

# Dihydropteridine Reductase as an Alternative to Dihydrofolate Reductase for Synthesis of Tetrahydrofolate in *Thermus thermophilus*

Valérie Wilquet,<sup>1†</sup> Mark Van de Castele,<sup>2‡</sup> Daniel Gigot,<sup>1</sup> Christianne Legrain,<sup>3</sup> and Nicolas Glansdorff<sup>1,2,3\*</sup>

Microbiology, Université Libre de Bruxelles,<sup>1</sup> Microbiology, Vrije Universiteit Brussel,<sup>2</sup> and J. M. Wiame Institute for Microbiological Research,<sup>3</sup> B-1070 Brussels, Belgium

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**A strategy devised to isolate a gene coding for a dihydrofolate reductase from *Thermus thermophilus* DNA delivered only clones harboring instead a gene (the *T. thermophilus* dehydrogenase [DH<sub>T<sub>r</sub>] gene) coding for a dihydropteridine reductase which displays considerable dihydrofolate reductase activity (about 20% of the activity detected with 6,7-dimethyl-7,8-dihydropterine in the quinonoid form as a substrate). DH<sub>T<sub>r</sub></sub> appears to account for the synthesis of tetrahydrofolate in this bacterium, since a classical dihydrofolate reductase gene could not be found in the recently determined genome nucleotide sequence (A. Henne, personal communication). The derived amino acid sequence displays most of the highly conserved cofactor and active-site residues present in enzymes of the short-chain dehydrogenase/reductase family. The enzyme has no pteridine-independent oxidoreductase activity, in contrast to *Escherichia coli* dihydropteridine reductase, and thus appears more similar to mammalian dihydropteridine reductases, which do not contain a flavin prosthetic group. We suggest that bifunctional dihydropteridine reductases may be responsible for the synthesis of tetrahydrofolate in other bacteria, as well as archaea, that have been reported to lack a classical dihydrofolate reductase but for which possible substitutes have not yet been identified.</sub>**

Dihydrofolate reductase (DHFR; EC 15.1.3) catalyzes the synthesis of tetrahydrofolate (FH<sub>4</sub>), a key metabolite involved in the synthesis of several amino acids, purines, and deoxythymidylate. The substrate of DHFR, dihydrofolate (FH<sub>2</sub>), is a product of thymidylate synthase (TS; *thyA* gene), which uses N<sup>5</sup>,N<sup>10</sup>-methylene-FH<sub>4</sub> as a substrate. *Escherichia coli* DHFR-null mutants (with a mutation in *dyrA* or *folA*) appear to be nonviable, even on rich medium, unless they are also TS deficient (1, 6); this finding suggests that TS activity is toxic in a *dyrA* background because it exhausts residual FH<sub>4</sub> production due to another enzyme proceeding at a slower pace than DHFR (4). This substitute enzyme could be a dihydropteridine reductase (DHPR; EC 1.6.99.7), since *E. coli* contains a DHPR able to catalyze the same reaction as DHFR at a comparatively low rate (16, 17). The metabolic role of prokaryotic DHPR is not known; it is possible that nonenzymatic oxidation of FH<sub>4</sub> produces a quinonoid FH<sub>2</sub> species which is regenerated to FH<sub>4</sub> by DHPR but not by DHFR (14). In the few proteobacteria (but not *E. coli*) in which aromatic amino acid hydroxylases have been reported to occur, a DHPR regenerates the reduced cofactor tetrahydropterin, which is oxidized during the hydroxylase reaction (21). The metabolic role of these hydroxylases, however, is not clear.

