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Biochemistry

Dihydrofolate reductase: thymidylate synthase, a bifunctional polypeptide from *Crithidia fasciculata*

(affinity chromatography/folate metabolism/protozoal enzymes)

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ABSTRACT **The molecular weight of dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) from protozoa has been reported to be 5- to 10-fold larger than the isofunctional enzyme of most other organisms studied, based on gel filtration. This enzyme from the protozoal flagellate *Crithidia fasciculata* has been purified to homogeneity and found to be a bifunctional protein with thymidylate synthase (5,10-methylene tetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45) activity. The purified protein, eluted from methotrexate-Sepharose columns by dihydrofolate, migrated as a single band on both nondenaturing and denaturing polyacrylamide gel electrophoresis. The monomer M_r is 56,700 ± 200. The native M_r was calculated to be 107,000 from a sedimentation coefficient of 5.9 and Stokes radius of 4.4 nm. Dihydrofolate reductase and thymidylate synthase activities of the rodent malaria organism *Plasmodium berghei* also copurified on Sephadex G-200 and methotrexate-Sepharose columns, suggesting that this unique bifunctional protein might occur throughout the Protozoa.**

Dihydrofolate (H_2folate) reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) is isolated from most organisms as a protein of M_r 18,000–22,000 (1). However, the apparent M_rs of the H_2folate reductases from certain parasite protozoans [malaria (2), trypanosomes (3), crithidia (3), and coccidia (4)] have been estimated to be 100,000–240,000, by gel filtration. Similar observations have been made for the enzyme from free-living protozoa (*Tetrahymena pyriformis*, *Euglena gracilis*, and *Acanthamoeba castellani*) (unpublished data). Thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45) is an enzyme closely linked in function to H_2folate reductase; the H_2folate produced from the thymidylate synthase reaction must be reduced to regenerate the tetrahydrofolate required for folate cofactor-requiring enzymes. Thymidylate synthase from bacterial and mammalian sources is usually found as a dimer of native $M_r \approx$ 60,000–70,000 (5); the enzyme from three parasite protozoa was found to be much larger on Sephadex columns [*Plasmodium berghei*, $M_r >$ 100,000 (6), *Crithidia fasciculata*, M_r = 175,000; *Trypanosoma cruzi*, M_r = 200,000 (7)]. Walter and Konigk (8) found that the levels of H_2folate reductase and thymidylate synthase in *Plasmodium chabaudi* varied in parallel during the course of a synchronous infection.

These reports suggested to us a possible association of H_2folate reductase and thymidylate synthase in the Protozoa. The results of this study demonstrate that the two enzyme activities in *C. fasciculata* are on a single polypeptide of M_r = 56,700 ± 200, which may exist as a dimer in the native state.

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MATERIALS AND METHODS

H_2folate reductase activity was determined by the spectrophotometric assay of Baccanari *et al.* (9), in 50 mM Na,K phosphate buffer (pH 7.0) and with the addition of bovine serum albumin (1 mg/ml) for the purified enzyme preparations.

Thymidylate synthase activity was determined by the tritium release assay as used by Al Chalabi and Gutteridge (7). The 200 μl reaction systems contained: 50 mM Tris·HCl (pH 7.8) (37°C), 5 mM formaldehyde, 1 mM H_4folate, 50 mM 2-mercaptoethanol, 0.1 mM [5-^{3}H]dUMP (8–10 × 10^3 cpm/nmol), and enzyme. Bovine serum albumin (1 mg/ml) was added when the purified enzyme was assayed. After incubation at 37°C (usually for 30 min), the reaction was terminated by the addition of 0.5 ml of Norit A slurry (7), and 0.25 ml of the supernatant resulting from centrifugation for 15 min at 10,000 × *g* was mixed with 3 ml of Aquasol-2 (New England Nuclear) for determination of radioactivity. For both enzymes, 1 unit of activity is equal to the production of 1 nmol of product per min.

