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Dihydrofolate Reductase of the Extremely Halophilic Archaebacterium *Halobacterium volcanii*

THE ENZYME AND ITS CODING GENE*

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***Halobacterium volcanii* mutants that are resistant to the dihydrofolate reductase inhibitor trimethoprim contain DNA sequence amplifications. This paper describes the cloning and nucleic acid sequencing of the amplified DNA sequence of the *H. volcanii* mutant WR215. This sequence contains an open reading frame that codes for an amino acid sequence that is homologous to the amino acid sequences of dihydrofolate reductases from different sources. As a result of the gene amplification, the trimethoprim-resistant mutant overproduces dihydrofolate reductase. This enzyme was purified to homogeneity using ammonium sulfate-mediated chromatographies. It is shown that the enzyme comprises 5% of the cell protein. The amino acid sequence of the first 15 amino acids of the enzyme fits the coding sequence of the gene. Preliminary biochemical characterization shows that the enzyme is unstable at salt concentrations lower than 2 M and that its activity increases with increase in the KCl or NaCl concentrations.**

The extremely halophilic archaebacteria of the genus *Halobacteriacae* are adapted to survive and grow at extreme salinities. In order to maintain an osmotic balance, these bacteria accumulate KCl intracellularly to concentrations that can reach 4 M (7). Therefore, their entire biochemical system is adapted to function at very high salt concentrations. Most of the enzymes of halobacteria are active and stable at high salt concentrations and become inactive below monovalent salt concentrations of about 2 M (14, 16). In all instances investigated it was found that the amino acid composition of these halophilic proteins has an excess of negatively charged amino acids over positively charged amino acids. It is, however, unclear how this fact influences the functional adaptation of the enzymes to high salinities. A thorough analysis of the interaction of the halophilic enzymes malate dehydrogenase and glutamate dehydrogenase of *H. marismortui* with salts demonstrated that these proteins bind unusual quantities of salt and water (30). This binding is found to be a specific property of the native enzyme, as it is significantly reduced upon denaturation. So far, lack of detailed determination of the three-dimensional structure of these halophilic enzymes hampers the elaborate analysis of these protein-salt-water interactions.

The enzyme dihydrofolate reductase (DHFR)[1] is an excellent subject for comparative studies on the relationships between structure and function. The three-dimensional structures of homologous dihydrofolate reductases of *Escherichia coli* (4), *Lactobacillus casei* (9), chicken (28), mouse (25), and human (19) were determined at high resolution. In spite of considerable variations in the amino acid sequences among these proteins, the three-dimensional structures show a high degree of homology. The catalytic mechanism of *E. coli* DHFR has recently been studied in detail (8). In addition, site-directed mutagenesis of the enzyme has been applied in order to answer questions with respect to the structure-function relationships of the protein that were raised by the high resolution x-ray structure (1, 5, 13, 27).

H. volcanii, which is usually sensitive to the dihydrofolate reductase competitive inhibitor trimethoprim, gives rise to spontaneous resistant mutants at frequencies of 10^{-10}–10^{-9}. In an earlier report it was demonstrated that these mutants are the result of gene amplifications (22). It was also shown that these amplifications are associated with an overproduction of a 20-kDa protein. It was thus hypothesized that the resistance to trimethoprim is the result of amplification of the gene coding for DHFR which in turn causes the overproduction of this enzyme.

This paper describes the purification and the nucleic acid sequence determination of the gene coding for *H. volcanii* dihydrofolate reductase. In addition, it describes the purification of the enzyme to homogeneity and presents data on the effect of salt concentration on the catalytic activity which clearly prove that the enzyme requires high salt concentration for its biological function.

EXPERIMENTAL PROCEDURES[2]

RESULTS AND DISCUSSION

Previous work (22) has shown that the spontaneous acquisition of resistance to trimethoprim by *H. volcanii* can be

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05088.