Trimethoprim (TMP) is a powerful inhibitor of prokaryotic

DHFRs, but when such a DHFR is overexpressed from a plasmid, it may confer TMP resistance on an *E. coli* host (5, 13, 19, 20). We have used this approach in order to isolate a *dyrA* gene from *Thermus thermophilus*. As described below, the gene selected in this way proved, however, to code for a dehydrogenase (*T. thermophilus* dehydrogenase [DH<sub>T<sub>r</sub>]) of the short-chain dehydrogenase/reductase (SDR) family (12) with both DHPR and DHFR activities. No *T. thermophilus* *dyrA* homologue could be isolated by this approach; also, there appears to be no DHFR gene in the *Thermus* genome (A. Henne, personal communication).</sub>

## MATERIALS AND METHODS

**Culture conditions.** *E. coli* strains were grown at 37°C in rich liquid medium 853 (19) or in solid medium with added 1.5% agar (Difco). Kanamycin (KAN) at 50 μg ml<sup>-1</sup> or KAN plus 10 mM TMP was added to the medium for bacteria harboring recombinant plasmids. *T. thermophilus* HB27 was grown at 72°C in a medium containing (per liter) 9 g of tryptic soy broth (Difco), 4 g of yeast extract (Difco), and 3 g of NaCl; the pH of this medium was adjusted to 7.5 (11).

**Cloning the DH<sub>T<sub>r</sub></sub> gene.** Restriction enzymes and T4 ligase were purchased from Boehringer Mannheim. *T. thermophilus* HB27 genomic DNA partially digested with the enzyme *Sau3A* was used to construct a genomic λZAP DNA library in the pBK-CMV vector (Stratagene) according to the manufacturer's instructions. This library was used for the transformation of *E. coli* strain XL1-Blue MRF (Stratagene) as described in Results.

**Enzyme assays.** DHFR activity was assayed as described in reference 19 except that the buffer was 50 mM potassium phosphate (pH 6.5) and the temperature was as indicated in the text. The DHPR assay was run with 50 mM potassium phosphate buffer at pH 7.0 and at 35°C with a 0.1 mM concentration of the cofactor (NADPH or NADH), 0.1 mM pteridine, and 6,7-dimethyl-7,8-dihydropterine in the quinonoid form (qPtH<sub>2</sub>), which was obtained from the 5,6,7,8-tetrahydro form and an equimolar amount of 2,6-dichloroindophenol. One unit of reductase activity was defined as the amount of activity required to convert 1 nmol of NADPH per min.

\* Corresponding author. Mailing address: Microbiology, Vrije Universiteit Brussel, B-1070 Brussels, Belgium. Phone: 32 2 526 72 75. Fax: 32 2 526 72 73. E-mail: nglansdo@vub.ac.be.

† Present address: Department of Human Genetics, KULeuven/VIB, B-3000 Leuven, Belgium.

‡ Present address: Diabetes Research Center, Vrije Universiteit Brussel, B-1090 Brussels, Belgium.

