Clostridium cylindrosporum formyltetrahydrofolate synthetase tetramers cross-linked with dimethyl suberimidate remained active in the absence of the monovalent cations normally required for enzymic activity and the tetrameric conformation. The modified enzyme was analyzed by sodium dodecyl sulfate electrophoresis, sedimentation velocity, and gel permeation chromatography. Under the experimental conditions used, the enzyme was only partially cross-linked; 74% of the enzyme was cross-linked dimer or monomer. Nonetheless, the modified enzyme is able to retain enzymic activity and the tetrameric structure under conditions where native enzyme would be completely dissociated and inactivated. The result suggests that cross-linked dimers strongly associate with each other and with monomers. Flame emission spectroscopy indicates that cross-linked enzyme contains two monovalent cations per tetramer.

Formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase (ADP-forming), EC 6.3.4.3) catalyzes the reaction: 1 formate + Mg-ATP + (−)-H$_4$folate ↔ Mg-ADP + P$_i$ + NADH$_2$folate. Like many other enzymes (1), the enzyme isolated from Clostridium cylindrosporum requires the presence of monovalent cations for optimal activity (2-6). The active form of the enzyme is a tetramer. In the absence of an effective monovalent cation (such as NH$_4^+$, K$^+$), the tetramer dissociates into inactive monomers; the complete loss of activity probably is due to a loss of the H$_4$folate binding site (7). The tetramers can be stabilized in the absence of an effective monovalent cation at 20°C by multivalent anions such as sulfate (4). The sulfate-stabilized tetramer is enzymically active but has a sharply increased $K_a$ value for formate (3).

There is an absolute requirement for certain monovalent cations for the reassociation of inactive monomers into active tetramers (4-6). The reassociation has been studied extensively by sedimentation and gel filtration and by measuring the rate of return of enzymic activity (4-6, 8-10). This report describes an exploration of the role of the monovalent cation in enzymic activity and conformation using formyltetrahydrofolate synthetase modified so that the subunits were physically constrained from dissociation.

**EXPERIMENTAL PROCEDURES**

Unless otherwise noted, reagents were ACS reagent grade or better. Formyltetrahydrofolate synthetase was crystallized and assayed by the method of Rabonowitz and Pricer (11). Lyophilized C. cylindrosporum was generously donated by Professor Richard H. Himes (University of Kansas). Only crystalline enzyme with an initial specific activity of >400 μmol/min·mg of protein was used. Enzyme was routinely diluted in ice-cold 0.05 M potassium maleate, 0.1 M 2-mercaptoethanol, pH 7.5, before assay. Tetrahydrofolic acid (H$_4$folate) was produced by catalytic hydrogenation of folic acid (Sigma) over Adam’s catalyst (MCB) in neutral aqueous solution (12).

Cross-linking was performed according to Davies and Stark (13) at room temperature in 0.1 M Tris-HCl, 0.1 M 2-mercaptoethanol, 50 mM K$_2$SO$_4$, pH 8.0 (pH at room temperature). Dimethyl suberimidate (Sigma) was added as a dry powder (1 g/g of protein); after 2 h, cross-linking was interrupted by dialysis. When dimethyl suberimidate was added as a solution, there was considerably less cross-linking, probably attributable to the instability of suberimidate in aqueous solutions of pH 7 to 9 (14), (11). Cross-linking was performed according to Davies and Stark (13) at room temperature in 0.1 M Tris-HCl, 0.1 M 2-mercaptoethanol, pH 8.0. Enzyme was desalted and dialyzed on a Sephadex G-25 column (1 x 13 cm) previously equilibrated with the same buffer. The separation of (NH$_4$)$_2$SO$_4$ from crystalline enzyme was demonstrated by testing the effluent with BaCl$_2$. The fractions containing the protein (as estimated by $\lambda_{280}$) were pooled. Enzyme was reassociated by addition of K$_2$SO$_4$ (50 mM, final concentration). The active tetramers were then cross-linked in the presence of K$_2$SO$_4$. Potassium was subsequently removed by dialysis against 0.1 M Tris-HCl, 0.1 M 2-mercaptoethanol, pH 8.0.