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[1] The abbreviations used are: DHFR, dihydrofolate reductase; h-DHFR, halobacterial dihydrofolate reductase; MES, 4-morpholinepropanesulfonic acid; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

[2] Portions of this paper (including "Experimental Procedures," Figs. 1–3, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

correlated to an amplification of a particular region in the genome. In addition, all the resistant mutants were shown to overproduce a protein of about 20 kDa. These two observations suggested that the basis for the trimethoprim resistance is an amplification of the gene coding for DHFR which in turn causes an overproduction of the enzyme. In order to substantiate this hypothesis, the DHFR of an overproducing strain has been purified to homogeneity as described under "Experimental Procedures." The purification methodology was specially designed for halophilic enzymes which are usually unstable at low salt concentrations. The use of ammonium sulfate-mediated chromatography in the purification of halobacterial enzymes was originally introduced by Mevarech *et al.* (18) and became a standard method in the purification of halophilic enzymes.

The degree of purification of the homogeneous enzyme is 20-fold indicating that it comprises about 5% of the total cell protein. The amino acid sequence of the first 15 residues of the enzyme is,

NH_2-Met-Glu-Leu-Val-Ser-Val-Ala-
Ala-Leu-Ala-Glu-Asn-Arg-Val-Ile

In order to determine whether the amplified DNA sequence codes for DHFR, we have cloned the 1.9-kilobase long amplified DNA fragment of the trimethoprim-resistant mutant WR 215 into the plasmid pUC19 (pDR7). A 981 base pair long *Pst*I-*Kpn*I subclone of pDR7 (pDR7.2) was sequenced entirely using the strategy illustrated in Fig. 4. The nucleotide sequence of one of the strands is given in Fig. 5. Below the DNA sequence the deduced amino acid sequence is given, starting with Met and extending for 162 amino acids. The sequence of the first 15 codons is in agreement with the sequence of the first 15 amino acids of the DHFR given above. The first ATG of the coding region is preceded by a sequence 5′ GGAG which might serve as a ribosomal binding site as it is complimentary to the sequence 5′ CUCC at the 3′ end of the 16 S rRNA of this organism (10).

The codon usage of the DHFR gene is summarized in Table II. As can be seen there is a considerable bias toward codons of higher G+C as is expected from the fact that the overall G+C content of the DNA of *H. volcanii* is 66.5% (23).

A preliminary SI protection analysis to determine the 5′ end of the mRNA shows that the entire *Pst*I-*Kpn*I fragment is protected (data not shown). It seems, therefore, that the gene coding for DHFR is part of a long multicistronic message.

Comparison of the Primary Structure of H. volcanii DHFR to Structures of DHFRs from Other Sources—The primary structure of DHFRs from various sources were determined previously. Moreover, the crystal structures of DHFRs of *E. coli*, *L. casei*, and chicken liver were determined to high resolution (4, 28). The overall backbone folding of the various molecules is very similar even though the degree of amino-acid sequence homology is less than 30%. Using the three-dimensional structures, the primary structures of the DHFRs of *E. coli*, *L. casei*, and chicken liver were aligned (28). Based on this sequence alignment the amino acid sequence of DHFR of *H. volcanii* (h-DHFR) was aligned with the sequences of the other DHFRs as is shown in Fig. 6. The degrees of amino acid sequence homology between h-DHFR and the DHFRs of *E. coli*, *L. casei*, and chicken are 30, 23, and 26%, respectively. The alignments of the amino acid sequences of the different

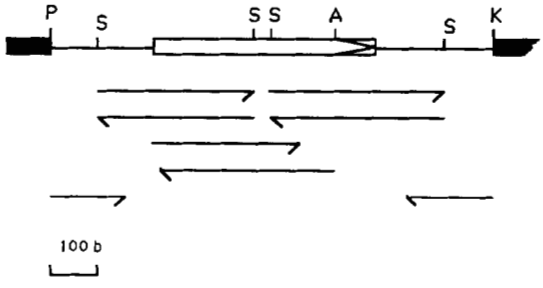

FIG. 4. **Partial restriction map and sequencing strategy of the gene coding for DHFR in *H. volcanii* and its flanking regions.** The *thicker line* represents vector sequences. The *large open arrow* in the map designates the region coding for the gene. The *arrows* beneath the restriction map illustrate the direction and the length of the sequence determined. The enzymes used in the map are *P*, *Pst*I; *S*, *Sal*I; *A*, *Ava*I; *K*,KpnI.