DrOAR	<b>M</b> THNEISPAPAR <b>V</b> AL	<b>V</b> TGSSRGLGRAMALR	<b>L</b> AQDGFTVAVHYGRG	<b>E</b> AEAQOVAADIRAAG	60
EcOAR	-----MNFEGK <b>I</b> AL	<b>V</b> TGASRGI <b>G</b> RAIAET	<b>L</b> AARGAKVIG-TATS	<b>E</b> NGAQATSDYLGANG	53
TtDH	-----M <b>R</b> TAL	<b>V</b> TGS <b>A</b> KGI <b>G</b> RAILLA	<b>L</b> AREGYAVAVHYRTS	<b>E</b> ALAEATRQEA <b>E</b> ALG	50
LmPTR1	-----MTAP <b>T</b> VP <b>V</b> AL	<b>V</b> TGAAKRLGRSIAEG	<b>L</b> HAE <b>G</b> YAVCLHYHRS	<b>A</b> AEANALSATLNARR	55
LmqDPR	-----MKNVL	<b>L</b> IGARGALGRAVANA	<b>F</b> ANGKWSITS---VD	<b>Q</b> AAAVQQGDECCA <b>V</b> N	47
RatDHPR	----MAAS <b>E</b> ARRVL	<b>V</b> YGGRGALGSR <b>C</b> VQA	<b>F</b> RARNW <b>V</b> AS---ID	<b>V</b> VENEASAS <b>V</b> IVKM	53
		<u><b>TGxxxGxG</b></u>			
DrOAR	<b>G</b> -AAQVFGADLSQPA	<b>N</b> AGT-----	-----LVEDVIAAL	<b>G</b> RLDVLV <b>N</b> NAGITRD	102
EcOAR	----KGLMLN <b>V</b> TDP <b>A</b>	<b>S</b> IES-----	-----VLEKIRAEF	<b>G</b> EV <b>D</b> ILV <b>N</b> NAGITRD	92
TtDH	<b>V</b> -KA <b>I</b> KVRADL <b>T</b> REE	<b>E</b> VDR-----	-----LVEEVRYHL	<b>G</b> GV <b>G</b> VL <b>V</b> N <b>V</b> GDYLY	92
LmPTR1	<b>P</b> NS <b>A</b> ITVQADLS <b>N</b> VA	<b>T</b> APVSGADGSAP <b>V</b> TL	<b>F</b> TRCAELVAAC <b>Y</b> THW	<b>G</b> RC <b>D</b> VLV <b>N</b> NASS <b>F</b> YP	115
LmqDPR	<b>P</b> ---ASSIEELQ <b>Q</b> AYK	<b>S</b> AVT-----	-----GL	<b>-</b> K <b>V</b> DAVIN <b>V</b> AG <b>G</b> WAG	80
RatDHPR	<b>T</b> ---DSFTEQADQ <b>V</b> T <b>A</b>	<b>E</b> VGK-----	-----LLGD	<b>Q</b> K <b>V</b> DA <b>I</b> LCV <b>A</b> GGWAG	89
				<u><b>NNAG</b></u>	
DrOAR	<b>G</b> LAI <b>R</b> MKDE-----	-----D	<b>W</b> DAVLQTNLSS <b>A</b> FAA	<b>C</b> RAAL <b>K</b> HMMK <b>N</b> RSGR	142
EcOAR	<b>N</b> L <b>L</b> MRMKDE-----	-----E	<b>W</b> ND <b>I</b> ETNLSS <b>V</b> FRL	<b>S</b> KAVMRAM <b>K</b> KRHGR	132
TtDH	<b>K</b> PI <b>E</b> EV <b>S</b> LE-----	-----E	<b>W</b> RW <b>I</b> LD <b>T</b> N <b>L</b> TAT <b>F</b> LL	<b>T</b> QRV <b>L</b> PLMVA <b>Q</b> GFGR	132
LmPTR1	<b>T</b> PL <b>L</b> RNDE <b>D</b> GHE <b>P</b> CV	<b>G</b> DREAMETATAD <b>L</b> FG	<b>S</b> NA <b>I</b> AP <b>F</b> FL <b>K</b> AFAH	<b>R</b> FAG <b>T</b> PA <b>K</b> HR <b>G</b> T <b>N</b> YS	175
LmqDPR	<b>G</b> S <b>V</b> AD <b>A</b> STAA-----	-----S	<b>T</b> ELMLR <b>Q</b> SL <b>F</b> SS <b>V</b> AA	<b>A</b> H <b>V</b> FS <b>T</b> Q <b>G</b> E <b>K</b> NG <b>L</b> LL	121
RatDHPR	<b>G</b> NA <b>K</b> SK <b>S</b> L <b>F</b> K-----	-----N	<b>C</b> DL <b>M</b> W <b>K</b> Q <b>S</b> IF <b>T</b> STIS	<b>S</b> HL <b>A</b> T <b>K</b> HL <b>K</b> E <b>G</b> GL <b>L</b> T	130
			<u><b>N</b></u>		
DrOAR	<b>I</b> V <b>N</b> VSS <b>V</b> VA--LAG <b>N</b>	<b>P</b> GQ <b>A</b> NYVASKAG <b>L</b> IG	<b>L</b> TRALAKE <b>Y</b> GG <b>R</b> GIT	<b>V</b> NA <b>I</b> AP <b>G</b> F <b>I</b> ES <b>D</b> MTA	200
