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Purification and immunochemical characterization of a dihydrofolate reductase-thymidylate synthase enzyme complex from wild-carrot cells

Ildiko Toth, Gabor Lazar and Howard M.Goodman

Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Massachusetts General Hospital, Boston, MA 02114, USA

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An enzyme complex with dihydrofolate reductase (DHFR, E.C.1.5.1.3.) activity was purified to apparent homogenity from wild-carrot cells. The complex has a mol. wt of 286 kd and contains five polypeptide chains of 95, 70, 50, 45 and 26 kd. The DHFR enzyme activity and methotrexate-binding site are on the 45-kd subunit. Folate analogs (methotrexate, aminopterin and formylaminopterin) as well as SH-group inhibitors [*p*-hydroxymercuribenzoate, 5,5′-dithiobis(2-nitrobenzoic acid), or *N*-ethylmaleimide] inhibit DHFR. Thymidylate synthase (TS, E.C.2.1.1.45) activity co-purified with the enzyme complex through each of seven steps and co-eluted from gel filtration columns with the DHFR activity at the mol. wt of the enzyme complex. Further identification of TS within the complex was achieved using a *Leishmania* DHFR-TS antisera which specifically inhibited the carrot TS, although it immunoprecipitated both TS and DHFR. Polyclonal antisera, raised against and specific for the complex as judged by Ouchterlony double diffusion tests and Western blot analysis, inhibited and immunoprecipitated both DHFR and TS. The *Leishmania* antisera also identified the 70-kd polypeptide within the purified complex as TS in a Western blot experiment. The functions of the other three polypeptides have not yet been established.

Key words: higher plants/multimeric protein/thymidylate synthesis

Introduction

Dihydrofolate reductase [5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase (DHFR)] and thymidylate synthase [5,10-methylenetetrahydrofolate:dUMP C-methyltransferase (TS)] catalyze consecutive reactions in the biosynthesis of thymidylate. Inhibition of DHFR by folate analogs depletes the tetrahydrofolate pool, thereby inhibiting the synthesis of thymidylate, purines and some amino acids, and leads to the cessation of DNA synthesis. Because of its central metabolic role, DHFR is a prime target for drugs used in anticancer, antibacterial and antimalarial studies.

In most prokaryotes and eukaryotes DHFR and TS activities are separated on independently coded polypeptides; DHFR is a monomer with a mol. wt between 15 and 33 kd while TS is usually a dimer of identical subunits each with a mol. wt of ~35 kd (Blakley, 1984; Santi and Danenberg, 1984). The single exception is the protozoan bifunctional DHFR-TS, where the two activities are located in separate domains of a large mol. wt (110kd) dimeric enzyme (Meek *et al.*, 1985).

In spite of their importance DHFR and TS have not been extensively studied in higher plants. No information on the purification or biochemistry of TS is available, while DHFR has only been characterized from two sources (Albani *et al.*, 1986; Reddy and Rao, 1976). Neither showed similarities to other prokaryotic or eukaryotic DHFRs. Soybean DHFR is a 140-kd multimer with four non-identical subunits (Reddy and Rao, 1976), while the carrot enzyme is a trimer of three identical subunits and has a native mol. wt of 183 kd (Albani *et al.*, 1986).

Here we describe the isolation and partial characterization of another type of DHFR from wild-carrot cells with a different mol. wt and subunit structure and presumably with a separate physiological role. This DHFR activity is localized on the 45 kd polypeptide of a multimeric, large mol. wt (286 kd) enzyme complex with five non-identical subunits. TS, which catalyzes a reaction functionally coupled with DHFR, is physically linked to the DHFR as part of the same multifunctional enzyme complex and is located on a 70-kd polypeptide.

Results

Purification of DHFR

A seven-step protocol was devised to purify DHFR from carrot cell suspension cultures (Table I). The enzyme is fairly unstable, but the continuous presence during the purification of 10% glycerol and 20 mM 2-mercaptoethanol helped to stabilize the enzyme. The methotrexate (MTX) affinity chromatrography, step 4, did not give as good purification as in the case of the soybean enzyme (Reddy and Rao, 1976), therefore chromatofocusing (step 7) was used as the last step in the purification protocol. In good agreement with the isoelectrofocusing data (Figure 1) DHFR eluted from the chromatofocusing column in two peaks: a narrow peak at I_p5.3 (fraction I) and a broad peak between I_p4.7 and 5.0 (fraction II). Fraction I was ~90% pure and could be purified to apparent homogenity on a preparative native polyacrylamide gel, while fraction II was resolved on native gels into three DHFR activity peaks (Figure 2). The major activity peak, represented by fraction I, was designated 'DHFR-3' according to its mobility on native gels (Figure 2). The peak fractions were excised from the gel, electroeluted and used for further analysis. Starting from 500 g of frozen tissue 0.35 mg of homogenous DHFR-3 was recovered.