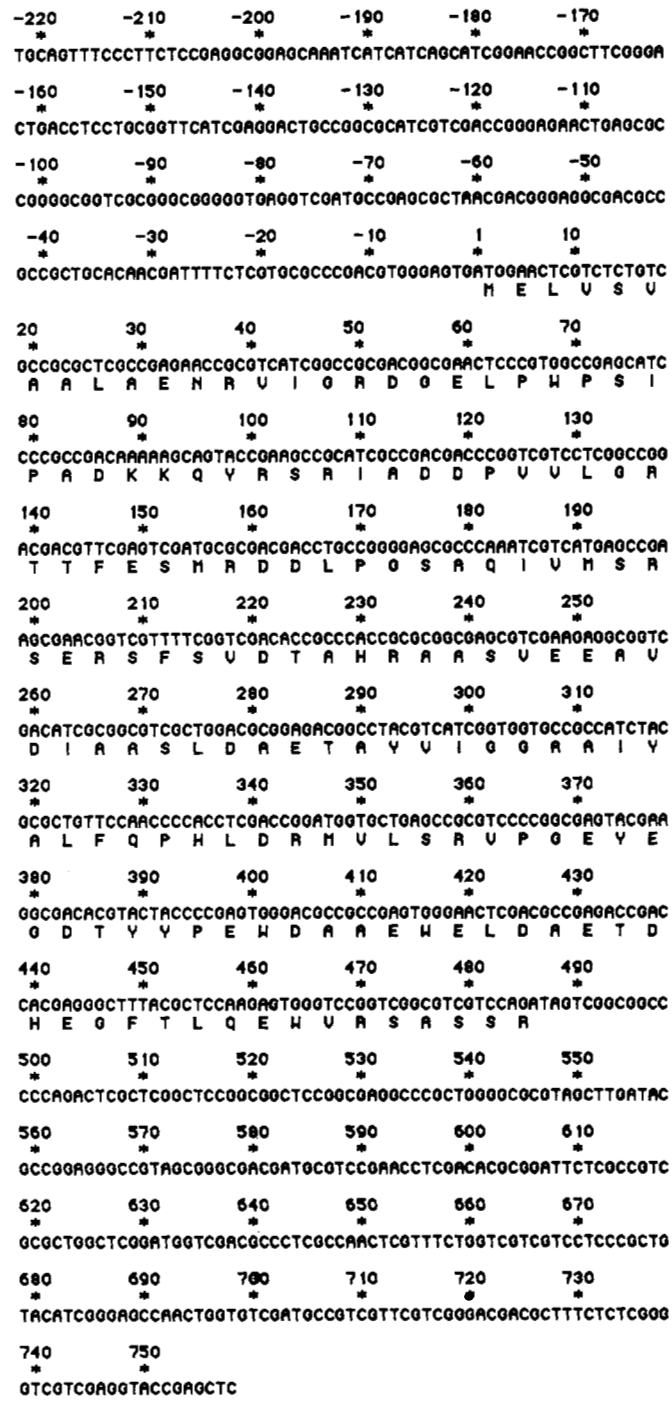

FIG. 5. **The nucleotide sequence and the deduced amino acid sequence of h-DHFR.** The coding region starts at position 1 as indicated.

TABLE II

Codon usage in the H. volcanii DHFR gene

TTT	Phe	2	TCT	Ser	1	TAT	Tyr	0	TGT	Cys	0
TTC	Phe	2	TCC	Ser	1	TAC	Tyr	6	TGC	Cys	0
TTA	Leu	0	TCA	Ser	0	TAA		0	TGA		0
TTG	Leu	0	TCG	Ser	6	TAG		1	TGG	Trp	4
CTT	Leu	0	CCT	Pro	0	CAT	His	0	CGT	Arg	0
CTC	Leu	7	CCC	Pro	4	CAC	His	3	CGC	Arg	6
CTA	Leu	0	CCA	Pro	0	CAA	Gln	3	CGA	Arg	2
CTG	Leu	4	CCG	Pro	4	CAG	Gln	1	CGG	Arg	4
ATT	Ile	0	ACT	Thr	0	AAT	Asn	0	AGT	Ser	0
ATC	Ile	7	ACC	Thr	2	AAC	Asn	1	AGC	Ser	7
ATA	Ile	0	ACA	Thr	0	AAA	Lys	1	AGA	Arg	1
ATG	Met	4	ACG	Thr	5	AAG	Lys	1	AGG	Arg	0
GTT	Val	0	GCT	Ala	0	GAT	Asp	0	GGT	Gly	2
GTC	Val	12	GCC	Ala	12	GAC	Asp	14	GGC	Gly	6
GTA	Val	0	GCA	Ala	0	GAA	Glu	6	GGA	Gly	0
GTG	Val	1	GCG	Ala	9	GAG	Glu	10	GGG	Gly	1