EcOAR	<b>I</b> ITIGSV <b>V</b> G--TM <b>G</b> N	<b>G</b> GQ <b>A</b> NYAA <b>K</b> AG <b>L</b> IG	<b>F</b> SK <b>S</b> LAREV <b>A</b> S <b>R</b> GIT	<b>V</b> N <b>V</b> VAP <b>G</b> FIET <b>D</b> MTR	190
TtDH	<b>I</b> V <b>N</b> LG <b>Y</b> AGAG <b>N</b> LLAR	<b>T</b> HIT <b>P</b> <b>Y</b> <b>V</b> I <b>A</b> K <b>T</b> G <b>V</b> IL	<b>Y</b> TK <b>A</b> I <b>A</b> K <b>R</b> FA <b>A</b> SGIT	<b>A</b> N <b>V</b> VAP <b>G</b> VA <b>E</b> NS <b>V</b> SK	192
LmPTR1	<b>I</b> IN <b>M</b> VD <b>A</b> MT <b>N</b> --Q <b>P</b> L	<b>L</b> G <b>Y</b> TI <b>Y</b> T <b>M</b> A <b>K</b> G <b>A</b> LE <b>G</b>	<b>L</b> TR <b>S</b> AA <b>E</b> LE <b>L</b> AP <b>L</b> Q <b>I</b> R	<b>V</b> NG <b>V</b> GP <b>L</b> SV <b>L</b> V <b>D</b> DM	233
LmqDPR	<b>L</b> T <b>G</b> AA <b>A</b> AV <b>S</b> ---PT	<b>P</b> GM <b>I</b> GY <b>T</b> A <b>K</b> SA <b>V</b> H <b>F</b>	<b>L</b> C <b>Q</b> SA <b>E</b> DP <b>S</b> VL <b>P</b> TD	<b>A</b> SV <b>L</b> AI <b>L</b> PT <b>I</b> L <b>D</b> PT <b>P</b> G	177
RatDHPR	<b>L</b> AG <b>A</b> KA <b>A</b> L <b>D</b> ---G <b>T</b>	<b>P</b> GM <b>I</b> GY <b>G</b> MA <b>K</b> GA <b>V</b> H <b>Q</b>	<b>L</b> C <b>Q</b> SLAG <b>K</b> NS <b>G</b> MP <b>S</b> G	<b>A</b> AA <b>I</b> AV <b>L</b> VP <b>T</b> L <b>D</b> TP <b>M</b>	186
		<u><b>YxxxK</b></u>		<u><b>PG</b></u>	
DrOAR	<b>K</b> LPED <b>T</b> KK <b>Q</b> Y <b>Q</b> AN <b>I</b> P	<b>L</b> AR <b>F</b> G <b>Q</b> PE <b>E</b> VA <b>A</b> L <b>V</b> A	<b>F</b> LASEGAG <b>Y</b> IT <b>G</b> Q <b>T</b> I	<b>G</b> VD <b>G</b> GM <b>N</b> PN-----	254
EcOAR	<b>A</b> LS <b>D</b> D <b>Q</b> RAG <b>I</b> L <b>V</b> Q <b>V</b> P	<b>A</b> GR <b>L</b> GG <b>A</b> Q <b>E</b> IA <b>N</b> AV <b>A</b>	<b>F</b> LAS <b>D</b> EAA <b>Y</b> IT <b>G</b> ET <b>L</b>	<b>H</b> V <b>N</b> GG <b>M</b> Y <b>M</b> V-----	244
TtDH	<b>P</b> LH-----E <b>I</b> P	<b>M</b> GR <b>L</b> AL <b>L</b> Q <b>E</b> IA <b>Q</b> AV <b>L</b>	<b>F</b> FF <b>V</b> REP-- <b>Y</b> L <b>T</b> G <b>Q</b> V <b>L</b>	<b>E</b> V <b>A</b> GG <b>W</b> N <b>L</b> -----	234
LmPTR1	<b>P</b> PA <b>V</b> W <b>E</b> G--HR <b>S</b> K <b>V</b> PL	<b>Y</b> QR <b>D</b> SS <b>A</b> AE <b>V</b> SD <b>V</b> VI	<b>F</b> LC <b>S</b> SK <b>A</b> K <b>Y</b> IT <b>G</b> TC <b>V</b>	<b>K</b> VD <b>G</b> GS <b>L</b> TR <b>A</b> -----	288
LmqDPR	<b>N</b> RS-----AM <b>P</b> HAD	<b>R</b> ST <b>W</b> TS <b>L</b> ED <b>V</b> A <b>Q</b> Q <b>I</b> V	<b>E</b> W <b>S</b> NG <b>S</b> RR <b>P</b> AS <b>G</b> SL <b>V</b>	<b>K</b> IV <b>T</b> EN <b>S</b> K <b>R</b> TR <b>F</b> IV--	229
RatDHPR	<b>N</b> RR-----SM <b>P</b> EAD	<b>F</b> SS <b>W</b> TP <b>L</b> EF <b>L</b> V <b>E</b> TF <b>H</b>	<b>D</b> WT <b>G</b> N <b>K</b> RP <b>N</b> SG <b>S</b> L <b>I</b>	<b>Q</b> V <b>V</b> TT <b>D</b> G <b>K</b> T <b>E</b> LT <b>P</b> AY <b>F</b>	241

