COMPARISON OF CHARACTERISTICS OF THYMIDYLATE SYNTHASES ISOLATED FROM EUKARYOTIC AND PROKARYOTIC CELLS

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The preparations of thymidylate synthase (TDS) from calf thymus, Gardner mouse lymphosarcoma and *E. coli* were investigated by affinity chromatography based on the formation of a ternary complex of the enzyme with tetrahydromethotrexate and deoxyuridine-5'-monophosphate (dUMP). The *E. coli* enzyme was a homogeneous protein of mol.wt. 70 000 and it was shown by reductive denaturation to contain one single chain of mol.wt. 35 000. The preparation isolated from the Gardner lymphosarcoma was a homogeneous protein of mol.wt. 72 000 containing a single subunit of mol.wt. 36 000. The enzyme from both sources yielded after treatment with 5-fluorodeoxyuridine-5'-monophosphate (FdUMP) and N^{5-10}-methylenetetrahydrofolate a negatively charged complex which was separated electrophoretically. Three proteins of mol.wt. 35 000 (component 1), 70 000 (component 2), and more than 250 000 (component 3), were isolated by affinity chromatography from calf thymus and separated by electrophoresis and gel filtration. Component 2 only was enzymaticaly active and yielded a complex when treated with 5-fluorodeoxyuridine-5'-monophosphate and N^{5-10}-methylenetetrahydrofolate. Components 1 and 3 were enzymatically inactive yet were immunologically related. The antiserum against component 3 precipitated in addition to component 3 also the active enzyme (component 2) and component 1. The antiserum against the active enzyme precipitated proteins 1 and 3. All three components were found to contain one chain of mol. wt. 35 000 after reductive denaturation. Components 1 and 2 contained this chain only whereas other, heavier chains were found in component 3.

Thymidylate synthase (EC 2.1.1.45) catalyzes reductive methylation of deoxyuridine-5'-monophosphate to thymidine-5'-phosphate utilizing N^{5-10}-methylenetetrahydrofolic acid as a donor of the single-carbon residue and as a reducing agent[1,2] at the same time. The enzyme is believed to set a limit to DNA biosynthesis and to represent a potential target enzyme in tumor chemotherapy[3-5]. Attention has been therefore focused on the investigation of the characteristics of the enzyme, especially on a comparison of enzymes isolated from various types of cells[6-8].

The possibility of obtaining large quantities of the enzyme protein from a L. *casei* mutant[9,10] resistant to 3',5'-dichloromethotrexate permitted the reaction mechan-

ism[11-18] and the primary structure of the enzyme consisting of 2 subunits of mol.wt. 35 000 (refs[19-23]) to be determined.

By contrast information on the enzyme from other bacterial and eukaryotic cells is meagre because of the small quantity of the enzyme protein available[24-32]. The technique of highly selective affinity chromatography described before[33-35] and based on the formation of a ternary complex of enzyme–tetrahydromethotrexate–dUMP in the column has permitted TDS to be obtained from enzyme poor eukaryotic cell sources in a single step and relatively high yields.

The present paper reports on the comparison of certain characteristics of TDS from *E. coli*, calf thymus, and Gardner lymphosarcoma since the differences among the enzymes, if any, may contribute to the formation of a rational base of selective inhibition of the tumor enzyme and possibly provide new information on the regulation of its activity.

EXPERIMENTAL

Chemicals: Deoxycytidine-5′-monophosphate, thymidine, deoxyuridine-5′-monophosphate, and thymidine-5′-monophosphate were from Koch-Light (Gr. Britain), methotrexate (MTX) of 99·5% purity from Lederle (USA). Cyanogum 41, acrylamide, N,N′-methylene-bis-acrylamide, Coomassie Blue R-250, mercaptoethanol, sodium dodecyl sulfate, cyclic AMP, cyclic GMP, ATP, and the protein standards were purchased from Serva (FRG). The ampholins were from LKB, Sepharose 4B, Sephadex G-200 superfine, and Sephacryl S-200 were from Pharmacia (Sweden). The sodium salt of 5-fluorodeoxyuridine-5′-monophosphate was kindly furnished by Dr W. Rode, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw. Folic acid and methotrexate (Lederle) were purified by precipitation at pH 4. Tetrahydrofolic acid and tetrahydromethotrexate were prepared by hydrogenation using hydrogen and a platinum catalyst[34].