Sedimentation velocity studies were performed in a Beckman model E analytical ultracentrifuge equipped with a phase plate and electronic speed control. The amounts of tetramer and monomer were determined by measurement of the areas under the schlieren peaks employing Simpson’s rule (15) and a Nikon micro comparator. Areas were corrected for radial dilution (16). Gel permeation chromatography was performed on a Sephadex G-150 column (38 x 1.5 cm) equilibrated with 0.1 M Tris-HCl, 0.1 M 2-mercaptoethanol, pH 8.0 (pH adjusted at 4°C). The column was operated at a flow rate of 8 to 10 ml/h under a 10-cm hydrostatic pressure head; 1.0-ml fractions were collected. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on 8.0 cm, 5% gels essentially as described by Weber and Osborn (17). Gels were scanned on a Gilford 2600-T G densitometer at 580 nm with a 0.1-mm slit.

Protein concentrations were determined by the Coomasie blue dye-binding method (18) using the molar absorptivity at 280 nm (19) for calibration. Potassium levels were quantitated by flame emission using a model 251 Instrumentation Laboratories AA/AE spectrophotometer. Cesium chloride (1000 ppm) was added to suppress flame formation.

**REFERENCES**

* This work was supported in part by National Science Foundation Research Initiation and Support Grant SER76-18113 for the purchase of the analytical ultracentrifuge and flame emission spectrophotometer. This material was presented in part at the 1978 Pacific Slope Biochemical Conference, Santa Barbara, Calif., and is taken in part from a thesis submitted by M. d R. in partial fulfillment of the requirements for the Doctor of Philosophy, Contribution of the Nevada Agricultural Experiment Station (Reno), Journal Series No. 495. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: Mg-ATP, Mg-ADP, the magnesium complex of ATP and ADP, Na$_2$ATP, the sodium salt of ATP; TrisATP, the Tris salt of ATP; H$_4$folate, tetrahydrofolic acid; SDS, sodium dodecyl sulfate.

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noise. Protein quenching or enhancement of the potassium signal was found to be negligible under our conditions ($\lambda = 766.5$ nm, 2 mg/ml of protein). The spectrophotometer was calibrated by adding standard aliquots of potassium to the protein solution and the response was found to be linear from 0 to 1 ppm.

RESULTS

As a first step, we determined the effect of dimethyl suberimidate cross-linking of formyltetrahydrofolate synthetase on the kinetic properties of the enzyme. Unfortunately, modification caused a drop in specific activity of between 50 and 80% (see Tables I and II). Although a large fraction of the enzyme activity was destroyed, the Michaelis constant of the three substrates was unaltered ($K_{M,ATP} = 0.079$ mM, $K_{\text{M},\text{FAD}} = 0.3$ mM, $K_{\text{M,formate}} = 4.9$ mM) nor was the stimulation by mono-

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme*</th>
<th>Assay*</th>
<th>[NH₄⁺]</th>
<th>Specific activity (mm μmol/min/mg) Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>Standard (37°C)</td>
<td>0</td>
<td>412 1.03</td>
</tr>
<tr>
<td>Cross-linked</td>
<td>Standard (37°C)</td>
<td>0</td>
<td>120 1.04</td>
</tr>
<tr>
<td>Native</td>
<td>'Tris substrates' (20°C)</td>
<td>0</td>
<td>49 3.5</td>
</tr>
<tr>
<td>Cross-linked</td>
<td>'Tris substrates' (20°C)</td>
<td>0</td>
<td>18 3.1</td>
</tr>
</tbody>
</table>

* Cross-linked enzyme was prepared as described. The specific activity before cross-linking was 468, and after cross-linking was 152. Native enzyme was prepared as if for cross-linking (as described), except that no dimethyl suberimidate was added. The data shown are for enzyme which was not dialyzed after cross-linking. After 116 h of dialysis (6 $\times$ 500 ml of 0.1 M Tris-HCl, 0.1 M 2-mercaptoethanol, pH 8.0), NH₄⁺ stimulation in Tris substrates increased to 4.8-fold.

'The standard assay mix is that described by Rabinowitz and Pricer (11) and contains 8 mM K⁺ and 50 mM Na⁺. The Tris substrate mix is identical except that Tris is the sole monovalent cation (3). TrisATP was prepared by passage of NaATP (Sigma) through a Dowex column (20). The assay temperature was lowered from 37°C to 20°C when Tris substrates were used to maintain the activity of the enzyme. Cross-linking of monomer units was monitored by electrophoresis on SDS-polyacrylamide gels. Fig. 1 shows a typical gel pattern which is similar to that observed for many other proteins (20). Electrophoresis of control (unmodified) enzyme yielded only a single band. The mobilities were consistent from experiment to experiment and gave the expected logarithmic dependence on molecular weight (17). The mobility of monomer derived from either native or cross-linked protein was identical. Under the conditions used in the present studies, the percentage of cross-linked tetramer was 4 to 10%. An appreciable amount of polymeric material was often observed at the top of the gel. The amount of this immobile material was greatly reduced if the duration during cross-linking was not allowed above 20°C.