FIG. 1. Multiple alignment of amino acid sequences of selected reductases belonging to the SDR family, in order of similarity: *D. radiodurans* 3-oxoacyl-(acyl-carrier-protein) reductase (DrOAR), the homologous protein from *E. coli* (EcOAR), *T. thermophilus* DHPR (TtDH), DHPR 1 (nonquinonoid) from *L. major* (LmPTR1), quinonoid DHPR from *L. major* (LmqDPR), and rat DHPR. Bold type indicates identical residues in at least four of the six sequences. Sites conserved in the SDR family are underlined.

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using a Pharmacia PhastSystem with a discontinuous buffer system and a continuous 8 to 25% gradient gel. Gels were stained with Coomassie brilliant blue. Protein standards (0.5 µg each on the gel; Pharmacia) used for the estimation of subunit molecular masses were phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and  $\alpha$ -lactalbumin (14.4 kDa).

**Native gel electrophoresis for enzyme activity staining.** The Bio-Rad Prep-Cell system was used at a basic pH. The running buffer was Tris-HCl at pH 10.0. The gel (about 5 ml) was composed of 1 volume of solution A (running buffer supplemented with 0.46 ml of *N,N,N',N'*-tetramethylethylenediamine/100 ml), 2 volumes of solution B (10% acrylamide, 2.5% *N,N*-methylene bisacrylamide), and 4 volumes of solution C (0.14% ammonium persulfate). The loading buffer contained 25 mM Tris, 100 mM glycine, 0.1% bromophenol blue, and 10% saccharose. About 5 µg of protein was loaded on the gel and run for 1 h at 150 V.

## RESULTS

**Isolation of the  $DH_{Tt}$  gene and analysis of the nucleotide sequence.** The  $\lambda$ ZAP *T. thermophilus* genomic library was plated for titration on rich medium containing KAN and for selection on plates containing KAN and TMP. For ca. 4,000 colonies screened (with a mean insert size of 2.8 kb and thus a fragment sampling covering six times the *T. thermophilus* genome), there were 20 colonies on the KAN-TMP plates (with insert sizes ranging from 1.5 to 4.0 kb). Crude extracts from overnight cultures of these recombinant strains were cleared by centrifugation, treated for 20 min at 70°C to inactivate resident *E. coli* DHFR activity, and assayed for DHFR activity at 50°C as described previously (19). All of these extracts displayed similar thermoresistant DHFR activities (on average, 2.5 U/mg

of protein), whereas neither the extract from the control *E. coli* *dyrA*<sup>+</sup> strain nor that from *E. coli* transformed with a plasmid carrying an unrelated gene (pTAD1GDH [3]) displayed measurable activity.

