Monovalent Cation Induced Reassociation of Formyltetrahydrofolate Synthetase Monitored by Rayleigh Light Scattering and Enzymic Activity†

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ABSTRACT: It has been previously established that formyltetrahydrofolate synthetase isolated from *Clostridium cylindrosporum* is reversibly dissociated and inactivated in the absence of certain monovalent cations. In the present paper, the reassociation of monomeric, inactive enzyme to form tetrameric, active enzyme was monitored by Rayleigh light scattering and enzymic activity. Light-scattering measurements confirmed that the active enzyme is composed of four subunits of equal weight. With the assumption that the results of analytical ultracentrifugation are correct—that monomers and tetramers are the only species ever present at appreciable levels—the amount of tetramer formed during reassociation was calculated from the light-scattering data. Evidence for the accumulation of catalytically active intermediates was obtained by comparing the rate of association of monomers (detected by light scattering) to the rate of return of enzymic activity. The accumulation of intermediates was most strikingly seen at low monovalent cation concentration at low ionic strength. Evidence is also presented that sedimentation favors reassociation of the enzyme. The reassociation data were fit to a second-order reversible rate equation. Interestingly, although the data were derived from the same experiments, the kinetic plot based on light-scattering measurements yielded a straight line function with an abrupt change in slope about 10 min after initiation of reassociation, while plots based on enzymic activity measurements gave a single slope.

Formyltetrahydrofolate synthetase [formate:tetrahydrofolate ligase (ADP), EC 6.3.4.3] is one of the many enzymes known to be activated by monovalent cations (Suelter, 1970). It catalyzes the reaction $MgATP^{2-} + HCOO^- + H_4folate = MgADP^- + P_i^{2-} + N^{10}formylH_4folate$[1] and has been purified to homogeneity from *Clostridium cylindrosporum* by Rabinowitz & Pricer (1962). The structure of the catalytically active form of the protein (Welch et al., 1971) appears to be a tetramer composed of four apparently identical subunits with a mass (tetramer) of (2.3–2.4) × 10^5 daltons (Himes & Rabinowitz, 1962a; Welch et al., 1971). Tetramers can be reversibly dissociated into catalytically inactive monomers by a variety of procedures (Scott & Rabinowitz, 1967; Welch et al., 1971; Harmony et al., 1974; Garrison et al., 1976). The quaternary structure and enzymic activity of the enzyme are maintained by monovalent cations; the most effective are NH_4^+, K^+, and Rb^+ (Scott & Rabinowitz, 1967; Welch et al., 1968, 1971). Thus far only monomers and tetramers have been observed; there is kinetic evidence for the formation of a dimer intermediate during reassociation.

Himes, Rabinowitz, and their co-workers have extensively studied the reassociation of formyltetrahydrofolate synthetase isolated from *C. cylindrosporum* and *C. acidi-urici* by following the return of enzymic activity (Himes & Harmony, 1973; Harmony et al., 1974, 1975; Harmony & Himes, 1975). We report here a study of the reassociation of synthetase monomers into tetramers by two independent methods: return of enzymic activity and increase in turbidity. To the best of our knowledge this is the first time the kinetics of reassociation of this enzyme has been studied by a method which does not depend on activity measurements.

Experimental Procedures

Reagents. Unless otherwise stated, reagents were ACS analytical grade. Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid (Sigma) over Adams catalyst (MCB) in neutral aqueous solution and purified on DEAE-cellulose (Schleicher & Schuell) according to Blakely (1957) and Samuel et al. (1970). Ammonium chloride (analytical reagent) was purchased from Mallinckrodt. Ammonium sulfate, ultrapure, was purchased from Schwarz/Mann. Adenosine triphosphate, 2-mercaptoethanol, dithiothreitol, and protamine sulfate were all purchased from Sigma. Concentrated Coomassie Blue protein reagents were purchased from Bio-Rad and used as directed.

N^{10}-Formyltetrahydrofolate Synthetase. N^{10}-Formylhydrofolate synthetase was purified from lyophilized *Clostridium cylindrosporum* (kindly donated by Professor Richard H. Himes, University of Kansas) and assayed as described by Rabinowitz & Pricer (1962). A unit of activity is defined as 1 μmol of product formed per min. The specific activities of crystalline preparations immediately after purification varied between 390 and 430 μmol min^{-1} (mg of protein)$^{-1}$.

Light Scattering. In this work a Brice-Phoenix Universal light-scattering apparatus (Model 200) was used. It was modified for 1 × 1 cm cells by substituting the collimating slits as indicated in the instruction manual of the instrument. The photomultiplier load resistor was modified according to the manufacturer's instructions to obtain a 10-fold increase in sensitivity. The photomultiplier output was measured as the voltage drop across the load resistor using a high-input resistance digital voltmeter (0.1-mV resolution, G. N. Wood, Model GM-300). The light source was a mercury lamp, and the 546- and 436-nm wavelengths were selected by filters. The value of the refractive increment for the synthetase was taken

† From the Division of Biochemistry, University of Nevada, Reno, Nevada 89557. *Received October 6, 1981.* Contribution of the Nevada Agricultural Experiment Station (Reno), Journal Series No. 536. This report is taken, in part, from a thesis submitted by M.d.R. in partial fulfillment of the requirements for the doctor of philosophy degree. The work was partially supported by the University of Nevada Research Advisory Board; the purchase of the analytical ultracentrifuge and related equipment was made possible by National Science Foundation Grant SER76-18113.

[1] Abbreviations: MgATP and MgADP, the magnesium salts of adenosine triphosphate and diphosphate; H_4folate, tetrahydrofolate; N^{10}formylH_4folate, N^{10}-formyltetrahydrofolic acid; P_i, orthophosphate; DEAE-, diethylaminoethyl-; SA/X, specific activity per fraction tetramer; DTT, dithiothreitol; τ, turbidity; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-3530$01.25/0

as 0.1954 at 436 nm and 0.1883 at 546 nm, values determined for bovine serum albumin (Halwer et al., 1951). Since the molecular weights of formyltetrahydrofolate synthetase monomers and tetramers were in excellent agreement with published results [see the sections Molecular Weight of Formyltetrahydrofolate Synthetase (Monomer and Tetramer)], these values of the refractive index increment were used throughout this work. Any resulting small systematic errors will not alter any of the significant findings reported here.