C. fasciculata (ATCC no. 11745) was grown in an undefined liver infusion/proteose peptone medium (10) for 2½ days at 26–28°C, to a cell density of 2–5 × 10^7 organisms per ml. Cells were collected by centrifugation, washed twice in cold Krebs-Ringer glucose solution, and resuspended to a cell density of 6–9 × 10^9/ml in 50 mM Tris·HCl, pH 8.4 (5°C)/1 mM EDTA/1 mM dithiothreitol. In the later experiments with larger volumes of medium (12–20 liters), 10 μM phenylmethylsulfonyl fluoride was included as an inhibitor of proteolytic enzymes. Cells were disrupted by passage twice through a French pressure cell at 18 × 10^3 psi (125 mPa), and the supernatant obtained after centrifugation at 100,000 × *g* for 60 min was taken as the crude extract.

Plasmodium berghei (NYU-2 strain) was grown, harvested, and extracted as described (11).

Sephadex G-200 columns were run in either 0.1 M Na,K phosphate, pH 7.0/1 mM dithiothreitol/1 mM EDTA (buffer A) or 50 mM Tris·HCl, pH 7.8 (5°C)/1 mM EDTA. The columns were calibrated with blue dextran ($M_r \approx 2 \times 10^6$), beef liver catalase ($M_r$ 232,000), *Escherichia coli* alkaline phosphatase (M_r 86,000), ovalbumin (M_r 45,000), and horse muscle myoglobin (M_r 17,800).

NaDodSO$_4$/polyacrylamide gel electrophoresis was run on gel slabs by the method of Laemmli (12), and stained with Coomassie blue (13). Samples were boiled for 5 min in upper gel buffer containing 2% NaDodSO$_4$/5% sucrose/1.25 M 2-mercaptoethanol. The Pharmacia low molecular weight calibration kit was used for standards, along with catalase (subunit

Abbreviations: H_2folate, 7,8-dihydrofolate; methylene H_4folate, N^5,N^{10}-methylenetetrahydrofolate; FdUMP, 5-fluoro-2′-deoxy-5′-uridine monophosphate.

M_r = 58,000) and bovine liver L-glutamic dehydrogenase (subunit M_r = 53,000).

Sucrose density gradients (5–20% sucrose in 0.1 M Na,K phosphate buffer, pH 7.0/1 mM dithiothreitol) were centrifuged in a Beckman SW 50.1 rotor with a Sorvall OTD 65 ultracentrifuge for 17 hr at 38,000 rpm. Fractions (3 drops each) were collected from the bottom of each tube and assayed for enzyme activity. Standard proteins were *E. coli* alkaline phosphatase, yeast alcohol dehydrogenase, and beef liver catalase (S = 6.4, 7.4, and 11.3, respectively). In one experiment a crude extract of crithidia was run in a Beckman SW 27 rotor in a Beckman model L3-40 ultracentrifuge for 72 hr at 27,000 rpm, with alkaline phosphatase and catalase as standards. The S values were calculated by the method of Martin and Ames (14).

Crude extract (55 ml) from the harvest of cells from 12 liters of medium was loaded onto a 5 × 30 cm column of DEAE-cellulose (Whatman DE-52) that had been equilibrated with 50 mM Tris·HCl, pH 8.7 (5°C)/1 mM dithiothreitol/1 mM EDTA. After washing with 1 liter of starting buffer at 1 ml/min, a gradient of 1250 ml of starting buffer and 1250 ml of starting buffer containing 0.25 M KCl was applied. H_2folate reductase and thymidylate synthase coeluted in a symmetrical peak at approximately 0.12 M KCl. Active fractions were pooled (252 ml) and dialyzed against three changes (2 liters each) of 50 mM KPO_4, pH 6.4/1 mM EDTA/1 mM dithiothreitol. Two-thirds of this material was mixed overnight with 4 ml of methotrexate-Sepharose affinity resin, supplied by D. Baccanari of these laboratories (9). The mixture was then packed into a column and washed with the dialysis buffer at 48 ml/hr until A_{280} was consistently <0.05. Additional protein was removed by washing with 50 mM Tris·HCl, pH 8.3 (5°C)/1 mM EDTA/1 mM dithiothreitol/1 M KCl. After the A_{280} was consistently <0.025, a 30-ml gradient of 0–1.4 mM H_2folate was applied in the same buffer. Both enzyme activities were eluted at <0.5 mM H_2folate. The peak fractions were pooled, concentrated 7-fold, and dialyzed against 500 vol of 50 mM Tris·HCl, pH 8.7 (5°C)/1 mM dithiothreitol/1 mM EDTA by repeated concentration and dilutions in an Amicon dialysis cell with a YM-10 membrane.

