10^{-4} \times r\) divided by the molar concentration of unbound MgATP. The protein concentrations were ○, \(5.9 \times 10^{-5} \mu\); ▲, \(7.5 \times 10^{-5} \mu\). Experiments were performed at 23° and pH 8.0.
sociation constant of 0.073 mM. The excellent agreement between the two methods confirms the results obtained by the method of partition equilibrium and establishes the validity of this method for studying adenine nucleotide binding to formyltetrahydrofolate synthetase. The remainder of the experiments reported in this paper were performed by this method at 37° and at pH 8.0.

When magnesium is omitted, ATP becomes a less specific ligand. Fig. 4 is a Scatchard plot of the binding of ATP in the absence of magnesium to formyltetrahydrofolate synthetase isolated from C. cylindrosporum. These data cannot be fitted to a straight line. Additional binding of ATP was observed at the higher concentrations tested in contrast to the results observed with MgATP. This indicates that ATP binds to more than one set of sites, and with different affinities. One set of sites might be the four MgATP binding sites. If one assumes that ATP binds at these sites with an affinity equivalent to that of MgATP, the experimentally observed binding could be corrected by subtracting an amount equivalent to that expected for MgATP binding. When this was done, the resulting additional binding could not be fitted to any interpretable pattern. Therefore, if ATP binds to the four MgATP sites, it must do so with a slightly lower affinity than MgATP.

A Scatchard plot of the binding of ATP to the formyltetrahydrofolate synthetase isolated from C. cylindrosporum is shown in Fig. 5. The behavior was similar to that observed with the C. cylindrosporum enzyme, but the difference in binding of MgATP and ATP was less pronounced. ATP exhibits additional binding sites only at very high concentrations. Extrapolation of the data for ATP binding at lower concentrations yields 3.7 binding sites, the same value as that observed for MgATP. The dissociation constant for ATP, obtained by this extrapolation, is 0.043 mM. This represents only slightly less affinity than that observed with MgATP and strongly suggests that ATP binds to the same sites as MgATP. The increased binding at higher concentration must represent binding at additional sites which cannot bind MgATP and which have a lower affinity.

The effects of the other substrates, formate and (dL)-tetrahydrofolate, on MgATP binding were each tested separately. This was done by repeating the direct binding experiment in the presence of saturating amounts of the second substrate. The presence of 1 mM (dL)-tetrahydrofolate resulted in a slight decrease in affinity for MgATP. The reagent solutions contained a low endogenous level of formate, which would effect an enzymic conversion of some of the MgATP to MgADP. Such an effect would be sufficient to explain the slight decrease in affinity that was observed. The presence of 20 mM sodium formate had no significant effect on MgATP binding.

Direct Binding Experiments with ADP—Scatchard plots for the binding of MgADP to the formyltetrahydrofolate synthetases isolated from C. cylindrosporum and from C. acidi-urici are shown in Fig. 6a. For both enzymes, there are 3.6 binding sites per tetramer, and there is no indication of binding to additional sites with lower affinity. The enzyme isolated from C. cylindrosporum binds MgADP with a dissociation constant of 0.12 mM. As with MgATP, the enzyme from C. acidi-urici binds MgADP with approximately twice the affinity of the enzyme from C. cylindrosporum. It has a dissociation constant of 0.065 mM.

In contrast to ATP binding, the absence of magnesium does not affect ADP binding to the formyltetrahydrofolate synthetase isolated from C. cylindrosporum (Fig. 6b). There is no indication of additional binding at high concentrations of ADP. There are 3.6 binding sites per tetramer. The dissociation constant for ADP, 0.13 mM, is within experimental error of that determined for MgADP. This strongly suggests that ADP and MgADP bind to the same set of sites.

The remainder of the experiments were performed with the formyltetrahydrofolate synthetase isolated from C. cylindrosporum.
a. Binding of MgADP to formyltetrahydrofolate synthetase. Data are given as a Scatchard plot with $r$ representing the moles of MgADP bound per mole of enzyme and $r /[\text{MgADP}]$ representing $10^{-4} \times r$ divided by the molar concentration of unbound MgADP. The data obtained with the enzymes isolated from C. acidis-urtici and C. cylindrosporum are represented by the open and solid figures, respectively. The protein concentrations were 0, $10.2 \times 10^{-6}$ M; $\bullet$, $6.0 \times 10^{-6}$ M; $\bigtriangleup$, $5.4 \times 10^{-6}$ M.

b. Binding of ADP to formyltetrahydrofolate synthetase. $r$, moles of ADP bound per mole of enzyme; $r /[\text{ADP}]$, $10^{-4}$ divided by the molar concentration of unbound ADP. The protein concentration (●) was $4.5 \times 10^{-6}$ M.