DHFR-3 could be resolved into five bands by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with mol. wts of 95, 70, 50, 45 and 26 kd (Figure 3). These five polypeptides could either be the subunits of DHFR, an enzyme complex in which a portion of the polypeptides constitutes the DHFR activity, or contaminants. The latter possibility seems unlikely since mol. wt determinations on Biogel A 0.5 columns of fractions from early and late purification steps indicated the continuous presence of a large, 250-kd protein with DHFR activity (data not shown). This mol. wt corresponds well to the sum of the mol. wts of the five polypeptides as determined by SDS PAGE.

Identification of DHFR activity within the enzyme complex

To differentiate between the two other possibilities for the presence of multiple peptides, DHFR-3 was separated on a

Table I. Purification of DHFR enzyme complex from WOO1C carrot cell line

Purification step	Total protein (mg)	Sp. act. (U/mg)	Total act. (U)	Purification (fold)	Yield (%)
Crude	4562	12.8	59546	–	100
Protamin sulfate (1)	3060	19.3	59000	1.5	99
30–55% $(NH_4)_2SO_4$ (2)	732	68.1	49776	5.3	84
DEAE–Sephacel (3)	328	130.2	42640	10.2	72
MTX-affinity (4)	36.2	380	13756	30	23
Biogel-P-100 (5)	14.4	700	10083	55	19.3
Thiol–Sepharose-4B (6)	2.4	3700	8880	289	14.9
Chromatofocusing (7)					
fr.I. pH 5.4–5.1	0.35	14000	4900	1094.0	8.2
fr.II. pH 5.1–4.8	0.42	10800	4536	844.0	7.6

U, Amount of enzyme required to produce 1 μmol tetrahydrofolate/min at pH 7.2 and 42°C.

specifically designed SDS–PAGE (see Materials and methods) and after renaturation and DHFR-specific-enzyme staining the DHFR activity was localized to the 45-kd polypeptide (Figure 4). The development of the DHFR-specific color reaction was inhibited by MTX, further confirming that the active and MTX-binding sites are on the 45-kd polypeptide (Figure 4). Therefore it would seem most likely that the large mol. wt form is an enzyme complex and DHFR is a 45-kd polypeptide.

Catalytic properties of the purified DHFR complex

The purified complex has high DHFR specific activity (14 000 U/mg) in the presence of 0.4 mM dihydrofolate and 0.3 mM NADPH at 42°C and pH 7.2. It has no activity in 50 mM phosphate buffer at pH 5.9, which are the optimum conditions for the previously described acidic DHFR (Albani *et al.*, 1986).

SH groups play an important role in maintaining the catalytic function of DHFRs (Reddy and Rao, 1976) and also are important in maintaining the DHFR activity of the complex as shown by inhibition of DHFR activity with the SH-group-specific inhibitors *p*-hydroxymercuribenzoate (pHMB), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide (NEM) with I_{100} values of 0.1 mM, 0.1 mM and 1 mM respectively.

Folate analogs were also potent inhibitors of DHFR activity with I_{50} values of 1.35×10^{-8} M for MTX, 1.4×10^{-7} M for aminopterin and 1.5×10^{-7} M for formylaminopterin. The MTX inhibition was pH dependent; 10^{-7} MTX which fully inhibited DHFR activity at pH 7.2 caused < 10% inhibition at pH 8.0. (This property was exploited in the MTX-affinity chromatography purification step, see Materials and methods.)

Identification and localization of thymidylate synthase in the DHFR enzyme complex

An attempt was made to ascertain the function(s) of the other polypeptides in the complex. Folate pathway enzymes with functionally coupled activities to DHFR were obvious possibilities. We guessed that TS might be one of these and direct assays for TS activity indicated that this was correct.