Nondenaturing polyacrylamide slab gel electrophoresis was run in the buffers described by Laemmli (12), with 7% polyacrylamide. Proteins were stained with Coomassie blue (13) or sliced into 2-mm sections and eluted overnight into 250 μl of buffer A and bovine serum albumin (1 mg/ml) for assay of H_2folate reductase and thymidylate synthase. Protein concentrations were determined with Coomassie blue (15) or the Lowry *et al.* (16) method as modified by Bensadoun and Weinstein (17), to eliminate interference by Tris and dithiothreitol. Protein concentrations for crithidial preparations were 10% higher when the modified Lowry method was used, compared to the Coomassie method, with bovine serum albumin as standard for both.

Tris base, Tris·HCl, *dl*-L-H_4folate, 5-fluoro-2′-deoxyuridylate (FdUMP), dithiothreitol, Na_4 EDTA, NADPH, enzymes for standards, bovine serum albumin, and dUMP were purchased from Sigma. Sephadex G-200 and the low molecular weight standards electrophoresis kit were from Pharmacia. 2′-Deoxy[5-^{3}H]uridylate was bought from Amersham/Searle, diluted to a specific activity of ≃5 Ci/mol, and purified on a charcoal/cellulose column (18). H_2folate (19) was stored in 5 mM HCl at −70°C. Pyrimethamine and trimethoprim were synthesized at Burroughs Wellcome.

RESULTS

The study was carried out with *C. fasciculata*, a protozoan easily cultured in large batches, which had been shown to possess a high molecular weight H_2folate reductase (3). H_2folate reductase and thymidylate synthase from this organism were eluted from Sephadex G-200 columns in a constant ratio. The apparent M_rs of the enzymes were 150,000–170,000 from calibrated columns. These two enzymes also were not separated when crude extracts were run on DEAE-cellulose columns (Fig. 1).

The coelution of H_2folate reductase and thymidylate synthase from columns that separate proteins on the basis of size and of charge indicated a probable association of these enzymes but provided no evidence on the nature of the type of bonds involved. It was thought that a noncovalent association could be revealed by binding the crithidial H_2folate reductase to a