Competitive Binding Experiments—MgADP is a competitive inhibitor of MgATP (15). The $K_d$ for MgADP is approximately equal to the $K_m$ for MgATP. To confirm the difference in dissociation constants for MgADP and MgATP and to prove that they both bind to the same enzyme site, a competitive binding experiment was performed. [3H]ADP was used as the radioactive ligand, and the concentration of MgADP and enzyme were chosen to give approximately half-maximal saturation. Increasing amounts of MgATP were then added to identical samples, and their effect on MgADP binding was determined. The concentration of unbound MgATP was calculated as described under "Methods." The results are shown in Fig. 7. A least squares analysis of the slope of this line gave a value of 1.57, which is equal to the dissociation constant for MgADP divided by dissociation constant for MgATP. When measured independently, the ratio of the two binding constants is 1.60.

The dissociation constant for AMP was determined by competition with MgADP. The effect of increasing the concentration of AMP on the apparent dissociation constant for MgADP is shown in Fig. 8. The fact that the data fit a straight line indicates that both ligands bind to the same site. From the slope of this plot, the dissociation constant for AMP was calculated to be 1.2 mM. This represents a 10-fold decrease in affinity compared to MgADP binding.

In a similar manner, the dissociation constants for ribose 5'-phosphate and for adenosine were determined by competition with MgATP. The dissociation constants calculated for the binding of ribose 5'-phosphate and adenosine were 8.6 mM and 17 mM, respectively.

The effect of inorganic phosphate on the binding of MgADP and of MgATP was determined by performing competition experiments with each nucleotide. The results are shown in Fig. 9. Inorganic phosphate at concentrations as great as 9 mM has no effect on the binding of MgADP. However, the presence of only 1.5 mM inorganic phosphate displaces a significant amount of MgATP from the enzyme. The fact that the data fit well to a straight line suggests that Pi and MgATP share a common binding site. The site for the γ-phosphate of MgATP probably also serves as the binding site for inorganic phosphate in the reverse reaction. The apparent dissociation constant for inorganic phosphate calculated from its effect on MgATP binding was 7.1 mM.

Experiments with Methyleneediphosphonate Analogues of Adenine Nucleotides—The ability of various methyleneediphosphonate analogues of adenine nucleotides to serve as inhibitors or substrates in enzymic reactions has been studied extensively (36, 39–41). In general, they have been found to be either poor sub-
FIG. 8. Competition of binding of MgADP to formyltetrahydrofolate synthetase as a function of unbound AMP concentration. The data are plotted as $10^4 \times [MgADP] \times (n - r/r)$ (the apparent molar dissociation constant for MgADP) versus $10^3 \times$ molarity of unbound AMP, $r$, moles of MgADP bound per mole of enzyme; $n$, the number of MgADP binding sites. The total concentration of MgADP was fixed at $1.08 \times 10^{-4}$ M, and the concentration of AMP was varied as indicated. Protein concentration was $6.0 \times 10^{-5}$ M.

\[
K_D = 1.2 \text{ mM}
\]

FIG. 9. Effect of inorganic phosphate on MgADP and MgATP binding to formyltetrahydrofolate synthetase. The data are plotted as $10^4 \times [A] \times (n - r/r)$ (the apparent molar dissociation constant for either MgATP or MgADP) versus $10^3 \times$ molarity of unbound inorganic phosphate ($P_i$), $r$, moles of either MgATP or MgADP bound per mole of enzyme; $n$, the corresponding number of binding sites. The total concentrations of MgATP and MgADP were fixed at $10.4 \times 10^{-5}$ M and $9.6 \times 10^{-5}$ M, respectively. The concentration of $P_i$ was varied as indicated. The protein concentrations were $\bullet$, $6.5 \times 10^{-5}$ M; $\Delta$, $5.4 \times 10^{-5}$ M.

\[
K_{D-P_i} = 7.1 \text{ mM}
\]

stimates or they have weaker affinities than the natural materials. With formyltetrahydrofolate synthetase, the $\beta$-$\gamma$-methylene analogue of ATP could be used to examine the effect of nucleotides on the binding of folate substrates without initiating an enzymic reaction.\(^4\) In order to determine what concentrations were sufficient to saturate the enzyme, the affinity for the various methylenediphosphonate analogues of adenine nucleotides was measured.