The transmittance of the neutral filters and the value of the constant "a" were determined periodically and found to be unchanged. For molecular weight determinations the value of the y intercept of an Hc/τ_{exc} vs. c plot was calculated from linear regression analysis of the data points. The excess turbidity (τ_{exc}, the difference between the turbidity of the solution and the solvent) was calculated as described in the instruction manual of the instrument. The protein concentration (c) is in grams per milliliter; H is a collection of constants including the refractive increment [see, for example, Timasheff & Townend (1970)].

Determination of the "fraction tetramer" (X) at any time (t) during the course of a reassociation was based on excess turbidities. To date, only monomers and tetramers of formyltetrahydrofolate synthetase have been reported. The excess turbidity observed at any time (τ_{obsd}) is therefore assumed to be due to that of monomers and tetramers only. At any concentration e

$$\tau_{obsd} = e\frac{d\tau_Q}{dc} + (c_T - e)\frac{d\tau_M}{dc}$$

or

$$\tau_{obsd} = X\tau_Q + (1 - X)\tau_M$$

where

$$X = \frac{\tau_{obsd} - \tau_M}{\tau_Q - \tau_M}$$

and e = the concentration of tetramers in grams per milliliter, c_T = the total concentration of enzyme in grams per milliliter, X = fraction tetramer, τ_M = the excess turbidity of a 100% monomer solution of concentration c_T, and τ_Q = the excess turbidity of a 100% tetramer solution of concentration c_T. The values of τ_M and τ_Q were obtained from calibration plots of τ_{exc} vs. c for pure solutions of monomer and tetramer. The "fraction tetramer" obtained in this way is a weight fraction.

The fraction tetramer values at the beginning of the reassociation agreed with the composition of the solution determined by sedimentation velocity experiments. In the absence of activating cations, ultracentrifugation did not alter the distribution between monomer and tetramer. The two methods also agreed at the end of the experiment as long as about 80% of the protein had been converted to tetramer (see Results).

For extrapolation of the turbidity (and fraction tetramer) and specific activity of a reassociation experiment to infinite time, the reciprocals of the values were plotted as a function of the reciprocal of time and extrapolated to $1/t = 0$ by a least-squares fit as described by Harmony et al. (1974).

Equilibrium Constant. The equilibrium constant was calculated from the stoichiometry of the reaction determined by Harmony & Himes (1975):

$$4M + 2NH_4^+ \rightleftarrows Q$$

$$K_{eq} = \frac{[Q]}{[M]^4[NH_4^+]^2}$$

where M represents monomer and Q represents tetramer.

The equilibrium concentrations were determined as follows: the fraction tetramer (or fraction monomer) obtained by extrapolating the light-scattering data to infinite time was multiplied by the protein concentration and divided by the appropriate molecular weight as follows:

$$[T] = \frac{Xc_T}{227\,000}$$

$$[M] = \frac{(1 - X)c_T}{56\,700}$$

Kinetic Analysis. In the experiments reported here, an appreciable fraction of the monomers had not reassociated after attainment of equilibrium. Therefore, a reversible second-order rate equation was used to analyze the sample data.

The rate of formation of tetramer (Q) from monomer (M) is

$$\frac{d[Q]}{dt} = k_1[M]^2 - k_2[Q]$$

where

$$4M \underset{k_2}{\overset{k_1}{\rightleftarrows}} Q$$

On the basis of the findings of Harmony and Himes, the rate equation contains a second-order dependence on monomer concentration. Monovalent cation is considered to be in large molar excess and essentially constant over the reassociation experiment; therefore, the concentration of monovalent cation is combined with the rate constant. Although the reaction rate is second order with respect to monomer concentration, four monomers are required for each tetramer formed. This stoichiometry is accounted for in the derivation of the rate equation. Defining $[M]_0$ as the initial monomer concentration and $[Q]_{eq}$ as the concentration of tetramer at equilibrium and making the appropriate substitutions to eliminate [M] and k_2 from the equation, one obtains

$$\frac{d[Q]}{[M]_0^2 - \left(\frac{[M]_0^2}{[Q]_{eq}} + 16[Q]_{eq}\right)[Q] + 16[Q]^2} = k_1\,dt$$

Integration yields

$$F(Q) = \frac{1}{\sqrt{-q}} \ln \frac{(32[Q] - b + \sqrt{-q})(b + \sqrt{-q})}{(32[Q] + b + \sqrt{-q})(b - \sqrt{-q})} = k_1 t$$

where

$$q = 64[M]_0^2 - \frac{[M]_0^2}{[Q]_{eq}} + 16[Q]_{eq}^2 < 0$$

$$b = -\frac{[M]_0^2}{[Q]_{eq}} - 16[Q]_{eq}$$

When the basic ideas outlined above are used, the following analogous expressions can be derived for three other possibilities. When the reaction is kinetically second order in monomer but the reassociation is irreversible, then

$$[Q]/[[M]_0([M]_0 - 4[Q])] = kt$$

When the formation of tetramer is kinetically first order in

monomer, the rate equation is

$$\frac{1}{4} \ln \frac{[M]_0}{[M]_0 - 4[Q]} = kt$$

if the reassociation is irreversible and

$$\frac{[Q]_{eq}}{[M]_0} \ln \frac{[Q]_{eq}}{[Q]_{eq} - [Q]} = k_1 t$$

if the reassociation is reversible.