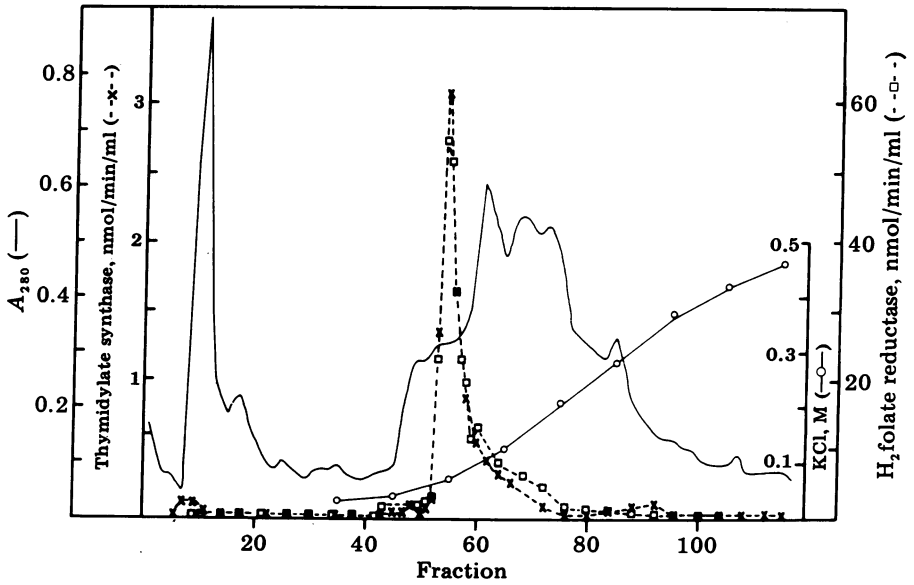

FIG. 1. DEAE-cellulose chromatography of H_2folate reductase and thymidylate synthase from *C. fasciculata*. A crude extract was incubated 60 min at 5°C with 10 mM $MgCl_2$, bovine DNase I (0.07 mg/ml), and bovine RNase A (0.07 mg/ml) and centrifuged at 100,000 × *g* for 60 min. The supernatant was dialyzed overnight against 500 vol of 50 mM Tris·HCl, pH 8.4 (5°C)/1 mM EDTA, and 7.7 ml of the extract was applied to a 1.5 × 30 cm column of DE-52 equilibrated in the same buffer. After a wash with 130 ml of starting buffer, a gradient of 0–500 mM KCl in buffer (500 ml) was run.

Table 1. Purification of H_2folate reductase and thymidylate synthase

Fraction	Total volume, ml	Total protein, mg	Thymidylate synthase Units	Thymidylate synthase Units/mg	H_2folate reductase Units	H_2folate reductase Units/mg
Crude extract	55	1161	2277	2.0	22,060	19
DE-52 pool, dialyzed	252	82.9	1084	13.1	16,590	200
MTX-Sepharose pool, concentrated*	2.0	0.666	310	465	7,642	11,474

MTX, methotrexate.
* The actual values measured were one-third less for this fraction because only two-thirds of the DE-52 pool was used in this step.

methotrexate-Sepharose affinity gel and eluting those proteins not specifically bound by using a high-salt wash. Thymidylate synthase from several mammalian and bacterial sources can bind significantly to folate analog-affinity columns only in the presence of dUMP (20–22). The results with thymidylate synthase and H_2folate reductase from *C. fasciculata* were strikingly different; both enzymes bound to the affinity resin, remained bound through a wash containing 1 M KCl which removed most extraneous proteins, and were eluted together by H_2folate (Table 1; Fig. 2). These same results were observed with crude extracts or with preparations partially purified by Sephadex G-200 or by DEAE-cellulose column chromatography. Yields from the affinity column were 47.9 ± 5.9% (mean ± SEM; $n = 5$) for H_2folate reductase and 39.9 ± 7.8% ($n = 5$) for thymidylate synthase. The enzyme preparations from the affinity columns were most stable after dialysis and concentration when stored at 5°C in buffer A containing 1 mg bovine serum albumin per ml.

Purified protein was analyzed for purity by polyacrylamide gel electrophoresis with and without $NaDodSO_4$. A single band of protein was found by both methods (Fig. 3). On nondenaturing gels, the protein staining band corresponded to the gel slices that demonstrated both enzymatic activities (Fig. 3A). The mean (±SEM) monomer M_r, as determined on 7%, 8.6%, 10%, and 12% $NaDodSO_4$ acrylamide gels, was 56,700 ± 200 ($n = 5$). These results demonstrate that the crithidial H_2folate reductase and thymidylate synthase activities reside on a single polypeptide chain—i.e., that it is a bifunctional protein.

The apparent M_r of 150,000 for the crithidial enzyme is based on gel filtration data, a method that does not actually determine M_r but rather the effective radius of a molecule (23). The Stokes radius determined from the calibrated Sephadex G-200 columns was 4.4 nm. From the sucrose density gradient centrifugation experiments, an S value of 5.9 was determined for the purified enzyme (S = 5.7 for the enzyme in a crude extract). A M_r of 107,000 was calculated, with a frictional coefficient of 1.43, by the method of Siegel and Monty (23). This M_r is almost twice that of the monomer.