AMP-PCP,\(^5\) the $\beta$-$\gamma$-methylene analogue of ATP, was found to be a very strong competitive ligand. The presence of $0.005$ mM AMP-PCP caused a significant decrease in the amount of MgADP bound in the presence of $0.13$ mM unbound MgADP. Because of the very low concentration of AMP-PCP required to displace MgADP, it was not possible to quantitatively interpret the results of the AMP-PCP competition. Qualitatively, the dissociation constant for AMP-PCP was estimated as being at least 10-fold less than that for MgADP.

The effect of AMP-PCP on the enzyme kinetics was also examined. AMP-PCP was found to be a strong competitive inhibitor of ATP. Double reciprocal plots of initial velocity versus ATP concentration at various levels of AMP-PCP are shown in Fig. 10. The lines clearly intercept on the ordinate. The kinetic inhibition constant for AMP-PCP was found to be $0.01$ mM. This is more than 20 times less than the $K_m$ for ATP or the $K_i$ for ADP. Therefore, formyltetrahydrofolate synthetase has a strong affinity for AMP-PCP. In order to decide whether this was a result of the methylene group itself or its position in the

\[^4\] N. P. Curthoys and J. C. Rabinowitz, manuscript in preparation.

\[^5\] The abbreviations used are: AMP-PCP, adenylyl methylene-diphosphonate; AMPCP-P, $\alpha$-$\beta$-methylene analogue of ATP; AMPCP, $\alpha$-$\beta$-methylene analogue of ADP.
ATP molecule, the kinetic properties of α-β-methylene analogues of ATP and ADP were examined.

AMPCP-P, the α-β-methylene analogue of ATP, could be either a substrate or an inhibitor of formyltetrahydrofolate synthetase. When 1 mM AMPCP-P was substituted for ATP in the standard assay, there was no absorbance change at 350 nm after 10 min. The same amount of enzyme produced an absorbance change of 0.1 when 1 mM ATP was used as the substrate. If AMPCP-P is a substrate for formyltetrahydrofolate synthetase, it is utilized at less than 0.01 times the rate of an equivalent concentration of ATP. Conversely, AMPCP-P was found to be a competitive inhibitor with respect to ATP and had a $K_i$ of 0.028 mM. This is slightly greater than the $K_m$ for ATP or the $K_i$ for ADP.

When 1 mM AMPCP, the α-β-methylene analogue of ADP, was added to an enzyme assay mixture containing 1 mM ATP, there was no inhibition of enzymic activity. Therefore, the effect of AMPCP on MgATP binding was examined by a competitive binding experiment. Increasing concentrations of AMPCP caused a linear increase in the apparent dissociation constant for MgATP. From this effect, a dissociation constant of 1.9 mM was calculated for AMPCP. This is slightly greater than the dissociation constant for AMP.

**Discussion**

The method of partition equilibrium was readily applicable to the investigation of the binding of ATP and related compounds to the formyltetrahydrofolate synthetase isolated from *C. cylindrosporum* and *C. acidurici*. This method was both rapid and required only a minimal amount of enzyme and ligand. The catalytic activity of the enzyme was not affected by its presence in the dextran and polyethylene glycol polymers. The excellent agreement between the MgATP binding results obtained by this method and by equilibrium dialysis establishes the validity of partition equilibrium for studying adenine nucleotide binding to formyltetrahydrofolate synthetase. It also indicates that the presence of polymers does not affect the binding properties of this enzyme and suggests that the results obtained through the use of this method are directly comparable with those obtained in standard buffered solutions. In fact, the high concentration of polymers may produce an aqueous environment more directly analogous to in vivo conditions.

The competitive binding experiment using MgATP and MgADP indicates that both nucleotides bind to the same site. The results of direct binding experiments with both MgATP and MgADP suggest that there are four identical, noninteracting nucleotide sites per mole of enzyme. Formyltetrahydrofolate synthetase has a tetrameric structure (24). The available experimental evidence suggests that the four subunits are identical or very nearly identical (24, 28). The finding of four nucleotide sites per tetramer suggests that each subunit has one site for nucleotide binding. Further support for this conclusion was obtained by investigating the binding of nucleotides to monomeric subunits.