Sample Preparation. Crystalline formyltetrahydrofolate synthetase was collected by centrifugation and resuspended in 0.4 mL of 0.1 M Tris-HCl and 0.1 M 2-mercaptoethanol, pH 8.0 (at room temperature), and desalted on a 13 × 1 cm Sephadex G-25 column previously equilibrated with this buffer and calibrated with blue dextran. This exchange of an effective monovalent cation (like NH_4^+ and K^+) by an ineffective one (like Tris H^+) causes the enzyme to dissociate into monomers with concomitant loss of activity (Himes & Harmony, 1973). The protein concentration was determined by absorbance at 280 nm with the molar absorptivity determined by Himes & Cohn (1967) in a Hitachi–Perkin-Elmer UV–vis spectrophotometer (Coleman 111). The separation of sulfate from the protein was verified by use of $BaCl_2$. Fractions containing dissociated protein were pooled and set aside for enzyme activity assay. In most of the experiments described in this work the protein concentration of the pooled fractions was between 4 and 6 mg/mL.

For the reassociations in NH_4Cl, aliquots of NH_4Cl in the Tris-HCl–2-mercaptoethanol buffer were added to the cuvette. Ionic strength, when necessary, was adjusted with various amounts of NaCl [for the reassociations in $(NH_4)_2SO_4$, Na_2SO_4 was used instead].

Attempts were made to clarify the solution by filtration. Membrane filters from several manufacturers were prewashed with the Tris–mercaptoethanol buffer. However, filtered protein failed to reassociate as well as the unfiltered controls. To clarify the solutions for light scattering, we routinely centrifuged the protein, the buffer, and the NH_4^+ solutions at 39000*g* for 65 min at 0 °C in a Servall RC-2 refrigerated centrifuge. For some experiments the dissociated protein was diluted to a working concentration of 1 mg/mL with Tris-HCl–mercaptoethanol buffer prior to centrifugation. Depending on the particular experiment, the buffer or protein solution was transferred to a preweighed light-scattering cuvette which was then covered with parafilm. The concentrated protein solution and salt solution were transferred in a similar way into dust-free containers. All glassware used in the light-scattering experiments had been acid washed, rinsed with dust-free water [prepared by distillation as described by Timasheff & Townend (1970)], and dried in a drying oven at 90 °C underneath dust-free beakers.

The solvent-filled (or protein-filled) cuvette was weighed to determine the amount of solvent (or protein solution) and was then placed in the light-scattering chamber. The intensities of the transmitted and the scattered light (436 nm) were read at 0° and 90°. When the light-scattering cuvette initially contained only buffer, appropriate amounts of concentrated protein solution were transferred into the cuvette with a dust-free pipet and the contents mixed by inversion. It was then weighed to determine the exact amount of protein added. The scattering of the protein was now taken and recorded as the 0-min value. A sample of this solution was removed, diluted as below, and set aside for assay of enzymic activity. For molecular weight determinations and construction of τ_{exc} vs. c calibration plots, the protein was added in successive aliquots delivered from dust-free micropipets. The volume delivered was confirmed by weighing the cuvette and contents at the end of the experiment.

Molecular Weight Determination of Bovine Serum Albumin. The molecular weight of bovine serum albumin (BSA) was determined by light scattering to ensure that the apparatus constants had been adequately calibrated and that dust would not be a significant source of error. Bovine serum albumin was dissolved in 0.1 M Tris-HCl–0.1 M 2-mercaptoethanol, pH 8.0, to a final concentration of approximately 5 mg/mL. The solution was clarified for light scattering by centrifugation as described above. Additions of protein solution to the solvent-filled light-scattering cuvette were made with dust-free micropipets. Data were collected by using both the green and blue lines of the mercury lamp. A least-squares fit of the data gave a weight of 66 400 (green line, 546 nm) and 63 400 (blue line, 436 nm). Published molecular weights of bovine serum albumin range from 65 400 to 77 000. The values determined in this work are in excellent agreement with those determined by sedimentation diffusion and osmotic pressure measurements (Halwer et al., 1951; Creeth, 1952; Loeb & Scheraga, 1956; Scatchard & Pigliacampi, 1962; Edsall et al., 1950).

Reassociation. All reassociations were performed at 21 ± 1 °C. A known amount of NH_4^+ was added with a dust-free micropipet to start a reassociation. The contents of the cuvette were mixed by inversion, and the intensities of the transmitted light and the scattered light were read at the indicated times. At various time intervals during the reassociation, samples were withdrawn from the cuvette with dust-free micropipets and diluted in 10.0 mL of 0.05 M potassium maleate–0.1 M 2-mercaptoethanol, pH 7.5 buffer for subsequent assay of enzymic activity. This buffer stabilizes the enzyme (Rabinowitz & Pricer, 1962), and the high dilution effectively quenches the reassociation reaction. These tubes were stored on ice until they were assayed. When changes in the readings of the transmitted and scattered light became negligible (generally 150–200 min), the reassociation was considered to have reached equilibrium, and the cuvette was removed from the light-scattering chamber.

It was established that addition of various amounts of NH_4^+ does not significantly alter the value of the turbidity obtained with the Tris-HCl–mercaptoethanol buffer alone.

Generally the amounts of monomer and tetramer in the clarified stock protein solution and in the final reassociated protein were analyzed by sedimentation velocity.

Ultracentrifugation. Sedimentation velocity experiments were performed at 250000*g* (60 000 rpm) in a Beckman Model E ultracentrifuge equipped with a phase plate and electronic speed control. All cells used contained 12-mm KEL-F centerpieces. Schlieren and Rayleigh interference photographs were read in a Nikon microcomparator. Scans using absorption optics were made at 280 nm with a Beckman photoelectric scanner and a monochromator equipped with a high-intensity light source. Concentrations were corrected for radial dilution (Chervenka, 1973) in all cases.

Protein Determinations. Unless stated otherwise, protein concentration was determined by measuring the absorbance at 280 nm (Himes & Cohn, 1967). Occasionally the Coomassie Blue dye method (Bradford, 1976) or a modification of the method of Lowry described by Sutherland et al. (1949) was used. When the later methods were used, the color yield was standardized with the A_{280} method.

Results

Molecular Weight of Formyltetrahydrofolate Synthetase (Monomer). Enzyme was dissociated into monomers and

clarified for light scattering by centrifugation in concentrated solution as described under Experimental Procedures. The protein concentration of the stock monomer solution was 7.92 mg/mL, and the specific activity was 0.7 μmol min^{-1} (mg of protein)$^{-1}$. The scattering ratio was measured at 546 and at 436 nm. A least-squares analysis of the data gave masses of 54 000 (436 nm) and 59 300 daltons (546 nm), yielding an average mass of the protein of 56 700 daltons.