Bovine serum albumin (1 mg/ml) stabilized both enzyme activities during storage of purified preparations of 5°C. Bovine serum albumin also was required in the reaction mixtures for both activities to obtain a linear response of reaction rate to enzyme concentration. The turnover number of the H_2folate reductase activity, from Ackerman–Potter plots (24) with methotrexate at pH 6.3, was 1280 mol of H_2folate reduced per min per mol of methotrexate bound. In two separately purified enzyme preparations, the data from Ackerman–Potter plots indicated only ≈0.5 mol (0.46, 0.51) of methotrexate bound per 57,000 M_r subunit, suggesting that there was 1 mol of methotrexate bound per dimer. The apparent K_m values determined for thymidylate synthase were 3.3 μM for dUMP and 400 μM for *dl*-methylene H_4folate; those for H_2folate reductase were 6.7 μM for NADPH and <2 μM for H_2folate. The 50% inhibitory concentrations of standard inhibitors were in the expected ranges for each inhibitor, based on published data (3, 7, 10)—thymidylate synthase: FdUMP (preincubated 15 min in reaction mixtures without dUMP), 5 nM; FdUMP (not preincubated), 27 nM; methotrexate, 27% inhibition at 100 μM and H_2folate reductase: pyrimethamine, 6.6 μM; trimethoprim, 15 μM; methotrexate, ≈1 nM (dependent on enzyme concentra-

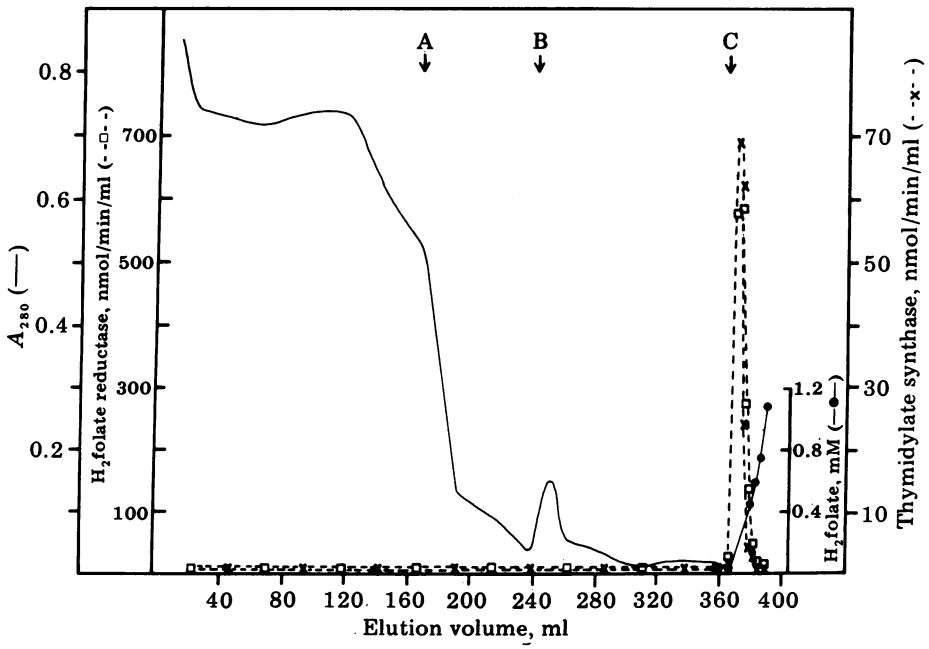

FIG. 2. Methotrexate-Sepharose affinity chromatography of an enzyme preparation from *C. fasciculata* pooled from a DE-52 column. At arrow A, the column was washed with equilibration buffer at pH 6.4; at arrow B, high pH/high-salt buffer was applied; at arrow C the H_2folate gradient was started.