Enzyme: The ammonium sulfate precipitated TDS fraction of *E. coli*, calf thymus, and the Gardner lymphosarcoma were prepared as described before[35]. The *E. coli* enzyme was purified by affinity chromatography on a Sepharose 4B derivative with immobilized aminoethyltetrahydromethotrexate using the procedure described before[35]; a modification[36] of the method was employed to obtain the enzyme from the Gardner lymphosarcoma and calf thymus. The active fraction obtained was concentrated in an Amicon ultrafiltration apparatus to 2 ml and in the Centriflo apparatus to 100—300 μl.

Gel filtration: Preparative gel filtration of fractions of the calf thymus enzyme was effected on a Sephacryl S 200 column in 0·2M sodium phosphate buffer, pH 7·2, at a flow rate of 4·5 ml/h. The fractions (2 ml) were analyzed for protein content and enzymatic activity and their purity was checked by polyacrylamide gel electrophoresis. The molecular weight determinations were carried out by thin layer gel filtration on Sephadex G-200 superfine using the apparatus of Pharmacia and the method of Anderson and Stoddart[37].

Polyacrylamide gel electrophoresis was carried out in the discontinuous arrangement according to Davis[38] using a 7·5% polyacrylamide gel and Tris-glycine buffer. Electrophoresis in sodium dodecyl sulfate was performed by the technique of Weber and Osborn[39] in 7·5% polyacrylamide gel and 0·1M phosphate buffer, pH 7, containing 1% of sodium dodecyl sulfate, at a current of 7 mA per sample. The proteins were denatured before electrophoresis in 4M urea in the pre-

sence of 0·125M mercaptoethanol, 6 h at 37°C. The molecular weight of protein chains was determined with the aid of protein standards using the procedure described[39].

Isoelectric focusing: The gel was prepared by mixing together 8·9 ml of 8·4% solution of Cyanogum 41, 0·8 ml of glycerol, 1·6 ml of 4% Ampholine, 0·4 ml of 1% solution of tetramethylenediamine, and 0·6 ml of 1% solution of ammonium persulfate. Four parts of Ampholine were used for the pH-range 5—7 and one part for pH 3—10. The separation was effected in tubes 8 cm long of 5 mm i.d. in 0·01M phosphoric acid (anolyte) and 0·02M-NaOH (catholyte) at a potential gradient of 62 V/cm at 4°C. After the focusing had been finished the gels were fixed in 20% sulfosalicylic acid and stained with Coomassie Blue R-250.

Immunologic experiments: Four rabbits weighing 1 800—2 200 g were immunized with 150 μg of the individual components of the calf thymus enzyme, dissolved in 0·5 ml of physiological saline and emulgated with an equal volume of the complete Freund adjuvant. The immunization was carried out 4times in 2-day intervals. Three weeks after the last injection of the antigen blood was removed and the serum obtained was used for the immunoprecipitation experiments, carried out by the Ouchterlony technique, and for immunoelectrophoresis[40,41].

Preparation of ternary complex with 5-fluorodeoxyuridine-5'-monophosphate[20,21]: the individual fractions (250 μl) were incubated with 635 nmol of FdUMP and 113 nmol of methylenetetrahydrofolate in a volume of 250 μl at 4°C, 5 h. The samples were dialyzed in collodium bags against 350 ml of 20% solution of sucrose containing 0·1M mercaptoethanol, concentrated to 100 μl by ultrafiltration, and separated by isoelectric focusing or by polyacrylamide gel electrophoresis. The solution of N^{5-10}-methylenetetrahydrofolate was prepared by dissolving 6 mg of tetrahydrofolic acid in 1 ml of 1M mercaptoethanol by adding 5% of $NaHCO_3$ and 75 μl of 0·3M formaldehyde.