Carrot TS, like TS from other sources (Meek *et al.*, 1985), was inherently unstable and could only be stabilized with a complex mixture of protease inhibitors (see Materials and methods). Under the optimal conditions 3–5 U of TS activity could be measured in crude extracts of WOO1C cells, a value which is 10-fold higher than the only other reported TS activity measurement on plant cells (soybean; Ohyama, 1976). In the original DHFR enzyme purification protocol no special attention was paid to protecting TS activity. However, enough measurable TS activity was present to show that TS co-purified with DHFR up

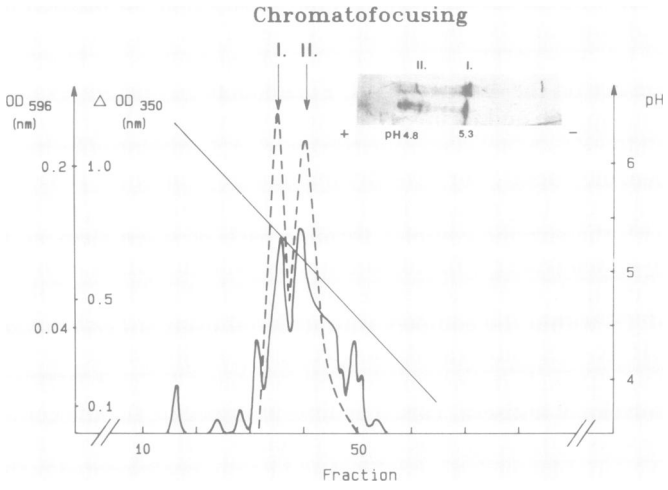

Fig. 1. Elution profile of DHFR from the chromatofocusing column. Two milligrams Thiol–Sepharose purified sample (step 6) were applied on the column and eluted with a pH gradient between pH 6.0 and 4.0 (see Materials and methods). The elution was monitored by the Bio-Rad protein micro assay (OD 596, solid line) and DHFR activity (OD 350, dashed line). Fractions I and II identify the two DHFR-activity peaks eluted from the column. Insert: isoelectrofocusing of DHFR from the WOO1C carrot cell line. Twenty micrograms MTX-affinity-purified sample (step 4) were electrofocused (see Materials and methods). After 2 h the gel was stained for DHFR enzyme activity by incubating in assay buffer containing 40 mM Tris pH 7.2, 0.4 mM dihydrofolate, 0.3 mM NADPH and 0.5 mg/ml MTT in the dark at 42°C. The presence of enzyme activity was indicated by the formation of a blue formazane band. Control experiments performed by the omission of any of the components from the assay mix or in the presence of 10^{-4}M MTX did not result in the appearance of the blue band.

to the chromatofocusing step (data not shown). Another indication of the association of TS and DHFR was provided by gel filtration (Figure 5) where the two activities co-eluted at a mol. wt corresponding to that of the complex [a part of both the DHFR and TS activities also co-eluted in the void volume of the Biogel A 0.5 column (Figure 5)].

Confirmation of the presence of TS in the complex was achieved using antisera raised against the *Leishmania* bifunctional DHFR–TS (Meek *et al.*, 1985). In enzyme activity inhibition experiments the *Leishmania* antisera recognized only TS, but not DHFR, from carrot. However, it immunoprecipitated both activities, indicating the physical association of the two enzymes. The inhibition of carrot TS by the antisera must reflect the presence of common epitope(s) in the active site of the carrot and *Leishmania* enzymes. This is not surprising since thymidylate

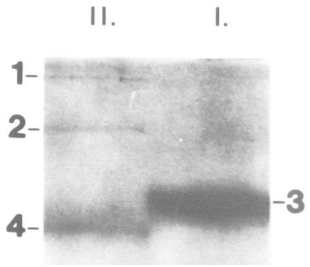

Fig. 2. Separation of chromatofocused fraction I and II on native PAGE. Twenty micrograms of purified and concentrated samples from the chromatofocusing (step 7) fractions I and II were separated on a 10% PAGE in the cold room with constant current (20 mA) for 4 h. The gel was stained for enzyme activity as detailed in the legend to Figure 1. DHFR enzyme forms are numbered according to their mobility: 1, 2 and 4 designate the three DHFR enzyme forms in chromatofocused fraction II and 3 identifies the major DHFR activity eluted in the chromatofocused fraction I.

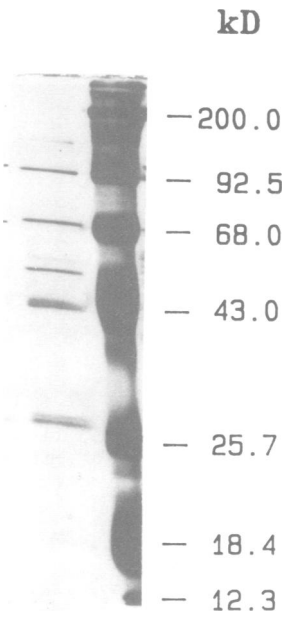

Fig. 3. SDS–PAGE electrophoresis of the purified DHFR-3 enzyme complex. Five micrograms of the purified DHFR-3 protein were separated on a 10% SDS–PAGE and stained using the silver method. The DHFR-3 complex has five components with mol. wts of 95 kd, 70 kd, 50 kd, 45 kd and 26 kd (**lane A**). The mol. wt markers were: myosin h-chain (200 kd, phosphorylase b (92.5 kd), bovine serum albumin (68 kd), ovalbumin (43 kd), α-chymotrypsinogen (25.7 kd), β-lactoglobuline (18.4 kd) and lysosyme (12.3 kd) (**lane B**).

synthase exhibits an unusually high degree of sequence and structural conservation from prokaryotes to human (Santi and Danenberg, 1984; Takeishi *et al.*, 1985).