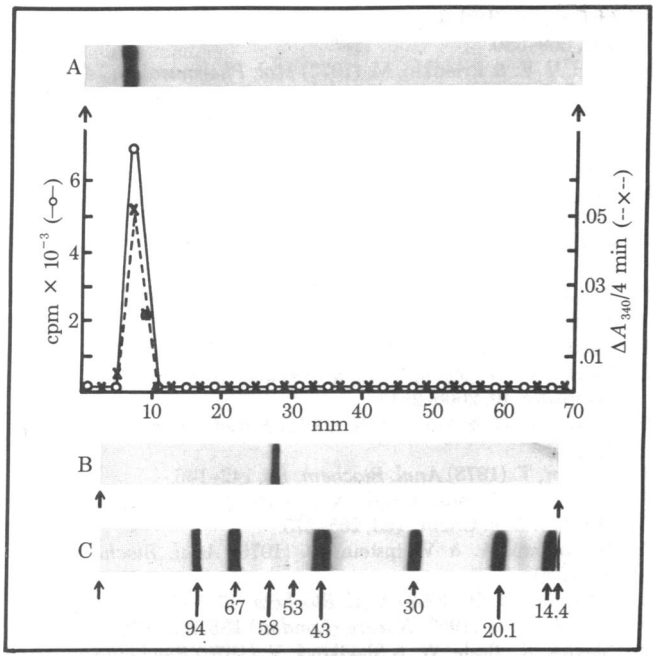

FIG. 3. Slab gel electrophoresis of purified H_2folate reductase and thymidylate synthase from *C. fasciculata*. (*A*) Stained gel and plot of enzyme activities of slices; 7% polyacrylamide gel electrophoreses of 6.2 μg of purified protein. o, Thymidylate synthase activity; x, H_2folate reductase activity. (*B*) Purified protein (4.1 μg) on NaDodSO$_4$/7% polyacrylamide gel electrophoresis. (*C*) M_r markers (×10^{-3}) run on the same gel slab as B; "58" and "53" represent the positions of the catalase subunit and L-glutamic dehydrogenase subunit, run on adjoining slots on the gel. The unlabeled arrows indicate the top (left) and the bottom (right) of each gel.

tion). Pyrimethamine, trimethoprim, and folate, each with and without NADPH, and H_2folate, all at 100 μM, did not inhibit thymidylate synthase activity. Similarly, dUMP, dTMP, FdUMP, methylene H_4folate, and FdUMP plus methylene H_4folate, all at 100 μM, did not affect the H_2folate reductase activity in the standard assay.

Fig. 4 shows the coelution of thymidylate synthase and H_2folate reductase of *P. berghei* from a Sephadex G-200 column. In this experiment, the apparent M_r was estimated as 150,000. Previous runs with other *P. berghei* crude extracts sometimes resulted in asymmetrical peaks, suggesting the presence of more than one form of the combined enzyme. The active fractions from the column were pooled, mixed overnight with 1 ml of methotrexate-Sepharose, poured into a column, and washed and eluted as described for the crithidia preparation. Both enzyme activities bound and were not eluted during the low-pH or the high-pH/high-salt wash, but both were eluted by 0.6 mM H_2folate. The yields were low (15% for H_2folate reductase, 12.5% for thymidylate synthetase), and the pooled active fractions lost 50% activity upon storage at 5°C overnight.

DISCUSSION

The finding of a multimer of a polypeptide chain of M_r 57,000 which contains catalytic sites for H_2folate reductase and for thymidylate synthase explains the unusually high M_r value observed by gel filtration for the H_2folate reductase of *C. fasciculata*. The assignment of both enzymatic activities to a single protein is dictated by the finding of a single band on NaDodSO$_4$/polyacrylamide gel electrophoresis at several acrylamide concentrations. Although Sephadex G-200 filtration data indicated an apparent M_r of 150,000–170,000, calculation of the M_r from averages of the Stokes radius and of the sedimentation coefficient (23) gave a value of 107,000. The apparent discrepancy is explained by the value of 1.43 for the frictional coefficient, indicative of an asymmetrically shaped molecule. These values must remain tentative because the partial specific volume was not determined and an average value was assumed (14); however, the M_r value calculated suggests that the native protein is a dimer.