The activity of TDS was determined by the modified method of Wahba and Friedkin[44], the activity of thymidine kinase and dCMP deaminase was assayed by the techniques described[42,43].

RESULTS

The isolation of TDS by affinity chromatography based on the formation of a ternary complex of the enzyme with tetrahydromethotrexate and dUMP was highly selective and specific. One protein, homogeneous when subjected to polyacralamide gel electrophoresis and showing a mol. wt. of 70 000, was isolated from the enzyme fraction of *E. coli* precipitated by ammonium sulfate. Electrophoresis in sodium dodecyl sulfate (after preceding denaturation in 4M urea containing 1% of mercaptoethanol), showed the presence of one subunit of mol.wt. 35 000. After the enzyme of p_I 5·5 had been preincubated with an excess of 5-fluorodeoxyuridine-5'-phosphate and methylenetetrahydrofolate, it was converted into two stable complexes separable by isoelectric focusing (p_I 3·9 and 4·1) (Fig. 1*).

The Gardner lymphosarcoma TDS was obtained by the standard technique using the affinity column; the preparation contained an admixture of proteins of low electrophoretic mobility. When the protein emerging from the affinity column in 0·05M phosphate was adsorbed to a DEAE-cellulose column (1 × 12 cm) and subsequently eluted with 0·5M phosphate, a homogeneous enzyme protein of mol.wt. 72 000 was

* See insert on the p. 742.

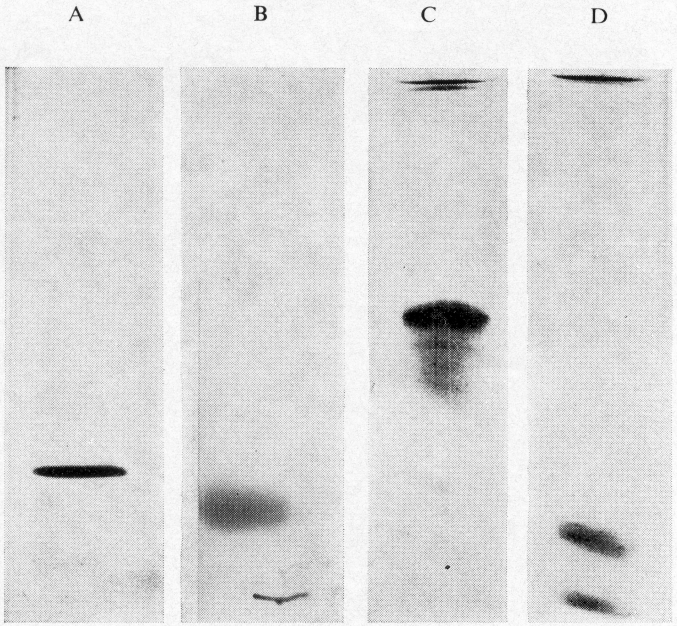

FIG. 1

Polyacrylamide gel electrophoresis of *E. coli* TDS. A native enzyme; B enzyme after denaturation by 4M urea in polyacrylamide gel containing 1% of sodium dodecyl sulfate; C isoelectric focusing enzyme; D isoelectric focusing of complex with FdUMP and methylenetetrahydrofolate

obtained; the alter contained one subunit of mol.wt. 36 000 as demonstrated by polyacrylamide gel electrophoresis after preceding denaturation in 4M urea. When treated with FdUMP and methylenetetrahydrofolate the enzyme protein was convertible into a complex showing a higher mobility on polyacrylamide gel electrophoresis (Fig. 2).