In Western blot analysis of the purified enzyme complex the *Leishmania* antisera recognized the 70-kd polypeptide (Figure 6).

Immunochemical characterization of the DHFR–TS enzyme complex

The properties of the antibody raised against the purified DHFR-3 enzyme complex were investigated using Ouchterlony double diffusion tests (Figure 7), enzyme inhibition and immunoprecipitation experiments (Figure 8), and Western blot analysis (Figures 9 and 10).

The DHFR antisera produced only one strong precipitine band with either the purified DHFR-3 complex or crude extract (Figure 7). Neither purified bovine nor chicken DHFRs were recognized by the complex specific antisera.

The antisera inhibited both DHFR and TS enzyme activities from WOO1C carrot crude extract and from purified DHFR enzyme complex and immunoprecipitated both activities (Figure 8).

In Western blot analysis the antibody recognized a few minor DHFR enzyme forms from the crude extract and only one strong band from the purified complex when they were separated on native PAGE (Figure 9). The specificity of the antisera was assayed by separation on SDS–PAGE where the antibody recognized the same five polypeptides in the crude extract as in the purified enzyme complex (Figure 10).

Immunoaffinity purification of the complex

Immunoaffinity chromatography using the polyclonal antisera was used in place of steps 5–7 of the purification protocol (i.e. the

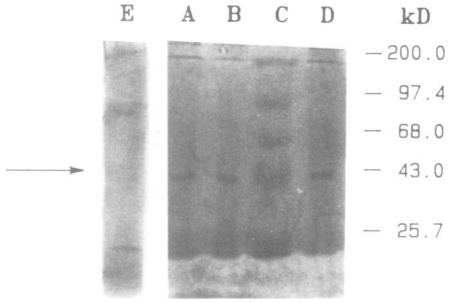

Fig. 4. Recovering of DHFR activity after SDS–PAGE. Fifty micrograms of MTX-affinity-purified (step 4) DHFR complex were dissolved in a sample buffer containing 50 mM Tris pH 6.8, 2 mM EDTA, 1% SDS (BDH), 1% 2-mercaptoethanol, 10% glycerol, 0.025% bromphenol blue and 10 μg/ml β-lactoglobulin (protective agent) and incubated at room temperature for 30 min. It was then separated on a SDS–PAGE according to the method of Laemmlie (1970) except that the separating gel contained 10 μg/ml BSA as a protective agent. After the electrophoresis the gel was extensively dialysed against multiple changes of 50 mM Tris pH 7.2, 20 mM 2-mercaptoethanol to renature the proteins. After 3 h of dialysis the gel was stained for DHFR enzyme activity. **Lanes A, B** and **D** are samples from three independent purifications: **lane E** is DHFR enzyme activity stained in the presence of 10^{-4}M MTX; **lane C** are the mol. wt markers.

MTX-affinity-purified fraction was applied to the column) and resulted in a complex of comparable purity. This material was separated on an SDS–PAGE and the 45-kd DHFR polypeptide subjected to amino acid analysis and amino acid sequencing. The relatively high methionine content (2.1%), was similar to that

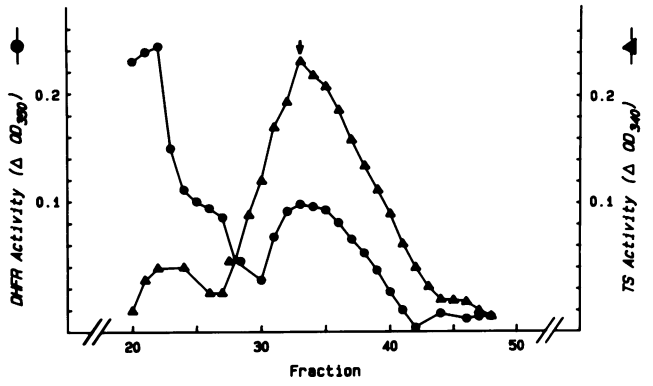

Fig. 5. Co-elution of DHFR and TS activities from a Biogel A 0.5 gel filtration column. Crude extract (1.5 ml) was applied to the column. TS and DHFR activities were assayed immediately after the elution of the fractions. The arrow indicates co-eluting DHFR and TS activities with a mol. wt of 250 kd.