The smallest DNA insert that could be recovered from the cognate phagemids (pDAT1-20) by *Eco*RI/*Pst*I digestion and that proved to be able to confer resistance to TMP was 1.7 kb long. It contained only one 702-bp open reading frame (ORF) encoding a putative polypeptide of 234 amino acids, named  $DH_{Tt}$ . This ORF was found by PCR amplification to be present in all recombinant pDATs. The GC content was high (69%), as expected for a *Thermus* gene. Surprisingly, the  $DH_{Tt}$  sequence showed no similarity to that of any DHFR but instead with sequences from representatives of the SDR family (Fig. 1). The sequence contains residues that are highly conserved throughout the SDR family, such as the NAD<sup>+</sup>/NADP<sup>+</sup> co-factor-binding motif (GXXXGXG, positions 8 to 14), the NNAG motif (NNVG in  $DH_{Tt}$ , positions 85 to 88), and the YXXXK motif (residues 153 to 157), claimed to be crucial for substrate binding and catalysis by SDR family proteins (Fig. 1) (7, 8).

**Overexpression and characterization of the  $DH_{Tt}$  gene product.** The  $DH_{Tt}$ -encoding gene was subcloned from pDAT5 into pET24a, an IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside)-inducible expression vector, yielding plasmid pTTDH. The ORF was amplified by PCR by using oligonucleotides designed to bring the ATG start codon in frame with the vector *Nde*I cloning site. The highest DHFR specific activity was obtained from cells grown at 37°C up to a density of  $4 \times 10^8$  cells/ml and

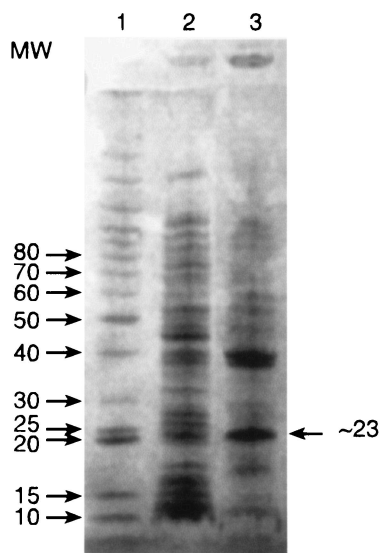


FIG. 2. Partial purification of  $DH_{7T}$  after cold precipitation, as shown by SDS-PAGE. Lane 1, molecular weight (MW) markers (in thousands, indicated on the left); lane 2, *E. coli* (pTTDH) clarified crude extract; lane 3, cold precipitate resuspended in 25 mM Tris-HCl (pH 10).

then induced by 1 mM IPTG and further incubated at 22°C for 4 h. The specific activity after 15 min of incubation of the extract at 50°C was 14.9 U/mg of protein at 35°C in *E. coli* pTTDH, compared to 2.9 U/mg of protein in pDAT5. This activity proved insensitive to 600  $\mu$ M methotrexate and 10  $\mu$ M TMP, concentrations which are fully inhibitory for *E. coli* DHFR (5).

Attempts at purifying the protein were hampered by low solubility, probably due to the high content of hydrophobic residues (50% L+V+I+M+F+A+P+W). *E. coli* pTTDH extracts kept for a few hours at 4°C formed a white precipitate that contained the activity and could be partly solubilized at pH 10; further dilution of such preparations under assay conditions fully solubilized them. SDS-PAGE of the cold precipitate gave a major band (Fig. 2) with a molecular mass of about 23  $\pm$  3 kDa, consistent with the 25.4-kDa value calculated from the sequence. Moreover, Edman degradation showed this band to consist of only one protein with an N-terminal amino acid sequence exactly like that deduced from the gene: MRTALV TGSAAK. Fast protein liquid chromatography (data not shown) gave a broad estimate of 50 to 90 kDa for the native protein; the high pI (>8.9) of this protein prevents quantitative electrophoresis by standard methods. The native enzyme is probably a dimer (like rat DHPR and several other members of the SDR family [15]), but the possibility of the occurrence of tetramers cannot be excluded.