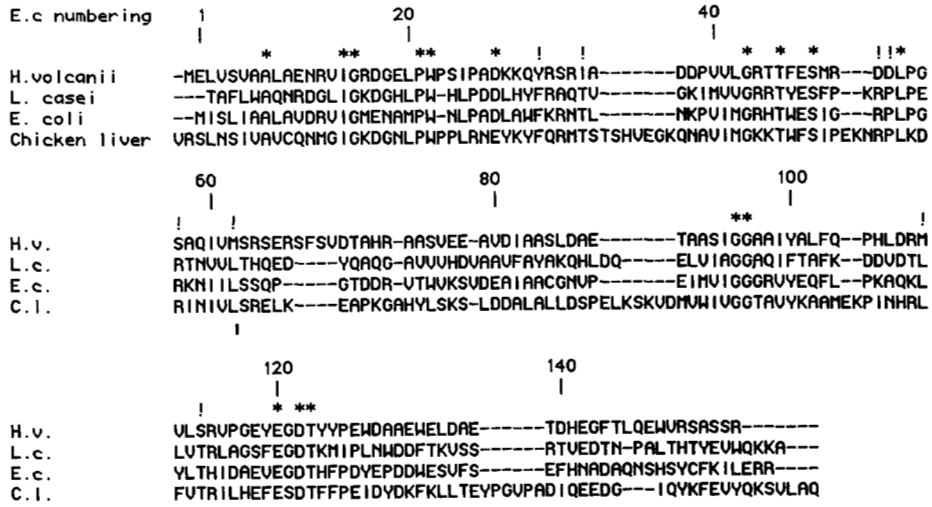

FIG. 6. **Alignment of the amino acid sequence of *H. volcanii* DHFR with the amino acid sequences of DHFRs of *E. coli*, *L. casei*, and chicken liver.** The *E. coli numbering* is shown above the sequences. Highly conserved residues which are conserved also in h-DHFR are marked by (*) and highly conserved residues that are not conserved in h-DHFR are marked by (!).

enzymes require the introduction of deletions and insertions which are generally located in loops that connect elements of secondary structure. The larger insertions in the chicken liver enzyme are conserved among all eukaryotic enzymes so far studied and therefore might have phylogenetic significance. If so, the existence of the insertion in h-DHFR (at a position corresponding to 66 of the *E. coli* enzyme) which is unique to this archaebacterial enzyme might serve a similar phylogenetic role. The structure Pro-Trp-Pro in avian DHFR at a position equivalent to amino acid 21 of *E. coli* is also conserved in all eukaryotic DHFRs and missing from all bacterial DHFRs except from that of *H. volcanii*.

Some amino acid residues are conserved in almost all the known DHFRs (*E. coli*, *L. casei*, *Streptococcus faecium*, chicken liver, bovine liver, porcine liver, human, *Leishmania*) as summarized in Refs. 2, 28. Most of these residues are found in *H. volcanii* DHFR as well. The function of some of the residues which are conserved also in h-DHFR were inferred from the crystal structures of *E. coli*, *L. casei*, and chicken liver DHFRs. For instance, in *E. coli* Ala-7, Ser-49, and Leu-54 are probably involved in the binding of dihydrofolate and the inhibiting analog methotrexate (4, 17). The role of Asp-27 (or the corresponding Glu in eukaryotic DHFR) is the protonation of the N-5 nitrogen of the dihydrofolate molecule. Replacement of this Asp by Ser or Asn reduce the catalytic activity of the enzyme drastically (13, 27). The bond between Gly-95 and Gly-96 has an unusual *cis* configuration which seems to have an essential role in the conformation of the enzyme. Replacement of Gly-95 by Ala abolishes the activity entirely (27).