Sedimentation velocity analysis of the monomer stock solution revealed the presence of 5% high molecular weight material (s_{20} = 8.9 S) which corresponded to the tetramer (Welch et al., 1971). The other 95% of the protein sedimented with a coefficient (s_{20}) of 4.0 S, indicating it was monomer (Welch et al., 1971). At the end of the molecular weight determination the contents of the light-scattering cuvette were analyzed by sedimentation velocity using Rayleigh interference optics. Only one component which sedimented as monomer was found. The 5% tetramer present in the stock solution was probably dissociated upon dilution in the light-scattering cuvette. Welch et al. (1971) determined the mass of the monomer (sedimentation equilibrium in 6.0 M guanidine hydrochloride) as 59 800 ± 100 daltons. Scott & Rabinowitz (1967) obtained a value of 58 000 daltons by sedimentation equilibrium. The average molecular weight of the monomer determined here by light scattering is one-fourth of that determined for the tetramer (see below).

Molecular Weight of Formyltetrahydrofolate Synthetase (Tetramer). Crystalline enzyme was prepared for light scattering as described under Experimental Procedures. The Tris–mercaptoethanol buffer contained 50 mM $(NH_4)_2SO_4$ to prevent the enzyme from dissociating into monomers. Crystalline enzyme was collected, dissolved in 0.1 M Tris-HCl–0.1 M 2-mercaptoethanol–0.05 M $(NH_4)_2SO_4$, pH 8.0 buffer, and passed through a G-25 column equilibrated with the same buffer. The protein peak was pooled and found to contain 6.23 mg/mL enzyme.

The scattering ratio was measured at 546 and 436 nm. A least-squares fit of the data gave a mass of 224 000 (546 nm) and 230 000 daltons (436 nm) for an average value of 227 000 daltons. This value is 4 times higher than that of the mass obtained for the monomer (56 700). Again, the literature values for the mass of the tetramer are in excellent agreement with the 227 000 dalton value: 238 000 ± 8000 daltons by sedimentation equilibrium (Welch et al., 1971) and 230 000 daltons by sedimentation diffusion (Himes & Rabinowitz, 1962a,b). Enzyme which was dissociated, then fully reassociated, behaved identically with the "native" enzyme described above.

Reassociations. Several reassociations were performed in the presence of varying concentrations of NH_4Cl. Samples were prepared for light scattering, and the return of enzymic activity and the increase in turbidity were measured as described under Experimental Procedures. For technical reasons, it was possible to follow the turbidity at one wavelength only; the blue line was used to obtain higher sensitivity. The data for reassociations performed at the extremes of NH_4Cl concentration used in the present work are shown in Figure 1. The progress of the reaction is expressed as enzymic activity (Figure 1A), turbidity (Figure 1B), and fraction tetramer (Figure 1C).

Qualitatively, reassociations under all conditions were similar. All of the reassociations leveled out after 50 or 60 min, and after this time the activities increased gradually. The variation in turbidity at zero time reflects differences in protein concentration which varied from experiment to experiment.