**Enzymatic specificity of the  $DH_{7T}$  protein.** The enzyme (cold precipitate) exhibited no detectable activity with either 6-bioperin (the best substrate for *Leishmania* PTR1 [10]) or DL-6-methyl-7,8-dihydropterine (a substrate for *E. coli* DHPR [16]) under all conditions tested (pHs 5.5 to 8.5 with different buffers in the presence of salts) (Table 1). Activity could be detected only with qPtH<sub>2</sub>. The enzyme was active with NADPH. With NADH, the background oxidation was too elevated for the

TABLE 1. Specific activity of DHPR from *T. thermophilus* with various substrates<sup>a</sup>

Substrate	Sp act (U/mg of protein)
6-Bioperidin.....	<0.05
PtH <sub>2</sub> .....	<0.05
qPtH <sub>2</sub> .....	26.2 $\pm$ 2.7
Potassium ferricyanide.....	<0.05
FH <sub>2</sub> .....	5.2 $\pm$ 1.1

<sup>a</sup> Activity was measured at 35°C in 50 mM potassium phosphate buffer (pH 7.5) in the presence of 0.1 mM NADPH and 0.1 mM pteridine.

detection of activity (but see the discussion of the next experiment). In contrast to *E. coli* DHPR, which is a flavoprotein (16) and exhibits pteridine-independent oxidoreductase activity with potassium ferricyanide, the *Thermus* enzyme did not exhibit such activity (Table 1); it thus appears similar to mammalian DHPRs, which do not contain a flavin prosthetic group. When assayed under the same conditions, the activities of FH<sub>2</sub> (DHFR) and qPtH<sub>2</sub> (DHPR) in the presence of NADPH are present at an  $\sim$ 1:5 ratio (Table 1).

**Activity staining on native gel electrophoresis gels.** The activity staining method used 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), which is reduced by the product of the enzymatic reaction to a formazan with a maximum  $\lambda$  of 560 nm. To allow the migration of the solubilized protein, basic gels were prepared (see Materials and Methods). *Thermotoga maritima* DHFR was used as a control, since it also has a high pI, has a rather similar  $M_r$ , and possesses DHFR activity but no DHPR activity. One gel was loaded with  $DH_{7T}$  in lane 1 and with *T. maritima* DHFR in lanes 2 to 8; the second gel was loaded inversely. After the electrophoresis of both gels in parallel, gel pieces corresponding to the different lanes were cut (Fig. 3). For each gel, pieces 1 and 2 were stained with Coomassie blue and pieces 3 to 8 were incubated at 37°C in a solution containing 50 mM phosphate buffer (pH 7.0), 0.5 mM NADH or NADPH, and 5 mg of MTT/ml. In addition, incubation solutions for lanes 5 and 6 contained 0.5 mM FH<sub>2</sub>, but lanes 7 and 8 contained 0.5 mM qPtH<sub>2</sub> (formed from 0.5 mM PtH<sub>4</sub> and 0.5 mM 2,6-dichloroindophenol). After 30 min of incubation, an intensely stained band appeared for *T. maritima* DHFR (Fig. 3A) incubated with FH<sub>2</sub> and NADPH (lane 6). Two hours later, a faint *T. maritima* DHFR band appeared on the gel pieces incubated with FH<sub>2</sub> and NADH (lane 5). At that time, bands were also detectable on the gel pieces containing  $DH_{7T}$  and incubated either with FH<sub>2</sub> and NADH or NADPH (Fig. 3B, lanes 5 and 6) or with qPtH<sub>2</sub> and NADH or NADPH (Fig. 3B, lanes 7 and 8).  $DH_{7T}$  exhibited DHFR and DHPR activities with both NADH and NADPH, but no preference for one of the cofactors was detectable under these conditions.

## DISCUSSION

The main points of this study can be summarized as follows. The *T. thermophilus* genome harbors a gene coding for a reductase ( $DH_{7T}$ ) which displays both DHPR and DHFR activities with NADH and NADPH as cofactors and possesses specific features of members of the SDR protein family. The