The formyltetrahydrofolate synthetase isolated from *C. acidurici* binds both MgATP and MgADP with approximately twice the affinity observed for the enzyme from *C. cylindrosporum*. The same 2-fold difference in the kinetic constant of ATP for the two enzymes was previously reported. For both enzymes, the $K_m$ value for ATP is approximately 3-fold greater than the respective dissociation constant. Both the kinetic and the partition equilibrium measurements were performed at 37° and at pH 8.0. The formate and tetrahydrofolate, present in the kinetic experiments, may cause a decrease in the affinity of the enzymes for MgATP. However, when examined individually, neither formate nor tetrahydrofolate had a significant effect on the binding of MgATP. Because of the unavailability of appropriate unreactive analogues for formate or tetrahydrofolate, it was not possible to examine the effect of the presence of both substrates on MgATP binding. However, previous kinetic experiments (15) suggested that MgATP is bound to the quaternary complex with a greater affinity than to either ternary complex. The difference in the kinetic constant and dissociation constant for each enzyme, therefore, suggests that the rate of dissociation of MgATP from the enzyme and of interconversion of the quaternary complex are of the same order of magnitude. An investigation of the equilibrium reaction rates (14) resulted in the same conclusion.

Previous investigations of the mechanism of cation activation showed that MgATP is the true substrate of formyltetrahydrofolate synthetase (37, 38). The binding experiments suggest that MgATP is a more specific ligand than ATP. MgATP binds to only one set of sites with no indication of additional binding at sites having a lower affinity, whereas ATP binds to more than one set of sites. This additional binding is more pronounced with the enzyme isolated from *C. cylindrosporum* than with the *C. acidurici* enzyme. The results suggest that part of ATP binding occurs at the specific sites for MgATP with a slightly decreased affinity. The additional binding is assumed to be due to nonspecific binding to sites having a lower affinity. The fact that ATP and MgATP bind to the same site agrees with the observation that ATP is a competitive inhibitor of the substrate, MgATP (37).

Competitive binding experiments were performed with the formyltetrahydrofolate synthetase isolated from *C. cylindrosporum* in order to measure its affinity for various compounds related to ATP. The dissociation constants measured at 37° and the corresponding apparent changes in free energy for the dissociation of ATP and related ligands are summarized in Table II. The table is arranged in order of decreasing affinity; AMP-PCP has the greatest affinity, whereas adenosine has the least. From this, it may be concluded that each of the various portions of the ATP molecule contributes to its over-all affinity. For example, the difference in $\Delta G$ values between adenosine and AMP suggests that the α-phosphate group contributes 1.6 kcal to the over-all affinity of ATP. Similarly, comparison of the $\Delta G$ values for AMP and ADP suggests that the β-phosphate group contributes 1.4 kcal, whereas the difference between the values for ADP and ATP suggests that the addition of the γ-phosphate adds...
only 0.3 kcal to the affinity. The results of this type of calculation are summarized in Table III.

Differences in AG values can be compared only if the reference energy level of all the compounds in solution is the same. Differences in magnesium chelation or hydration could affect the thermodynamic ground state of the various adenine nucleotides. Sufficient magnesium was present to chelate extensively only with ATP, ADP, and AMP-PCP; AMP was largely unchelated. MgADP and ADP bind to the enzyme with equal affinity, and even though ATP is a less specific ligand, it binds to the enzyme at the high affinity sites with an affinity similar to MgATP binding. This suggests that magnesium chelation does not significantly alter the thermodynamic ground state of ATP or ADP with respect to their binding to the enzyme surface. Experimental evidence suggests that ATP and AMP-PCP form similar magnesium chelates. AMP-PCP binds magnesium with an affinity approximately equal to that of ATP (42, 43), and magnesium chelation has similar effects in reducing the pK values for the second ionization of the γ-phosphate of ATP and AMP-PCP (43). The greater affinity of AMP-PCP could be related to a difference in hydration; the P—O—P bond of ATP could be hydrated to a greater extent than the corresponding P—O—P bond of AMP-PCP. X-ray analysis indicated that in imidodiphosphate tetra-anion, the imido proton is effectively buried between the two PO₄²⁻ groups and cannot hydrogen bond to the water molecules in the crystal lattice (42). This compound is a very similar structural analogue of pyrophosphate, and its failure to hydrogen bond suggests that oxygen of the P—O—P linkage of ATP is not hydrated. Supported by these observations, the comparison in the differences in ΔG values for the binding of ATP and related ligands appears to be valid. The results of such a comparison suggest that the nucleotide binding site is composed of subsites which have a specific binding interaction for each portion of the ATP molecule.

By comparison to the apparent differences in ΔG values attributable to each segment of the ATP molecule, the small difference in affinities between ADP and ATP is surprising. It suggests that the addition of the γ-phosphate contributes very little to the over-all ATP affinity. Because this is the reactive portion of the ATP molecule, one might expect a strong binding interaction for the γ-phosphate that would restrain its motion and could align the molecules in their most reactive conformation (44).