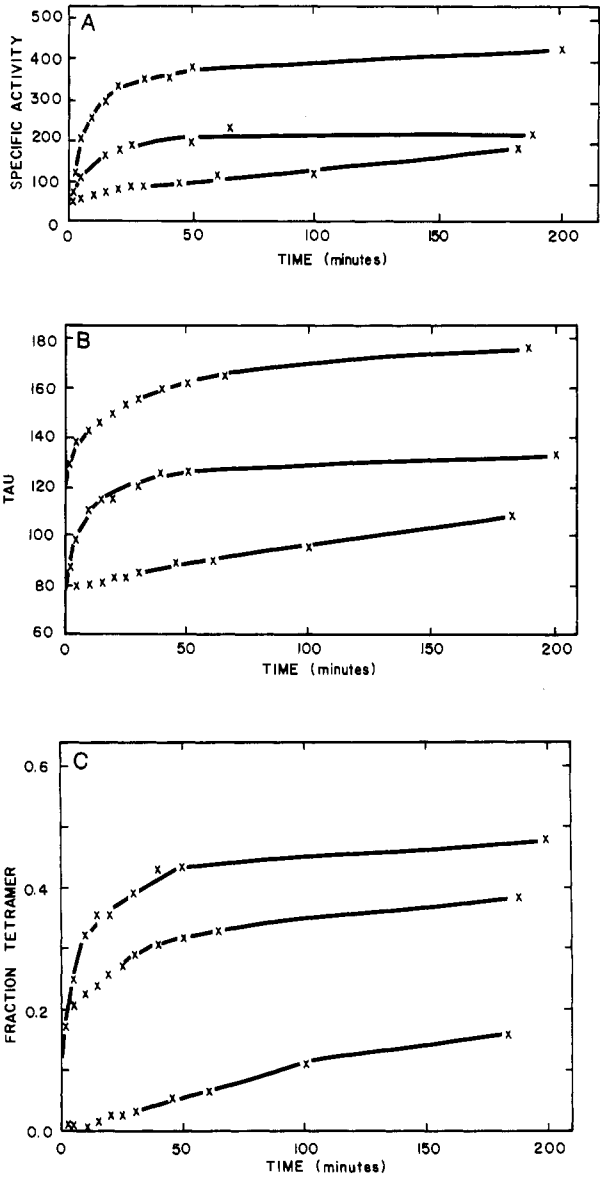

FIGURE 1: (A) Reassociation of formyltetrahydrofolate synthetase followed by enzyme activity. Experiments were performed as described under Experimental Procedures. Aliquots were removed from the light-scattering cuvette at the indicated times and diluted in 0.05 M potassium maleate–0.1 M 2-mercaptoethanol, pH 7.5, and stored on ice until assayed by the method of Rabinowitz & Pricer (1962). Reassociations were performed at (A) 38 mM (top curve), (B) 21.7 mM (middle curve), and (C) 6.8 mM (bottom curve) NH_4Cl. The protein concentrations and ionic strength are respectively the following: (A) 1.34 mg/mL, 0.105 M; (B) 0.88 mg/mL, 0.089 M; (C) 1.13 mg/mL, 0.074 M. The activities extrapolated to infinite time as described under Experimental Procedures are (A) 441, (B) 216, and (C) 190 μmol min^{-1} mg^{-1}. The equilibrium constants, estimated from the enzymic activity, are the following: 21.7 mM NH_4^+, 4×10^{17} M^{-5}; 6.8 mM NH_4^+, 4×10^{18} M^{-5}. An equilibrium constant could not be calculated for the 38 mM NH_4^+ experiments because the specific activity at the end of the experiment was greater than 410 μmol min^{-1} mg^{-1}. (B) Reassociation of formyltetrahydrofolate synthetase followed by light scattering (turbidity). The values of τ have been multiplied by 1×10^5. Experiments and position of the progress curves are those listed in (A). The values of τ at zero time (before addition of NH_4^+) were the following: 38 mM NH_4Cl, 105×10^{-5}; 21.7 mM NH_4Cl, 63×10^{-5}; 6.8 mM NH_4Cl, 76×10^{-5}. (C) Reassociation of formyltetrahydrofolate synthetase: fraction tetramer. The turbidity data in (B) were used to calculate the fraction tetramer as described under Experimental Procedures. The fraction tetramer extrapolated to infinite time as described under Experimental Procedures is (A) 0.50, (B) 0.38, and (C) 0.17. The equilibrium constants calculated according to the procedure outlined under Experimental Procedures are (A) 3×10^{17}, (B) 1×10^{17}, and (C) 2×10^{17} M^{-5}.

It has always been assumed (Himes & Rabinowitz, 1962a,b; Welch et al., 1971; Harmony et al., 1974; Harmony & Himes, 1975) that fully active tetramers had a specific activity of 400 ± 20 μmol min^{-1} (mg of protein)$^{-1}$, and previous reassociation studies have used this value as the end point of the reaction (Harmony et al., 1974; Harmony & Himes, 1975). According to those studies, the percent reactivation depends on the ionic strength and the concentration of the cation: As an example, in 30 mM NH_4Cl, at 0.1 M ionic strength, the percent reactivation was 90–100% complete. It was therefore surprising that, as judged by our light-scattering data, reassociations in 38 mM NH_4Cl were far from complete (Figure 1B,C). The value of the equilibrium constants calculated from the light-scattering measurements [$(1-7) \times 10^{17}$ M^{-5}, see legend to Figure 1C for data from some specific experiments] were generally reasonably close to the value of $(2-4) \times 10^{17}$ M^{-5} determined by Harmony & Himes (1975) and Harmony et al. (1975) from their measurements of enzyme activity. In contrast, the equilibrium constants based on our enzymic activity data varied over a wide range 4×10^{17}–2×10^{20} M^{-5} (see Figure 1A). Some equilibrium constants could not be calculated because the specific activity (based on total protein) exceeded the theoretical maximum of 400 μmol min^{-1} mg^{-1}.

It was our desire to test for the accumulation of active or inactive intermediates during the reassociation process. To do so we devised a simple function. Assuming tetramers to be the only active conformation and that all tetramers are active, we could calculate the specific activity of tetramers at any point in the reassociation experiment by dividing the specific activity by the fraction tetramer (SA_t/X_t). Further, we assumed for this calculation that only monomers and tetramers existed in any appreciable quantity—so that the increase in turbidity was due solely to tetramer formation. This assumption may be valid at the equilibrium point when ultracentrifugal analysis indicates that only tetramers and monomers are present (Himes & Harmony, 1973). It was our original intention that we could test for accumulation of intermediates by any deviation from the 400 μmol min^{-1} mg^{-1} of the specific activity per fraction tetramer. As hinted at above and discussed later, the situation is more complex because of the uncertainty of the value of the specific activity of the tetramers. Parts A and B of Figure 2 show the graph of the specific activity divided by the fraction tetramer as a function of time. A common feature of all the curves is the appearance of a maximum and the gradual decay to an equilibrium value. A horizontal line would be expected if tetramers were the only catalytically active species in solution and all tetramers had the same enzymic activity. In the presence of 6.8 mM NH_4Cl the maximum is larger than in the other reassociations, but the final value is comparable to the others. This high maximum could, in part, be due to errors in measuring the relatively low turbidities observed during the early part of the reaction; however, the consistent presence of these maxima strongly suggests the accumulation of active intermediates. Since the reassociation proceeded slowly in 6.8 mM NH_4Cl, it is plausible that the accumulation of intermediates under these circumstances is enhanced. In a similar experiment (6.8 mM NH_4Cl) done in the presence of dithiothreitol in place of 2-mercaptoethanol, results were essentially identical: a high specific activity maximum was observed, followed by the usual gradual decrease of the activity to the equilibrium value. Some time ago Harmony & Himes (1975) proposed that the reassociation had to proceed through a dimer intermediate to explain their kinetic data [these investigators determined that the reassociation is second order in monomer concentration (Harmony et al., 1974)]. It is possible that the dimer is catalytically active, although at present the nature of the intermediate suggested by the data of Figure 2 is not known. To the best of our knowledge, this is the first indication of the existence of catalytically active intermediates in the reassociation of formyltetrahydrofolate synthetase.