**TABLE II**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>DMS/protein (w/w)</th>
<th>Specific activity after cross-linking</th>
<th>Specific activity after dialysis</th>
<th>Length of dialysis (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>196</td>
<td>198</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>203</td>
<td>71</td>
<td>62</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>152</td>
<td>65</td>
<td>116</td>
</tr>
</tbody>
</table>

* Four buffer changes.

* Six buffer changes.

The cross-linking of monomer units was monitored by electrophoresis on SDS-polyacrylamide gels. Fig. 1 shows a typical gel pattern which is similar to that observed for many other proteins (20). Electrophoresis of control (unmodified) enzyme yielded only a single band. The mobilities were consistent from experiment to experiment and gave the expected logarithmic dependence on molecular weight (17). The mobility of monomer derived from either native or cross-linked protein was identical. Under the conditions used in the present studies, the percentage of cross-linked tetramer was 4 to 10%. An appreciable amount of polymeric material was often observed at the top of the gel. The amount of this immobile material was greatly reduced if the duration during cross-linking was not allowed above 20°C.

Sephadex chromatography or dialysis of unmodified enzyme against Tris-HCl causes dissociation and inactivation of formyltetrahydrofolate synthetase. The effect of cross-linking on the dissociation and inactivation was examined by first dialyzing cross-linked enzyme against a Tris buffer followed by Sephadex G-150 chromatography. A typical experiment is shown in Fig. 2. Some highly cross-linked material eluted as a shoulder at the void volume. There was a single peak of protein whose elution position corresponded to that of unmodified, tetrameric enzyme. In addition, there was a small peak of activity near the position expected for the monomer, but no protein peak was observed. When unmodified enzyme was similarly treated, no enzyme activity could be detected and a single protein peak eluted at a position corresponding to a molecular weight of roughly 60,000. In Fig. 2, the specific activity of each fraction was calculated by dividing the enzymatic activity by the protein concentration as determined by the absorbance at 280 nm (19). The specific activity of the peak fraction was essentially the same as that measured immediately after cross-linking. Cross-linking prevented the wholesale dissociation and inactivation of the synthetase. The decrease in specific activity on the low molecular weight side of the peak indicates the presence of dissociated, inactive materials.

The amount of chemical cross-linking of enzyme in each fraction was monitored by SDS electrophoresis of aliquots from several of the chromatography fractions. The distribution of cross-linked tetramers, trimers, etc., was essentially the same as that shown in Fig. 1. In general, the degree of cross-linking (e.g. amount of cross-linked tetramer) decreased slightly with increasing fraction number.

The dimethyl suberimidate-modified enzyme was also investigated by analytical ultracentrifugation (Fig. 3). When cross-linked enzyme was dialyzed for 48 h at 4°C, the sedimentation pattern showed only two components (Fig. 3A, lower pattern). Unmodified enzyme lost essentially all activity, whereas dialysis of cross-linked enzyme caused no change in activity (Table II, Experiment 1). For comparison, the sedimentation profile of the unmodified control enzyme is shown above that of the cross-linked enzyme (Fig. 3A, upper pattern). The SDS electrophoretogram indicated that the sample contained (on a weight basis) 4% cross-linked tetramer, 10% cross-linked trimer, 35% cross-linked dimer, and 50% monomer. The percentage by weight of the fast and slow sedimenting material is 67% and 33%, respectively. Therefore, although 85% of the modified protein was free to dissociate to monomer and dimer, the majority of the enzyme remained in the tetrameric conformation in the absence of the cations normally required to maintain the tetramer.

In other experiments, the effect of prolonged dialysis (62
and 116 h, Table II, Experiments 2 and 3) on the activity and conformation of cross-linked enzyme was examined. The amount of activity lost by dialysis increased with time, but dissociation was changed little. The cross-linked enzyme from Experiment 3 was subjected to sedimentation velocity at 25.4°C and 7.1°C (Fig. 3, B and C). The distribution of protein among the sedimenting components was not affected by temperature. Three components were observed; the fastest (63% of the total protein) and the slowest (24%) had sedimentation coefficients which correspond to tetramer and monomer, respectively. The intermediate component contained 14% of the total protein. Observed concentrations were not corrected for the Johnston-Ogston effect (21). Assuming the same dependence of the sedimentation coefficient on concentration as observed for the tetramer (22), the correction would be less than 15%. The protein composition by SDS electrophoresis was 10% tetramer, 16% trimer, 38% dimer, and 35% monomer. Again, more