The γ-phosphate of ATP probably binds to the site which binds inorganic phosphate in the reverse reaction. Competition experiments with P₁ showed that, whereas up to 9 mM P₁ had no effect on MgADP binding, the presence of 1 to 12 mM P₁ resulted in a linear increase in the apparent dissociation constant for ATP. This suggests that P₁ and ATP share a common site and that P₁ has an affinity such that 1 to 12 mM can displace some of the ATP from the enzyme surface. The apparent dissociation constant for P₁ calculated in this manner was 7.1 mM, corresponding to a 3.1-kcal interaction.

This estimate for the interaction of P₁ with the enzyme is probably low. The fact that P₁ had no effect on ADP binding suggests that its interaction occurs only at the site for the γ-phosphate of ATP. Because the remainder of the ATP molecule binds so strongly, it is reasonable to visualize sites containing P₁ and partially bound ATP. The effect of such abortive complexes would be to underestimate the affinity for P₁. Direct binding experiments with P₁ and measurement of the Kₙ for P₁ in the reverse reaction suggest that the dissociation constant for P₁ is approximately 2 mM. The fact that P₁ binds with such affinity and the effect of P₁ on MgATP binding suggest that the γ-phosphate of ATP does have a strong interaction with the enzyme. However, only 0.3 kcal of this interaction are available to increase the affinity of the enzyme for ATP. The remainder of this free energy may be used to strain the γ-phosphate of ATP towards the transition state required during the reaction mechanism (45).

Because it has not been possible to obtain evidence for the formation of any free or enzyme-bound intermediates (12-15), it has been proposed that the reaction catalyzed by formyltetrahydrofolate synthetase occurs by a concerted mechanism (12). However, 3²O studies have shown that there is an oxygen transfer from formate to inorganic phosphate (12). Whether this occurs with the aid of tetrahydrofolate or with the transient formation of formyl phosphate, the 3²O studies suggest a nucleophilic attack of formate on the terminal phosphate of ATP. Similar nonenzymic reactions result in an inversion of the phosphate group (46). In such an inversion, the phosphate group proceeds through a trigonal-bipyramidal intermediate, with the entering and leaving oxygens assuming axial positions and the other oxygens assuming equatorial positions (47). If the γ-phosphate of ATP were bound to the enzyme such that the phosphate oxygens were strained towards the steric positions required to form such an intermediate, the energy of activation for the over-all reaction would be greatly reduced. In a computer analysis of the active site of glutamine synthetase, an enzyme which catalyzes a reaction very analogous to that of formyltetrahydrofolate synthetase, it has been shown that the oxygen atoms required during the reaction mechanism (45).

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The unusually strong binding of AMP-PCP to formyltetrahydrofolate synthetase could support such an argument. The fact that this strong affinity exists only for AMP-PCP and not for the α-β-methylene analogues of ATP or ADP indicates that it results from the specific position rather than the general presence of the methylene group. X-ray studies of pyrophosphate and methylenediphosphonate crystals establish values of 1.63 A and 1.79 A for the P—O and P—C—P bond lengths and 128° and 117° for the P—O—P and P—C—P bond angles, respectively (42). In a model compound for a trigonal bipyramidal intermediate, the axial P—O bonds were estimated as 1.76 A, whereas the shorter equatorial bonds were 1.60 A (49). If the P—O bond were bound to the enzyme in such a way that the γ-phosphate was strained towards the conformation of such an intermediate, the axial P—O bond length would have to be increased 10%.

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### Table III

<table>
<thead>
<tr>
<th>Group</th>
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<tr>
<td>Adenine</td>
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<tr>
<td>Ribose</td>
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<td>α-Pi</td>
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<tr>
<td>γ-Pi</td>
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<tr>
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</tr>
<tr>
<td>γ-Pi of AMP-PCP</td>
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</tr>
</tbody>
</table>

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longer C–P bond and the more acute P–C–P bond angle might better position the oxygens of the γ-phosphate of AMP-PCP in the active site. These differences result in approximately a 0.4 A shift in the position of the oxygens. Because of the difference in positioning, some of the binding energy may no longer be required for straining the oxygens and could be used to increase affinity. This could explain the greater affinity for AMP-PCP.

The effect of using binding energy to strain the γ-phosphate of ATP towards the transition state would greatly reduce the energy of activation and increase catalysis significantly. This explanation is consistent with the experimental observations and suggests that strain may play an important role in the enzyme mechanism of formyltetrahydrofolate synthetase.

REFERENCES

Formyltetrahydrofolate Synthetase: BINDING OF ADENOSINE TRIPHOSPHATE AND RELATED LIGANDS DETERMINED BY PARTITION EQUILIBRIUM

Norman P. Curthoys and Jesse C. Rabinowitz