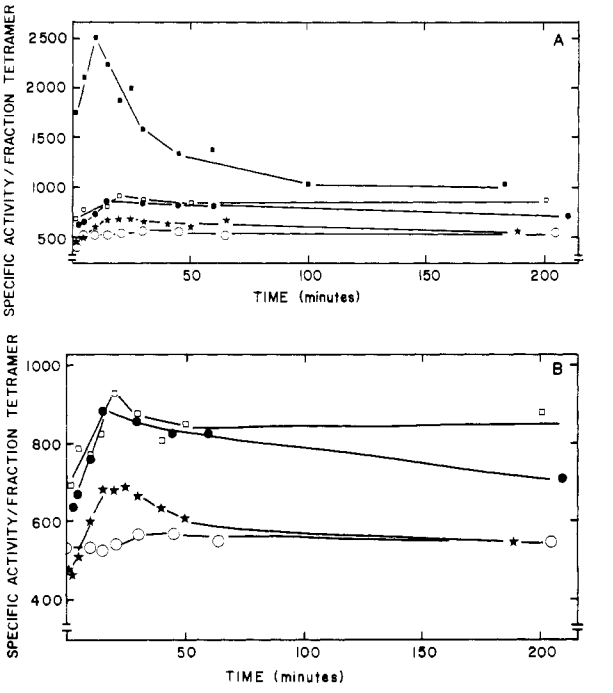

FIGURE 2: (A) Reassociation of formyltetrahydrofolate synthetase: specific activity per fraction tetramer. Data were calculated as described in the text. Experiments were conducted as described under Experimental Procedures. The NH_4Cl concentrations, ionic strength, and protein concentration during reassociation are as follows: (●) 38 mM, 0.105 M, 1.34 mg/mL; (○) 23 mM, 0.0902 M, 0.91 mg/mL; (★) 21.7 mM, 0.089 M, 0.88 mg/mL; (□) 9.9 mM, 0.0771 M, 1.06 mg/mL; (■) 6.8 mM, 0.074 M, 1.13 mg/mL. The lines are intended as a guide to the eye. The standard deviations were calculated and found not to exceed ±5% of the nominal value. (B) Enlargement of (A) to show detail. The lines are intended as a guide to the eye.

Harmony et al. (1974) reported that sulfate promotes the reassociation of the monomer. We also saw the same effect measured as either the return of activity or increase in turbidity. While the rate of association (measured as either turbidity or enzymic activity) increased with increased ionic strength, measurements at equal ionic strength of Cl^- and SO_4^{2-} showed SO_4^{2-} to be dramatically more effective at promoting both reassociation and reactivation. At high ionic strength (both in chloride and in sulfate) the variation of specific activity per fraction tetramer (SA_t/X_t) with time (Figure 3) was considerably different than that seen at low ionic strength, even though the NH_4^+ concentration was identical (6.8 mM). The value of this function increased rapidly from low values and leveled out with no hint of a hump. The results suggest that at high ionic strength, recovery of activity may lag behind the physical association of the protein.

Effect of Centrifugation on Reassociation. Because of the high values obtained for SA_t/X_t even at the end of the experiment (i.e., the reassociation appeared to be complete if judged by specific activity, but not by turbidity), it was important to obtain an independent measure of the fraction tetramer at the end of the reaction period. The composition of the reassociated protein solution determined from sedimentation velocity data agreed well with that determined from turbidity data as long as the protein was at least 75% tetramer. Table I lists some reassociation experiments which were an-

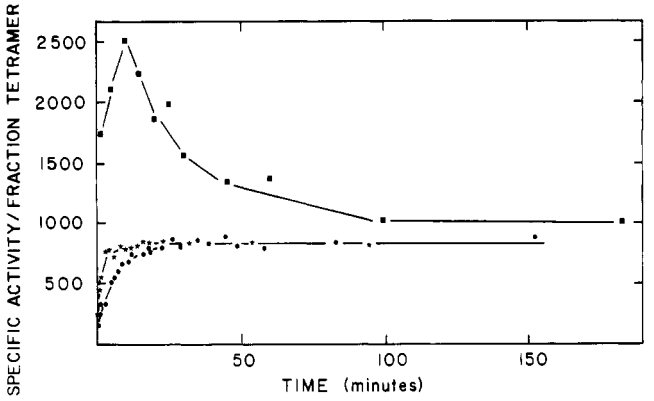

FIGURE 3: Reassociation of formyltetrahydrofolate synthetase: specific activity per fraction tetramer. Experiments as in Figure 2A. All reassociations were conducted in 6.8 mM NH_4^+. Top curve (■): Reassociation was initiated by addition of NH_4Cl; final ionic strength was 0.074 M. Middle curve (★): The solution in the light-scattering cuvette was made 60 mM in Na_2SO_4. The reassociation was initiated by addition of $(NH_4)_2SO_4$ to give a final NH_4^+ concentration of 6.8 mM. The protein concentration during reassociation was 1.04 mg/mL, and the ionic strength was 0.22 M. Bottom curve (●): Reassociation was initiated by addition of NH_4Cl (6.8 mM final NH_4^+ concentration) after sufficient NaCl was added to bring the final ionic strength to 0.23 M. The protein concentration was 0.99 mg/mL.

Table I: Fraction Tetramer and Specific Activity per Fraction Tetramer[a]

	light scattering		ultracentrifugation	
expt	fraction tetramer	SA/X[b]	fraction tetramer	SA/X[c]
A	0.97	439	1.00[d]	415
B	0.76	482	0.72	509
C	0.74	601	0.80	587
D	0.51	831	0.76	565
E[e]	0.31	829	0.57	524
F	0.21	896	0.61	547

[a] Reassociation experiments were performed as described under Experimental Procedures except as noted. Reassociation was induced by the addition of the following salts at the indicated final concentrations and ionic strengths: A, 20 mM $(NH_4)_2SO_4$, μ = 0.22 M; B, 4.9 mM $(NH_4)_2SO_4$, μ = 0.22 M; C, 4.8 mM $(NH_4)_2SO_4$, μ = 0.22 M; D, 3.0 mM $(NH_4)_2SO_4$, μ = 0.22 M; E, 6.8 mM NH_4Cl, μ = 0.74 M; F, 6.0 mM NH_4Cl, μ = 0.23 M. [b] Specific activity per fraction tetramer at infinite time. The fraction tetramer was calculated from light-scattering data. [c] Specific activity per fraction tetramer. The fraction tetramer was calculated from sedimentation velocity data using absorption optics. Specific activity was measured immediately before sedimentation velocity except in experiments E and F in which cases it was measured immediately afterward. [d] Sedimentation velocity using Rayleigh optics. [e] Reassociation in the presence of 10 mM DTT in place of 0.1 M 2-mercaptoethanol.