The enzyme preparation obtained from calf thymus by affinity chromatography contained 3 protein components of mol.wt. 35 000 (component 1), 70 000 (component 2), and over 250 000 (component 3), separable by electrophoresis or gel filtration. Component 2 only was enzymatically active and convertible into a complex of more negative p_I by the reaction with FdUMP and methylenetetrahydrofolate. Components 1 and 3 were not enzymatically active. The electrophoresis of the individual components denatured in 4M urea containing 1% of mercaptoethanol showed in the presence of SDS that all components contain a chain of mol.wt. 35 000. This chain was the only one in components 1 and 2 whereas certain heavier protein chains were also found in component 3. The distribution of the individual chains is given in Table I (Fig. 3*a*, 3*b*).

TABLE I

Distribution of chains in individual components 1—3 of calf thymus TDS

Component	Molecular weight (distribution in %)
1	35·000 (100)
2	35·000 (100)
3	35·000 (58·5), 49·000 (8·7), 71·000 (12·7) 111·000 (16·7), 130·000 (3·4)

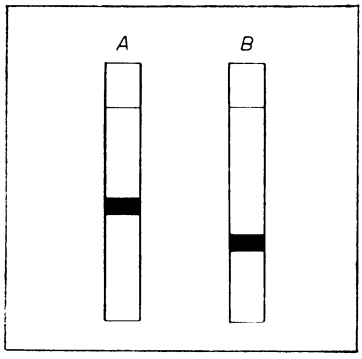

FIG. 2

Polyacrylamide gel electrophoresis of Gardner lymphosarcoma TDS. A native enzyme; B complex of enzyme with FdUMP and methylenetetrahydrofolate

The presence of the chain of mol.wt. 35 000 in the inactive components indicated their relationship with active component 2. This relationship was also proved by immunologic techniques. The antiserum was obtained by the immunization of rabbits by the individual components which had been separated by preparative gel filtration on Sephacryl S 200. The antiserum against the active enzyme (component 2) gave a precipitation (Fig. 4)* Ouchterlony test both with the active enzyme and also with components 1 and 3. The antiserum against component 3 precipitated in addition to component 3 also component 1 and the active enzyme (component 2). The same results gave immunoelectrophoresis (Fig. 5*a*, 5*b*). The immunoglobulin fraction which had been prepared from the antiserum against component 3 was bonded to Sepharose 4B. The immunoadsorbent thus prepared retained 20% of activity of the enzyme preparation obtained by affinity chromatography. We have not been able, however, to demonstrate an immunologic relationship between calf thymus TDS and TDS from *E. coli* and the Gardner lymphosarcoma.

To verify the hypothesis of the possible association of certain DNA synthetizing enzymes to form a multienzyme complex, the calf thymus TDS preparation purified by affinity chromatography was tested for the activity of dihydrofolate reductase, thymidine kinase, and deoxycytidine monophosphate deaminase. No activity of any of these enzymes was detected.

In an effort to find the possible precursor of the active form of the enzyme experi-

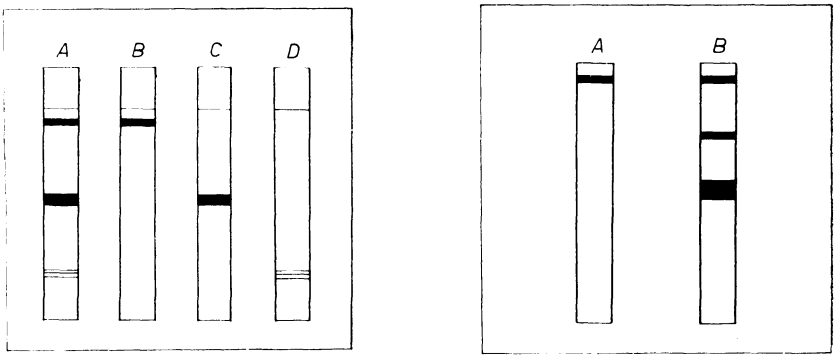

FIG. 3

Polyacrylamide gel electrophoresis of calf thymus TDS. *a* Separation in Tris-glycine buffer: A enzyme preparation obtained by affinity chromatography; B component 3; C component 2 (active enzyme); D component 1. The individual components were separated by gel filtration on Sephacryl S 200. *b* Isoelectric focusing in polyacrylamide gel, pH-range 3·5—9·0: A Active enzyme; B complex of enzyme with FdUMP and methylenetetrahydrofolate.