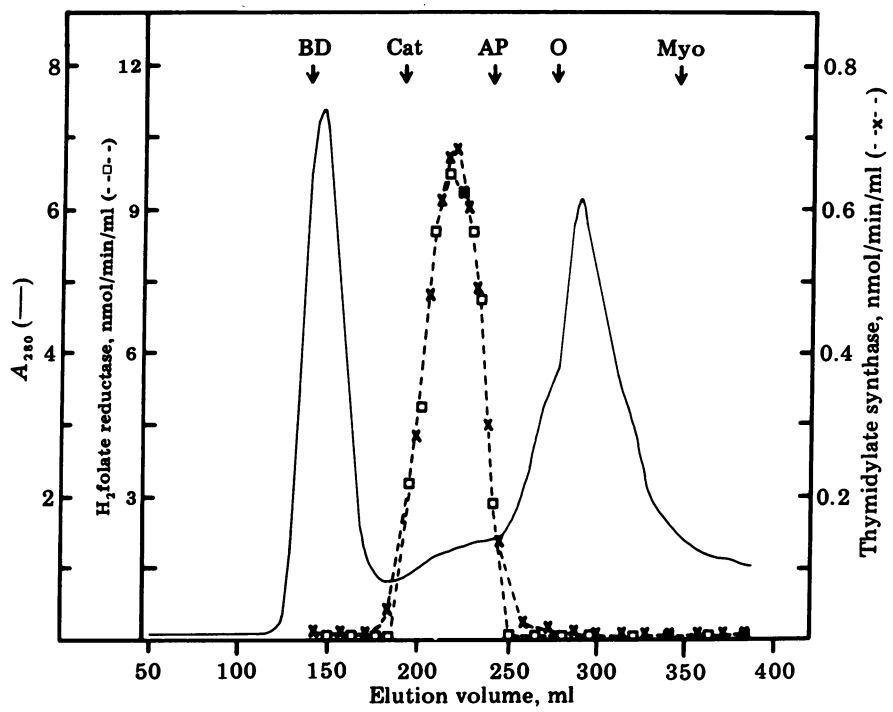

FIG. 4. Sephadex G-200 chromatography of H_2folate reductase and thymidylate synthase from *P. berghei*. A 2-fold concentrated *P. berghei* crude extract (12 ml) was chromatographed on a 2.5 × 78 cm Sephadex G-200 column, equilibrated and eluted, at 10 ml/hr, with buffer A. The arrows indicate the position of elution of markers from a separate run: BD, blue dextran; Cat, catalase; AP, alkaline phosphatase; O, ovalbumin; Myo, myoglobin.

A high M_r H_2folate reductase has been observed for all protozoal organisms studied in this regard but not in most other organisms studied, including representatives of bacteria (1), yeast (25), worms (26), insects (27), and vertebrates (1). *P. berghei* thymidylate synthase coelutes with H_2folate reductase from a Sephadex G-200 column and, most importantly, is eluted from a methotrexate-Sepharose affinity column by conditions usually specific for H_2folate reductase only. These findings lead to the hypothesis that an association of H_2folate reductase and thymidylate synthase may be found throughout the Protozoa, either through protein–protein association or through a bifunctional polypeptide. Complexes with other enzymes may also be formed in some cases, which could explain the observations of two peaks of H_2folate reductase sometimes found on Sephadex G-200 columns for some of the protozoa (ref. 3 and unpublished data). Serine hydroxymethyltransferase (EC 2.1.2.1) is an attractive candidate for inclusion in a multienzyme complex in those organisms that utilize folates mainly for the synthesis of thymidylate [e.g., malaria (2)], because it catalyzes the synthesis of the methyleneH_4folate required for the synthesis of thymidylate.

This investigation did not inquire into the functional interaction between the two enzymatic activities. The data suggest a dimer of a bifunctional polypeptide, with independent sites for catalysis for the two reactions. Presumably this arrangement would result in advantages to the cells in the regulation or efficiency (channeling) of these reactions (28), the consequences of which may prove to be a unique point of evolutionary divergence in the Protozoa.

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