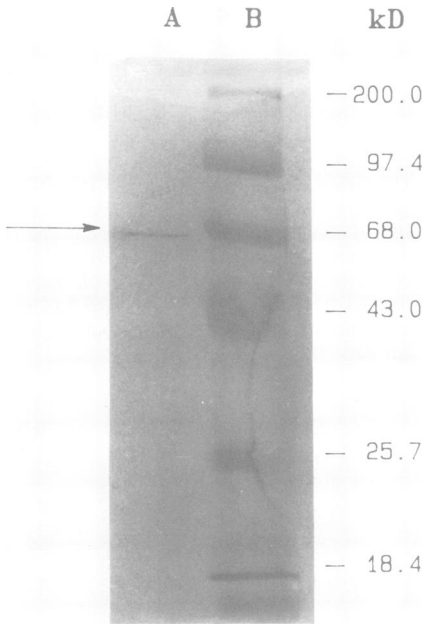

Fig. 6. Western blot analysis of purified DHFR enzyme complex with the *Leishmania* bifunctional DHFR–TS antisera. Twenty micrograms purified DHFR were probed with diluted (1:20) *Leishmania* antisera (**lane A**). No reaction could be detected from the crude extract (data not shown). Marker proteins are in **lane B**.

of DHFRs from other sources (Kumar *et al.*, 1980), but the 45-kd protein is N terminally blocked (data not shown).

Discussion

We have isolated a novel type of DHFR enzyme species from carrot, in a form which has not yet been described from any other prokaryotes or eukaryotes, specifically a DHFR activity located on a 45-kd polypeptide of a multimeric enzyme complex consisting of five subunits with a total mol. wt of 286 kd.

With regard to mol. wt the DHFR enzyme complex is comparable only with the two other known plant DHFRs described from soybean (Reddy and Rao, 1976) and carrot (Albani *et al.*, 1986), and shows no relationship to the small mol. wt monomeric DHFRs from diverse phylogenetic sources (Baccanari *et al.*, 1984; Chen *et al.*, 1984; Erickson and Mathews, 1971). The

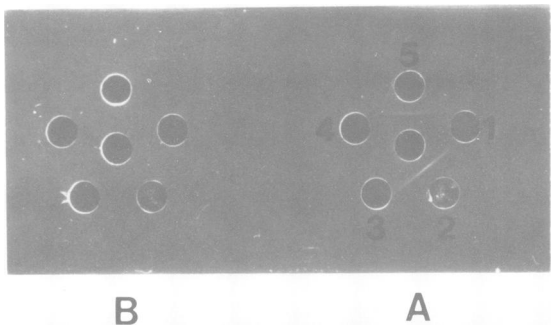

Fig. 7. Ouchterlony double diffusion test. The central well contains 10 μl of purified IgG from rabbit anti DHFR-3 antiserum (**A**) or pre-immune serum of the same rabbit (**B**). The wells contain the following: 1, BSA; 2, purified carrot DHFR-3; 3, purified chicken DHFR (Sigma); 4, purified bovine DHFR (Sigma); 5, crude extract from WOO1C carrot cells. Sharp preciptin bands appeared after overnight incubation at 4°C.

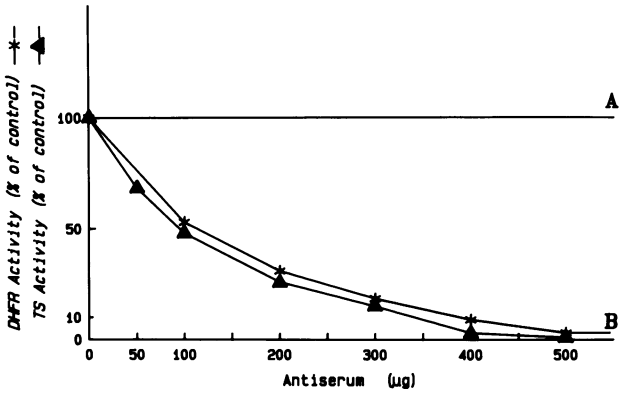

Fig. 8. Immunoprecipitation of carrot DHFR and TS activities by the DHFR-complex-specific antisera. Twenty micrograms MTX-affinity-purified (step 4) DHFR and 100 μg crude enzyme samples were incubated with increasing amount of antisera at 4°C. After 4 h the precipitate was pelleted by centrifugation and the DHFR and TS activities were determined (**B**). In the control experiment pre-immune serum was used using the same experimental conditions (**A**).

multimeric nature of the complex with DHFR activity resembles the soybean DHFR (Reddy and Rao, 1976) which consists of four different mol. wt subunits. With respect to pH optimum, temperature optimum, substrate specificity, SH-group inhibition and folate analogue sensitivity the carrot DHFR is also comparable only with the soybean enzyme (Reddy and Rao, 1976).