* See insert on the p. 742.

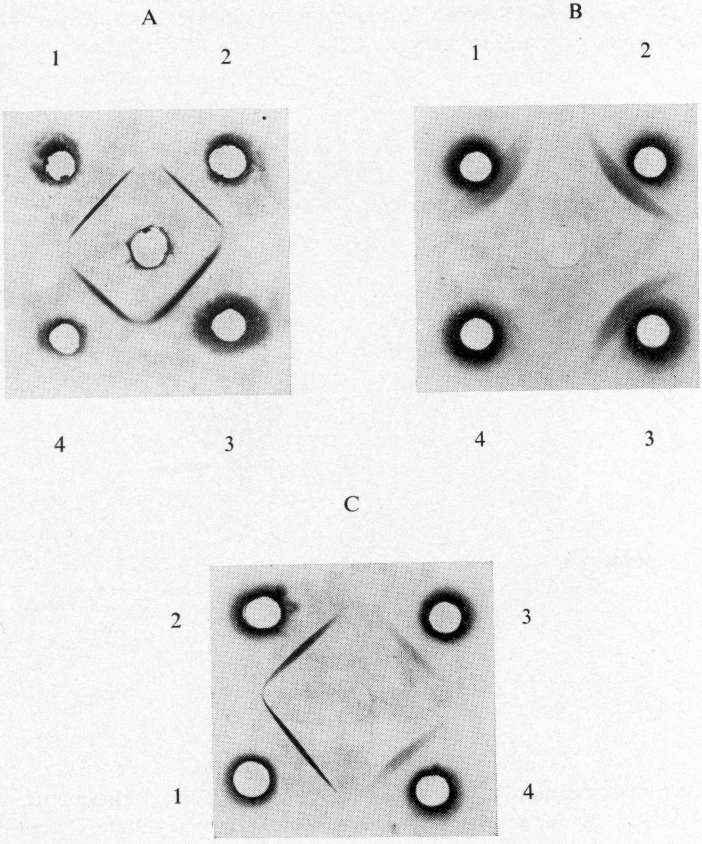

FIG. 4

Evidence of immunological relationship of calf thymus TDS components. Immunoprecipitation by the Ouchterlony technique. The central well contains: A component 1; B component 2 (active enzyme with a trace of component 1); C component 3. Rabbit antisera used: 1 and 2 antisera against component 3; 3 and 4 antisera against component 2 (active enzyme)

ments were made to convert component 3 into the active enzyme by limited proteolysis by trypsin, chymotrypsin and an acid proteinase from calf thymus (these enzymes were immobilized on an agarose support in enzite form) and by phosphorylation by protein kinase with simultaneous activation by cyclic adenosine monophosphate and guanosine monophosphate. The products of cleavage by the proteinases and of phosphorylation by protein kinase showed no thymidylate synthase activity.

DISCUSSION

So far no such differences have been found in the properties of TDS of various origin which would permit a selective inhibition of the tumor enzymes, even though the TDS from bacterial and eukaryotic cells somewhat differ in the degree of inhibition by tetrahydrofolate, isohomotetrahydroaminopterin, and tetrahydromethotrexate[33]. The TDS's from bacteria and various types of experimental tumors were found to differ in the activation by ATP and Mg^{2+} (ref.[27]) yet these data do not provide an adequate basis for a hypothesis explaining the regulation of enzyme activity or differences in the structures of enzymes of various origin.