alyzed by sedimentation velocity after light scattering. In those cases in which the reassociation had converted less than 75% of the monomers to tetramer (as judged by light scattering), further reassociation was observed in the ultracentrifuge. In the cases of experiments E and F the activity of the protein was determined after centrifugation and found to have increased substantially during the run. For example, in experiment F the activity of the protein before the run was 190 μmol min^{-1} mg^{-1}. After centrifugation, it had increased to 299 μmol min^{-1} mg^{-1} whereas a noncentrifuged control, assayed at the same time, had an activity of 206 μmol min^{-1} mg^{-1}. It should be pointed out that the specific activity per fraction tetramer obtained from sedimentation velocity data (Table I), although generally higher than 400 μmol min^{-1} mg^{-1}, was consistently lower than the value obtained from the light-

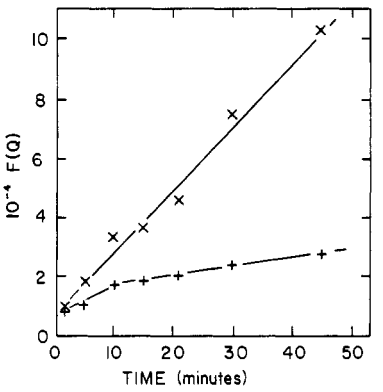

FIGURE 4: Reversible second-order rate plot for the reassociation of formyltetrahydrofolate synthetase in the presence of 38 mM NH_4Cl. See Experimental Procedures for the meaning and calculation of $F(Q)$. Upper curve (×): Data derived from measurements of enzyme activity. Lower curve (+): Data derived from light-scattering measurements.

scattering data. This is particularly true when less than 50% of the protein had been reconverted to tetramers (as judged by light scattering). The protein underwent further reassociation in the ultracentrifuge, but the specific activity per fraction tetramer decreased considerably. In analogous experiments, conducted in the absence of an activating cation, ultracentrifugation did not promote reassociation of partially dissociated synthetase (data not shown). Centrifugation-induced association is not unknown; for example, Gethner et al. (1977a,b) have reported the reassociation of tubulin in the preparative ultracentrifuge when spun at 35000 rpm for 1 h.

Rate Constants. Because of the complications outlined above, we have not yet performed a controlled systematic determination of rate constants under a variety of ionic and temperature conditions. The reassociation data were replotted according to the kinetic equations shown under Experimental Procedures. The reversible second-order rate equation gave the best fit under all conditions. Under certain reaction conditions, the irreversible second-order equation fit the data nearly as well. A typical plot is shown in Figure 4. Our data are therefore consistent with Harmony & Himes (1975) that the reassociation is second order in monomer concentration. Note that the reassociation as measured by enzymic activity gave a straight line throughout the experimental period but the light-scattering data gave a broken line. All experiments gave this general result; however, the actual position of the break varied between 5 and 20 min after addition of the NH_4^+. The data plotted according to the irreversible second-order rate equation had breaks at the same position. The data plotted according to the first-order rate equations gave two or more breaks. The reason for the break seen in Figure 4 may simply be the inadequacy of the reversible second-order rate equation to account for the light-scattering data; alternatively, there may be an actual change in the rate-limiting step of reassociation. In any case, it is clear that light scattering and enzymic activity are monitoring different events within the reactivation process. The rate constants from Figure 4 are 2100 M^{-1} min^{-1} (enzymic activity) and 1100 and 260 M^{-1} min^{-1} (light scattering). The rate constant derived from the enzymic activity data is questionable, as its value is sensitive to the value chosen for the specific activity of the fully reassociated tetramer. Rate constants based on light scattering presumably do not have this ambiguity. It must be kept in mind that these are apparent rate constants (see Experimental Procedures) and are expected to (and do) vary with cation concentration. Harmony & Himes (1975) report a rate of 800 M^{-1} s^{-1} (40 mM KCl; estimated from Figure 2 of their paper).

We replotted our data by using the equation employed by Harmony and Himes and obtained a value of 2250 M^{-1} min^{-1} in good agreement with the value obtained from our irreversible second-order rate equation and in good agreement with the value (after adjusting for the difference in units) indicated by Harmony et al. (1974, Figure 4). The magnitude of the discrepancy is larger than expected due to the higher ionic strength used by Harmony & Himes (1975). For comparison with another tetramer, Rudolph et al. report a rate constant of (1–4) × 10^4 M^{-1} s^{-1} for the reassociation of lactic dehydrogenase (Rudolph & Jaenicke, 1976; Rudolph et al., 1979).

Discussion

The monovalent cation induced reassociation of formyltetrahydrofolate synthetase monomers was studied extensively (MacKenzie & Rabinowitz, 1971; Harmony et al., 1974, 1975; Harmony & Himes, 1975) using the return of activity as a criterion for reassociation. In all their studies the specific activity of fully active, fully tetrameric enzyme was considered to be 410 μmol min^{-1} mg^{-1}. In this work, the reassociation reaction was studied by a method which is independent of enzymic activity measurements. The light-scattering studies reported here show hitherto unreported properties of formyltetrahydrofolate synthetase. Among these are (a) that centrifugation has a strong influence on the reassociation process, (b) the existence of tetramers of specific activity significantly greater than 410 μmol min^{-1} mg^{-1}, and (c) the accumulation of intermediates during the monovalent cation induced reassociation of monomers. Light-scattering measurements are also clearly in agreement with the previously reported association of four monomers in the presence of certain monovalent cations to form a tetrameric enzyme.

Effect of Centrifugation. Under conditions of low-activating cation concentration, where relatively large amounts of monomer remain, ultracentrifugation promotes the reassociation process. Centrifugation appears to have little effect when over 75% of the monomer has been converted to tetramer prior to ultracentrifugation. It is clear that ultracentrifuge studies performed here and in other laboratories contain a bias toward reassociation. The effect of centrifugation may be due to the effects of hydrostatic pressure or the separation of monomer and tetramer. Further experiments are required to resolve these possibilities.