Carrot, and probably higher-plant DHFRs in general, are functionally and structurally different from DHFRs from other sources as evidenced by their lack of antigenic cross reactivity with purified chicken and bovine DHFRs as well as the failure of a wide variety of DNA oligonucleotide probes representing the highly conserved functional domains of DHFR to hybridize in Northern blots to poly(A)$^+$ RNA of the WOO1C line (Lazar, unpublished).

Several folate-dependent enzyme activities are catalyzed by multifunctional enzymes (Smith *et al.*, 1980; Wasserman *et al.*, 1983) and multi-enzyme complexes (Henikoff *et al.*, 1986; Rowe *et al.*, 1978) in different organisms. This type of structural organization can allow more efficient biochemical and genetic regulation (Myoda and Funanage, 1985; Zelikson and Luzatti, 1977) and provide physiological advantages. The natural form of folate derivatives in eukaryotic cells are the folylpoly-γ-glutamates. The polyglutamate chain assists in the transfer of the

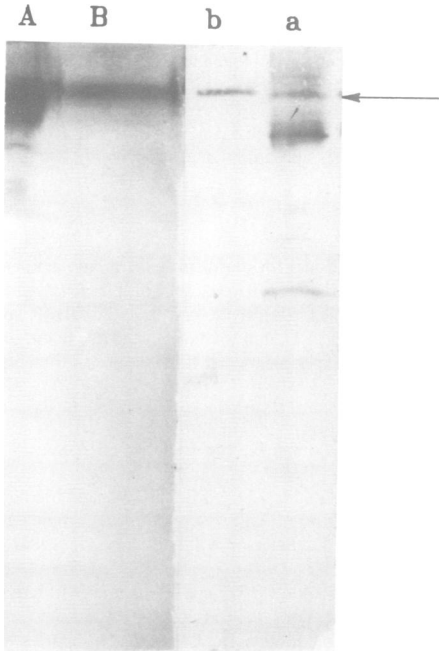

Fig. 9. Western blot analysis of DHFR using the DHFR-3-complex-specific antisera. Five micrograms of purified DHFR (**lanes B** and **b**) and 20 μg of crude extract (**lanes A** and **a**) were separated on a 10% native PAGE, part of the gel was stained for DHFR activity (left side, **lanes A** and **B**) and the other part of the gel was electroblotted to a nitrocellulose filter and probed with antibody (right side, **lanes a** and **b**). The arrow shows the DHFR-3.

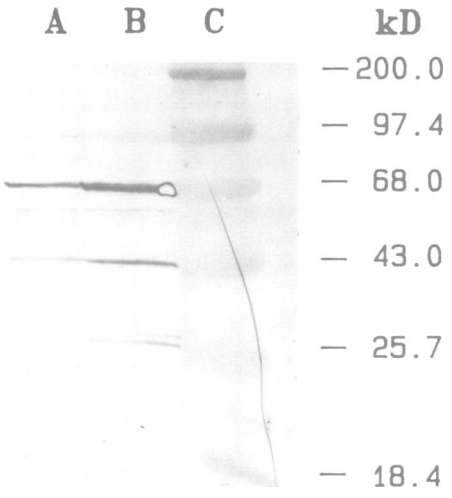

Fig. 10. Western blot analysis of DHFR enzyme complex after SDS–PAGE. Proteins were separated on a SDS–PAGE and visualized by Western blotting using anti-complex antiserum. Samples: 20 μg crude extract (**lane A**), 5 μg purified DHFR-3 (**lane B**), pre-stained protein markers (**lane C**).

folate portion of the molecule between consecutive active centers (MacKenzie and Baugh, 1980) thereby offering a possibility for efficient channelling.

Depending on the source of dihydrofolate, DHFR can perform two different functions: *de novo* synthesis or recycling of dihydrofolate released as the end-product of the TS-catalyzed uridylate–thymidylate conversion in the folate reduction pathway. In either case DHFR is associated with thymidylate synthesis (the *de novo* synthesis function providing tetrahydrofolate to the folate intermediary metabolism is more loosely connected to TS). On the other hand the efficient recycling function requires co-regulation of TS and DHFR. We have found that DHFR and TS are not only functionally and physically linked in carrot, but as in other species (Myoda and Funanage, 1985; Zelikson and Luzatti, 1977) are co-regulated as well (Lazar *et al.*, unpublished data). This strengthens the argument for a recycling function of DHFR within the complex.