FIG. 1. SDS electrophoresis of cross-linked formyltetrahydrofolate synthetase. The enzyme was cross-linked as described, then dialyzed for 116 h at 4°C against six changes of buffer (0.1 M Tris-HCl, 0.1 M 2-mercaptoethanol, pH 8.0, 500 ml at a time). Electrophoresis of 0.014-mg samples was as described by Weber and Osborn (17). Migration is from left to right. The gels were scanned as described under "Experimental Procedures." Peak A corresponds to the tetramer, B to the trimer, C to the dimer, and D to the monomer. The relative amounts of each component, expressed as percentages of the total area, are shown in the figure.

FIG. 2. Sephadex G-150 chromatography of cross-linked formyltetrahydrofolate synthetase. Specific activity of the enzyme was 379 μmol/min-mg after reassociation. Reassociated enzyme was cross-linked as described under "Experimental Procedures." After cross-linking, the specific activity dropped to 125 μmol/min-mg. Modified enzyme was then dialyzed for 1 h against two changes of buffer (0.1 M Tris-HCl, 0.1 M 2-mercaptoethanol, pH 8.0, 500 ml) at 4°C. The specific activity after dialysis was 129 μmol/min-mg. Gel permeation chromatography of a 3.6-mg sample was performed at 4°C as described under "Experimental Procedures." The specific activity of the peak fraction was 126 μmol/min-mg. Protein concentration of each fraction is reported as mg/ml.

The specific activity dropped to 125 μmol/min-mg. Mod-

Fig. 3. Sedimentation velocity of cross-linked formyltetrahydrofolate synthetase. The figures show typical sedimentation velocity experiments. Direction of sedimentation is from right to left in all cases. A, sedimentation velocity measured at 18.1°C. Control (upper) and cross-linked (lower) enzyme were dialyzed together for 48 h at 4°C against four changes of 500 ml of 0.1 M Tris-HCl, 0.1 M 2-mercaptoethanol, pH 8.0. The percentages of covalently cross-linked tetramer, trimer, and dimer are 4, 10, and 35, respectively, as determined by SDS electrophoresis. The sedimentation coefficients (s0) of the cross-linked enzyme (lower pattern) were 8.6 S and 4.1 S, those of the control were 9.3 S and 4.1 S. The picture was taken 24 min after attaining full speed (Experiment 1, Table II). B, enzyme was cross-linked as described and dialyzed for 116 h at 4°C against six changes (500 ml) of buffer (0.1 M Tris-HCl, 0.1 M 2-mercaptoethanol, pH 8.0). Sedimentation velocity was measured at 25.4°C. Covalently cross-linked tetramer was 10%, covalently cross-linked dimer was 38%, and covalently cross-linked trimer was 10% of the total. The sedimentation coefficients (s0) were 8.8 S, 6.1 S, and 3.9 S. The picture was taken 48 min after attaining full speed (Experiment 3, Table II). C, enzyme was prepared as described for B. Sedimentation velocity was performed at 7.6°C. The coefficients were 9.5 S, 6.5 S, and 4.0 S. The picture was taken 60 min after attaining full speed. D, unmodified enzyme dialyzed as described for B above. Sedimentation velocity was performed at 5.4°C. The coefficient (s90) was 4.2 S. The picture was taken 32 min after attaining full speed. than half of the material sediments as tetramer in the absence of monovalent cation. The control enzyme (Experiment 3, Table II; Fig. 3D) had only a single sedimenting component which corresponded to monomer. The amount of trimer as determined by SDS electrophoresis was in close agreement with that of the intermediate sedimenting species. Unfortunately, the data at hand do not permit an identification of the intermediate species as either dimer or trimer.

In a separate experiment, the effect of cross-linking per se on the sedimentation coefficient was measured. The enzyme was prepared as described under "Experimental Procedures" and divided into two equal portions. To one of these, dimethyl suberimidate was added, and the protein was cross-linked as described. The second portion was unmodified. Immediately after cross-linking (and before dialysis), both the cross-linked and the unmodified enzyme were centrifuged simultaneously in the same rotor. Only single sedimenting peaks were observed in each case. The sedimentation coefficients (s90) were 8.6 S (cross-linked enzyme) and 8.8 S (unmodified control). Although the protein concentration of the modified enzyme was slightly less than the control, the difference in s90 cannot be explained on the basis of the known effect of concentration on the sedimentation coefficient of the unmodified enzyme (22). The modest size of the change permits straightforward
interpretation of the sedimentation velocity experiments.