High Specific Activity. Surprisingly, the specific activity of the tetramer produced in the light-scattering experiments is much higher than 410 μmol min^{-1} mg^{-1}. The existence of these high activity tetramers is also supported by analytical ultracentrifugation of reassociated enzyme, although the effect of centrifugation itself slightly complicates the correlation. The existence of high specific activity tetramers is also indicated by the data of Scott & Rabinowitz (1967) and MacKenzie & Rabinowitz (1971, Figure 6).

Light scattering, as measured on the Phoenix-Brice apparatus, can only yield the weight average of all species in solution. In our analysis of the turbidity we assumed that only tetramers and monomers were present in significant amounts at any time; the assumption was based on analytical ultracentrifugation of partially reassociated enzyme, both here and previously reported (Scott & Rabinowitz, 1967). However, if catalytically active dimers or trimers are found in appreciable amounts, our analysis would ignore them, and the results would be interpreted as high specific activity tetramers. The fact that dimers or trimers have not been found may simply be an artifact: the conditions during sedimentation velocity experiments may not favor their existence.

The presence of tetramers of low specific activity is known. For instance, crystalline enzyme gradually loses activity upon storage (Garrison et al., 1976; Welch, 1969). When these crystals are dissolved and centrifuged, only tetramers are found; the specific activity remains low. The loss of activity appears to be reversible, however. After dissociation of the low specific activity enzyme by removing monovalent cation, we can routinely restore full activity by reassociation with NH_4Cl or $(NH_4)_2SO_4$. Buttlaire et al. (1972) have reported the reassociation of the synthetase to yield low specific activity tetramers. It would appear that reassociation and reactivation are not reciprocally linked phenomena.

Intermediates in the Reassociation Mechanism. The specific activity at a particular time was divided by the fraction tetramer at that time and the result plotted as a function of time to test for the presence of any intermediates during the reassociation. If the values of the specific activity were lower than 410 μmol min^{-1} mg^{-1}, the formation of an inactive intermediate would be indicated. Conversely, if the values were higher than 410 μmol min^{-1} mg^{-1}, an active intermediate would be indicated. The results indicate the presence of an active intermediate which is best seen at low ionic strength and low ammonium ion concentration. Under these conditions the overall reassociation reaction proceeded slowly and the accumulation of the transient species was enhanced. In the presence of chloride at high ionic strength or in the presence of sulfate, the reaction was much faster, and this intermediate was not observed. In fact, when the overall reassociation rate was fast, inactive intermediates seemed to accumulate early in the reaction.

The variable specific activities based on the fraction tetramer observed make the analysis of the data complex. It is not possible to determine the nature of the intermediate without additional experiments. Several possibilities exist; for example, monomers could become active upon binding of the monovalent cation, and the dimers themselves may be active or the tetramers may have a very high specific activity immediately after formation which gradually decays to the equilibrium value. The second-order dependence of reassociation rate reported by Harmony et al. (1974) and in this paper tends to eliminate the first possibility. In support of the last idea, A. Guida and W. Welch (unpublished results) observed that high specific activity tetramers (ca. 500 μmol min^{-1} mg^{-1}) produced by reassociation in the presence of ammonium ion gradually lost their high activity over a period of a few days and fell to a constant value of approximately 400 μmol min^{-1} mg^{-1}. The formation of tetramers of higher than expected specific activity is also supported by a comparison of the equilibrium constants from the present data obtained with NH_4Cl and those of Harmony & Himes (1975) and Harmony et al. (1975) using KCl. Equilibrium constants obtained from light-scattering data were in reasonable agreement with the data of Harmony (Harmony & Himes, 1975; Harmony et al., 1975) which are based on enzymic activity. Our equilibrium constants based on the enzyme activity and calculated in the same manner as those of Harmony were as much as 2 orders of magnitude greater than those reported by Harmony. It would appear, therefore, that the physical reassociation is proceeding as described by Harmony but that we are obtaining protein of remarkably higher enzymic activity. The comparison of apparent equilibrium constants does not, of course, rule out the accumulation of enzymically active dimers.

Earlier work showed a correlation between changes in A_{291} and reactivation (Welch et al., 1971). The spectral change was interpreted as a shift of aromatic residues to a less polar

environment as the enzyme reactivated. A similar result has been obtained for the *C. acidi-urici* enzyme (Harmony et al., 1974). On the basis of the present data, the aromatic residues may indeed monitor formation of the active site but not directly the association of the monomer units. It would be interesting to monitor simultaneously changes in A_{291} and turbidity.

Jaenicke (1979) and Zettlmeissl et al. (1979) using lactate dehydrogenase demonstrated multiple reassociation pathways for multiple-subunit enzymes. In their case, reassociation involved refolding of denatured subunits and formation of enzymically inactive aggregates. In this work the dissociation of formyltetrahydrofolate synthetase does not involve any gross conformational change of the subunits (Welch et al., 1971) or formation of inactive aggregates, but we feel the principle could possibly be the same: Reassociation to form active tetramers may involve a complicated or even branched pathway and the existence of tetramers of differing specific activities. The ready reversibility of the process makes the clostridial formyltetrahydrofolate synthetase system advantageous for the study of structure–function relationships in proteins.

This report is consistent with the earlier work on the monovalent cation mediated reassociation of formyltetrahydrofolate synthetase and extends the observations by monitoring the quaternary conformation directly. It is apparent that the reassociation process is considerably more complex than suggested by the monitoring of enzymic activity alone. We feel that quantitative values are premature based on the relatively limited number of experiments presented here; however, the qualitative results appear to be clear. The most significant findings demonstrate the presence of enzyme forms of extremely high specific activity. The nature of these forms and their relationship to the conformation and catalytic activity of the enzyme are currently under study.

Acknowledgments

We thank Professor Richard H. Himes for generous gifts of lyophilized *C. cylindrosporum* and helpful discussions, the Chemistry Department of the University of Nevada for kindly allowing us to use and modify their light-scattering apparatus, and Sherry Stevens and Suzanne Carlton for their help in preparation of the manuscript.

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