The existence of an acidic DHFR in carrot (Albani *et al.*, 1986) with different mol. wt, subunit structure, kinetic parameters and immunochemical properties suggest that in carrot and in the lower plant yeast (Zelikson and Luzatti, 1977) the *de novo* and recycling functions of DHFR may be assigned to different enzyme species. It is still not known whether in carrot the two functionally different DHFRs are located in different compartments (e.g. mitochondria and cytoplasm) as they are in yeast cells (Zelikson and Luzatti, 1977).

The function of the other complex-forming proteins and the role of the whole enzyme complex remains to be elucidated. Functionally interdependent but temporal association between DHFR, TS and another two to eight enzymes exist in the multienzyme complex replitase (Allen *et al.*, 1983; Prem Veer Reddy and Pardee, 1980; Noguchi *et al.*, 1983) which has a function of channelling the reduced nucleotides in the close vicinity of the replication fork. The co-elution of DHFR and TS activities in the void volume of the Biogel A 0.5 column (Figure 5; >500 kd) suggests that the two activities may be further non-covalently associated with other enzymes in a large mol. wt superstructure.

The physiological role of the enzyme complex is probably related to thymidylate synthesis. We hope that further studies on the biochemical and physiological role of the enzyme complex can provide information about the organization and regulation of the thymidylate pathway and its relationship with cell cycle regulation at the G_1,S boundary.

Materials and methods

Cell and culture conditions

The origin and maintenance of cell suspension cultures of the wild-carrot WOO1C line have been described previously (Sung, 1979; Sung, *et al.*, 1981).

Purification of DHFR enzyme complex

Cells were harvested from log phase suspension cultures, frozen in liquid N_2 and ground in TGM buffer (50 mM Tris pH 7.2, 10% glycerol, 20 mM 2-mercaptoethanol) in the presence of 0.1% polyvinyl-polypyrolidone and 1 mM phenylmethane sulfonyl fluoride. When TS enzyme activity was assayed the TGM buffer contained in addition 1 nM 1,10-phenantroline, 1 mM benzamide, 20 μg/ml leupeptine, 50 μg/ml soybean trypsin inhibitor and 50 μg/ml aprotinin. The crude extract was centrifuged at 15 000 *g* for 10 min and the supernatant was protamine sulfate precipitated (step 1) according to the method of Reddy and Rao (1976). The supernatant was further fractionated by $(NH_4)_2SO_4$ precipitation (30–55% saturation) and the pellet dialysed extensively against TGM buffer (step 2). The dialysate was applied to a DEAE Sephacel column (bed volume 80 ml) equilibrated with TGM buffer (step 3). DHFR was eluted with a 400 ml linear gradient of 50–300 mM KCl in TGM buffer. The active fractions were pooled and (step 4) adsorbed in batch for 30 min to a MTX–agarose-4B affinity resin (Pierce Chemical Company), washed with 10 vol TGM buffer and 10 vol 0.15 M NaCl in TGM buffer. The specifically bound proteins were eluted with 2 vol TGM buffer (pH 8.0) containing 0.5 mM dihydrofolate. The eluate was precipitated with $(NH_4)_2SO_4$ (90% saturation), resuspended in 50 mM Tris pH 7.2 and (step 5) applied to a Biogel P-100 column (bed volume 20 ml). The active fractions, which eluted in the void volume with 50 mM Tris pH 7.2, were (step 6) applied to a Thiol–Sepharose-4B column (Pharmacia Fine Chemicals, bed volume 10 ml) using a flow rate of 10 ml/h, washed with 3 vol 50 mM Tris pH 7.2 (flow rate 30 ml/h), and the specifically bound proteins eluted with 50 mM L-cysteine in 50 mM Tris pH 7.2. The eluant was dialysed overnight against 6000 ml of 25 mM histidine–HCl buffer pH 6.2 containing 10% glycerol and 20 mM

2-mercaptoethanol. The dialysate was subsequently (step 7) applied to a chromatofocusing column (Pharmacia, Inc.) (bed volume 20 ml, equilibrated with dialysis buffer, loading rate 50 ml/h) and DHFR eluted with a 1:8 mix of Polybuffer^{TM}96 (pH 4.0) and 10% glycerol, 20 mM 2-mercaptoethanol according to the technical manual supplied by the manufacturer, Pharmacia Inc. DHFR activity, which eluted in two separate peaks, was pooled separately, concentrated with the use of an Amicon ultrafiltration membrane and stored at −20°C. All enzyme purification steps were carried out in the cold room.