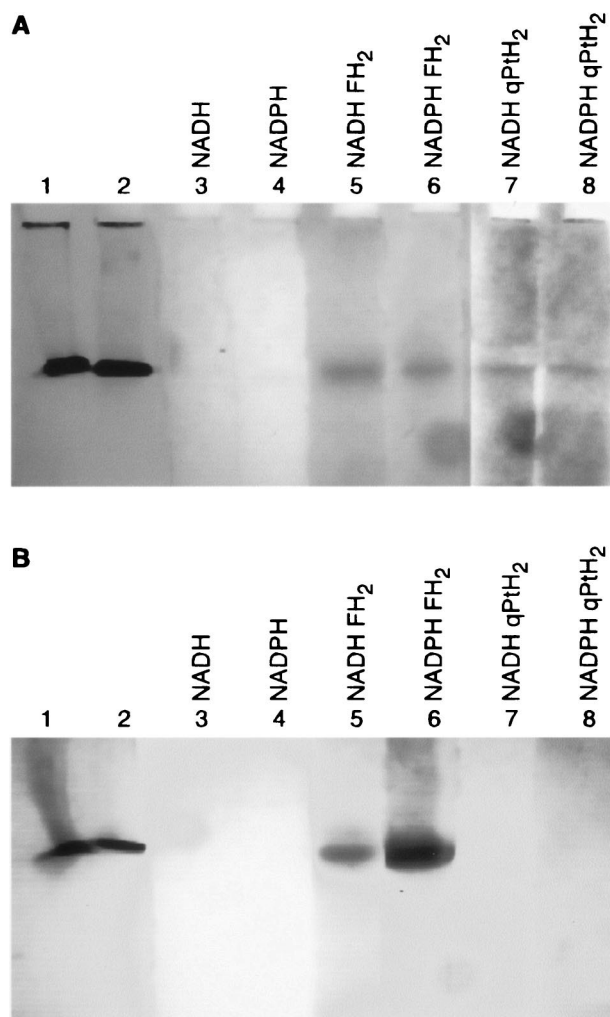


FIG. 3. Activity staining of  $DH_{Tt}$  (A) and *T. maritima* DHFR (B) on electrophoresis gels. After electrophoresis of the proteins (see the text), gel pieces were either stained with Coomassie blue (lanes 1 and 2) or incubated as described in the text and as indicated above the lanes in the presence of substrates and/or cofactors and 5 mg of MMT/ml.

DHFR activity of this reductase is considerable, i.e., about 20% of the DHPR activity detected with  $qPtH_2$  as the substrate.  $DH_{Tt}$  has no pteridine-independent oxidoreductase activity and is insensitive to concentrations of methotrexate and TMP that fully inhibit *E. coli* DHFR. Moreover, none of the clones that we could isolate from an apparently representative *Thermus* library expressed a true DHFR, and most significantly, no corresponding *dyrA* gene appears to exist in the *T. thermophilus* genome; indeed, BLAST probing of the complete genome (A. Henne, personal communication) did not reveal any sequence homologous to the corresponding genes from *T. maritima* and *Deinococcus radiodurans* (which is the closest known relative of *Thermus* [18]). It seems that the essential role of DHFR in cellular metabolism is challenged by our findings, since an unrelated dehydrogenase of the SDR family may fulfill the function classically assumed by a DHFR. It has already been reported that a number of bacteria and some

archaea appear to lack a DHFR gene (9), but the substitute function had not yet been identified in any of these organisms. A bifunctional DHPR/DHFR such as  $DH_{Tt}$  is clearly a good candidate for the agent responsible for this substitute function. Moreover, just as thymidylate may be synthesized by a classical ThyA protein or by a quite unrelated ThyX flavoprotein (9), it appears that the concomitant reduction of  $FH_2$  or various pteridines may be carried out by an enzyme without a flavin prosthetic group (like  $DH_{Tt}$  or the nonquinonoid pteridine reductase PTR1 of *Leishmania major* [2]) or by an unrelated flavoprotein, as in *E. coli*. The evolutionary origin of these functional redundancies is an intriguing question; temperature by itself is not likely to be the discriminating evolutionary factor, since *T. maritima* DHFR and  $DH_{Tt}$  are both thermophilic enzymes.

The role of  $DH_{Tt}$  as dihydropteridine reductase in *Thermus* is not known. In particular, whether *Thermus* possesses aromatic amino acid hydroxylases using a tetrahydropterin as a cofactor (see the introduction and reference 21) remains to be investigated.

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