The use of the highly selective technique of affinity chromatography permitted the TDS of *E. coli* and the Gardner lymphosarcoma to be isolated as a homogeneous, enzymatically active protein. Similar results were obtained by a modified technique with the Ehrlich ascites tumor[27], human tumor cells, *etc.*[32]. This fact contrasts with the finding of 3 protein components isolated from calf thymus which show immunologic and subunit relationship. The biological role of these proteins immunologically related

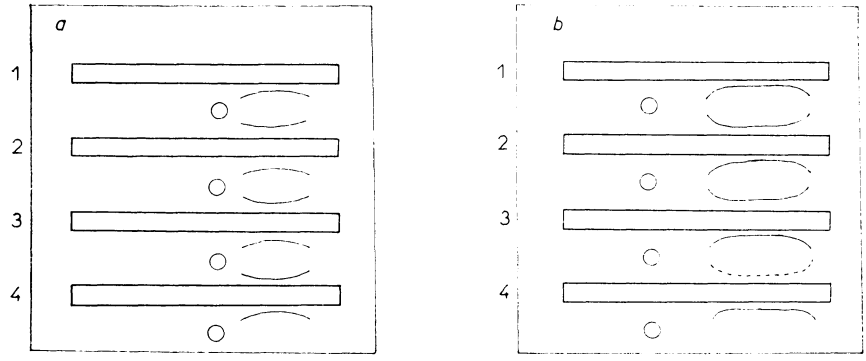

FIG. 5

a Immunoelectrophoresis of component 3; *b* immunoelectrophoresis of component 2. Component 2 is the active enzyme containing a trace of component 1. Antisera used: 1 and 2 antisera against component 3; 3 and 4 antisera against component 2 (active enzyme)

to TDS has not been elucidated. Component 1, which is microheterogeneous when subjected to polyacrylamide gel electrophoresis, could be regarded as a subunit of the enzyme partially degraded proteolytically and incapable of forming the active dimer. Nevertheless model experiments with proteolytic degradation of the enzyme by pepsin, chymotrypsin, and spleen cathepsin D resulted in fragments of other electrophoretic mobility. Component 3, containing heavier chains in addition to the chain of mol.wt. 35 000, can be regarded either as the associated form of TDS or its chains with other proteins or as the precursor form of the enzyme. The activity of certain other enzymes of the DNA synthesizing system in the preparations of calf thymus TDS has not been demonstrated.

Similar enzymatically inactive and immunologically related forms were found with dihydrofolate reductase whose function is linked with the function of TDS. A protein of mol.wt. 41 000 reacting with the antibodies against the active form of dihydrofolate reductase yet unable of binding substrates and methothrexate was found in the cells of human myeloid leukemia and in normal leukocytes[45,46]. Therefore its isolation by affinity chromatography was impossible. However, the immunologically related proteins of TDS from calf thymus obviously form a complex with tetrahydromethotrexate and dUMP and can therefore be obtained together with the active enzyme by affinity chromatography.

The existence of the precursor function of the high molecular weight and enzymatically inactive form of dihydrofolate reductase seems to be evidenced by the fact that this high molecular weight component is predominant in a synchronized culture of L 1210 cells during the stationary phase of cell division; its quantity decreases during the logarithmic phase of growth on behalf of the increase of the active form of the enzyme[47]. It can be postulated that a similar mechanism of activity regulation may govern TDS.

A series of papers[3-5] have reported an increase of TDS activity after the stimulation of cell division (*e.g.*, by hepatectomy) and a high activity in rapidly dividing cell populations. So far the data are meagre on the regulation of the activity of this enzyme by allostretic effect or by inhibition by the substrate and reaction products. The activation observed may be accounted for by the increased synthesis of the enzyme protein. The existence of immunologically related proteins in preparations of calf thymus TDS leads us to speculate that these enzyme derivatives may participate in the regulation of the activity of TDS in the thymus or other differentiated tissues as possible precursors of the enzyme associated with other protein chains (component 3) or its degradation products (component 1).

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