Isoelectrofocusing

Isoelectrofocusing was performed according to the LKB technical manual using 5% ampholine polyacrylamide plates (pH 3.5−9.5) with 150 V constant voltage at 4°C. After 2 h the pH gradient was determined and the gel was strained for DHFR enzyme activity.

Polyacrylamide gel electrophoresis

Ten per-cent polyacrylamide slab gel electrophoresis in denaturing and nondenaturing conditions was performed essentially as described by Laemmlie (1970).

DHFR activity was specifically stained by the method of Mell *et al.*, (1968). The active DHFR component of the enzyme complex was detected by the same specific enzyme staining after renaturation of the proteins on 10% SDS polyacrylamide gel made according to Lacks *et al.* (1979). Silver staining was performed according to Nielsen and Brown (1984).

Gel filtration

A Biogel-A 0.5 column (80 × 1.5 cm) was used to determine the mol. wt of the complex. The column was calibrated with thyroglobulin (670 kd), ferritin (440 kd), catalase (232 kd), aldolase (158 kd), chicken ovalbumin (44 kd) and horse myoglobin (17 kd). The elution buffer was 0.15 M NaCl in 50 mM Tris pH 7.5.

Enzyme assays

DHFR enzyme activity was determined spectrophotometrically according to the method of Reddy and Rao (1976). One unit of DHFR is defined as the amount of enzyme required to produce 1 μmol of tetrahydrofolate/min at pH 7.2 at 42°C. TS activity was assayed spectrophotometrically as described by Wahba and Friedkin (1961). One unit of TS activity is defined as the amount of enzyme required to produce 1 nmol of dihydrofolate/min at pH 7.4 at 30°C.

Protein concentrations were measured using the Bio-Rad assay according to the supplier's manual.

The inhibitor assays on DHFR were performed according to Reddy and Rao (1976) using the SH-group inhibitors 0.1 mM pHMB, 0.1 mM DTNB, or 1 mM NEM or the folate analogs MTX, aminopterin and formilaminopterin in a concentration range of $10^{-4}-10^{-9}$M.

Preparation of antiserum

The pooled first activity peak of DHFR from the chromatofocusing column (step 7; DHFR fraction I) was separated on a preparative native 10% polyacrylamide gel and stained for enzyme activity. The gel slice containing the active DHFR enzyme (DHFR-3, see text; ~50 μg protein) was emulsified in an equal volume of Freund's complete adjuvant and injected subcutaneously into a New Zealand white rabbit. Booster injections were administered 4 weeks after the initial injection. Two weeks after the last injection the animal was bled through the ear veins and the IgG fraction from the serum was purified on Protein A−Sepharose as described by the supplier (Bio Rad).

Antisera against the *Leishmania* bifunctional DHFR−TS (Meek *et al.*, 1985) was kindly provided by Professor D.V.Santi.

Immunochemical characterization of the DHFR enzyme complex

The ability of DHFR-complex-specific antisera and *Leishmania* DHFR−TS antisera to inhibit carrot DHFR and TS enzyme activities was determined by incubating an equal number of units of purified DHFR enzyme complex with increasing amounts of antibody in PBS buffer for 4 h at 4°C, centrifuging the precipitate (10 min., Eppendorf, 4°C), and assaying the enzyme activities in the supernatants. In control experiments pre-immune serum was used under the same conditions.

For immunoprecipitation 50 μl Protein A−Sepharose CL-4B (Pharmacia, Inc.) was added to each sample prepared as described above, the precipitate pelleted by centrifugation and the enzyme activities assayed in the supernatants.

For Western blot analysis proteins were transferred from 10% polyacrylamide gels to nitrocellulose filters (Schleicher & Schuell) as described previously (Towbin *et al.*, 1979) and probed with antisera according to Fisher *et al.* (1982) using Protein-A−horseradish peroxidase conjugate.

Immunoaffinity chromatography

The purified antibody was coupled to Affi-Gel 10 (Bio Rad, bed volume 3 ml) according to the method of Staehelin *et al.* (1981). Immunoaffinity chromatography was performed essentially as described by Server *et al.* (1985) using protein purified through step 4 (MTX-affinity purified). The proteins were precipitated from the eluants according to the method of Horigome and Sugano (1983).

Total amino acid analysis, protein sequencing

The immunoaffinity purified DHFR-complex-specific polypeptides were separated on a 10% SDS−PAGE and electroblotted to a freshly prepared polybrene-coated glass fiber sheet according to Vandekerckhove *et al.* (1985). The positions of the protein bands were visualized by fluorescamine (Fluka), the portion of glass-fiber sheet containing the immobolized (45-kd) protein excised, and subjected to total amino acid analysis and amino acid sequencing as described by Server *et al.*, (1985).

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