Properties of the cross-linked enzyme in the absence of exogenous monovalent cation may in part result from K+ "trapped" indirectly by the modification. To test this possibility, enzyme was cross-linked and dialyzed under conditions similar to Experiment 1, Table II. The enzyme lost 62% of the original activity during cross-linking and retained 92% of the latter activity after 48 h of dialysis. A control enzyme received exactly the same treatment (except for the chemical modification), was dialyzed in the same flask, and retained only 3% of original activity. Flame emission spectrophotometry revealed that control enzyme contained 0.26 ± 0.04 mol of K+/mol of tetramer (or 0.065/monomer unit) and cross-linked enzyme contained 1.6 ± 0.1 mol of K+/mol of tetramer. If one allows for the dissociation of cross-linked enzyme seen under these conditions (Fig. 3A) and the presumptive, concomitant loss of cation, the results are in excellent agreement with the value of two cations per tetramer obtained by Harmony and Himes (10) by analysis of the equilibrium constant.

**DISCUSSION**

Reassociation of inactive monomers to form active tetramers can occur by a variety of mechanisms. Two limiting cases are: (a) the sequential addition of monomers to form dimer, trimmer, and tetramer, or (b) the initial formation of dimer followed by association of dimers to form tetramer with no trimer intermediate produced. Combinations and variations of both mechanisms can be envisioned. The intermediates are presumed to be highly reactive as only monomers and dimers have been reported even under conditions where both monomer and tetramer coexist in appreciable quantities (2, 23). Partial cross-linking of the enzyme is sufficient to prevent dissociation of the tetramer when Tris is the sole exogenous monovalent cation but does not prevent a gradual loss of activity during prolonged dialysis (Fig. 3, A and B; Table II, Experiments 1 to 3). The gradual loss of activity appears to be a property of the protein itself rather than any inefficiency of cation removal by dialysis. Sephadex G-150 chromatography efficiently separates monovalent cations from the synthetase and produces complete loss of enzymic activity of unmodified enzyme. The slow inactivation observed during dialysis may be related to the reported instability of monomer (9) and/or to the formation of tetramers of low specific activity during long storage of crystalline enzyme (23).

The primary or covalent structure of *Clostridium cylindrosporum* formyltetrahydrofolate synthetase is apparently four identical polypeptides (4). Reaction of the enzyme with dimethyl suberimidate alters the structure by covalently joining two or more of the peptides. After modification, we can speak of tetramers which have a variety of covalent structures. The general types are diagrammed below:

![Diagram of covalent structures](image)

Structures A and B are cross-linked tetramers, C contains a cross-linked trimer, and D and E contain cross-linked dimers only. From SDS electrophoresis, it was possible to ascertain the covalent structure of the enzyme. Combining that information with the column and sedimentation experiments, it was possible to estimate the relative amounts of each tetramer. In the case of G-150 chromatography, the peak tetramer fraction was analyzed by SDS electrophoresis; in sedimentation experiments, the content was deduced from SDS electrophoresis of the mixture and the relative abundance of the sedimenting components. In both sedimentation experiments (Experiment 1, Table II) and chromatography experiments, 20% of the tetramers were Structure C, 32% were Structure D, and 41% were Structure E. The results indicated that cross-linked dimers were able to associate to form tetramers and that cross-linked dimer is able to bind two monomers (or at least retain two monomers) in the absence of exogenous monovalent cation. Harmony et al. (9) have shown that the rate-limiting step in the reassociation of *Clostridium cylindrosporum* formyltetrahydrofolate synthetase is dimer formation and that this step, at least, requires certain monovalent cations. The "trapping" of the dimer intermediate by chemical cross-linking caused retention of the monovalent cation. It appears that association of dimers with each other does not require additional monovalent cation. Existence of significant amounts of Structure C may indicate that dissociation of tetramer into dimer is obligatory for dimer dissociation to monomer. However, the existence of an appreciable amount of Structure E suggests that dimer may be able to bind monomer without cation. Work is continuing using the cross-linking technique for the study of the association process.

**Acknowledgments**—We wish to express our gratitude to Dr. Richard H. Himes for generous gifts of lyophilized *Clostridium cylindrosporum* and helpful discussions, to Marcy Welch for making the drawings, to Kathleen Schegg for help with the stimulation experiments and preparation of the TrisATP, and to Dr. Pat Beaulieu and Gale Starch for guidance in the emission spectrophotometry.

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