There are, however, residues that are conserved in all the DHFRs except for h-DHFR. These replacements are Leu-24 to Ile, Phe-31 to Tyr, Thr-35 to Ile, Arg-57 to Ser, Leu-62 to Met, and Thr-113 to Ser. Among these amino acids roles were suggested for Phe-31 and Thr-113 (5, 17). Phe-31 forms part of the hydrophobic core of DHFR, and its side chain is involved in the binding to various parts of methotrexate (20). Replacement of Phe-31 by the smaller amino acid Val reduces the binding of dihydrofolate and methotrexate. Moreover, it destabilizes the protein, probably by disrupting the close packing found in the interior of the protein. The effect of the replacement of Phe-31 by Tyr or Val on the catalytic properties of the *E. coli* enzyme was studied (6). It was found that these replacements cause a 2-fold increase in V_{max}, probably by accelerating the rate of dissociation of the product tetrahydrofolate from the enzyme. In *H. volcanii*, Phe-31 is naturally replaced by Tyr. This replacement of one aromatic side chain by another aromatic side chain might have only minor effect on the stability of the enzyme but considerable effect on its activity.

Thr-113 forms a network involving also a water molecule and Asp-27 by contributing a hydrogen bond with its hydroxyl

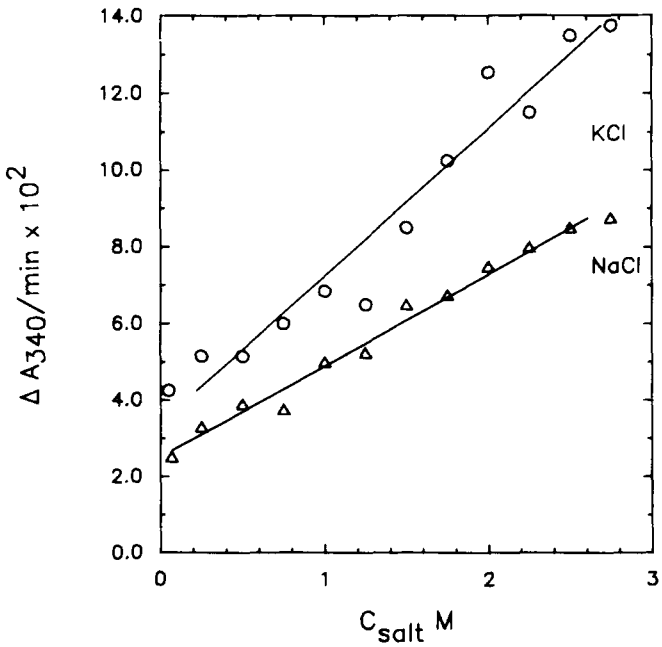

FIG. 7. **Enzymatic activity of h-DHFR as a function of salt concentration.** The activity was measured at the indicated salt concentrations in MES buffer, pH 6. Measurements were taken in the first two minutes after dilution.

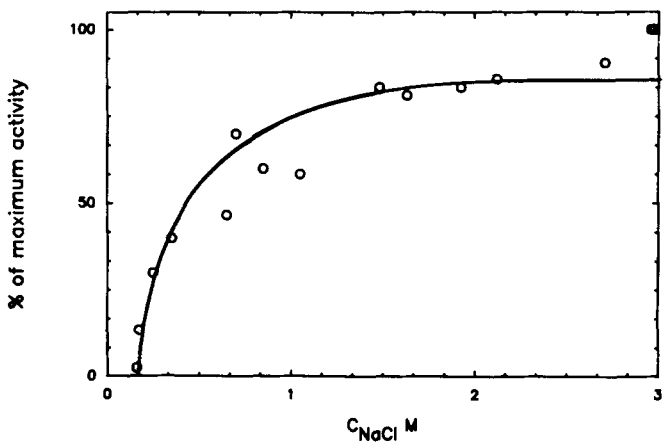

FIG. 8. **Stability of h-DHFR as a function of salt concentration.** Aliquots of the enzyme were diluted into solution containing the indicated salt concentrations and kept in these solutions for 24 h at 24 °C. Then the residual activity was measured in a solution containing 2 M NaCl.

side chain. Its replacement by Val decreases the binding of dihydrofolate 25-fold and causes a destabilization in the protein structure, although not affecting the k_{cat}. In *H. volcanii* this residue is replaced by a similar residue, Ser, which can participate in a hydrogen bond similar to Thr.

Most of the halophilic proteins are highly acidic (21). h-DHFR is also very acidic, having an excess of 15 acidic residues over basic residues. In comparison, in the enzymes from *E. coli* and *L. casei* there are 5 and 4 acidic residues in excess, respectively, and in the chicken enzyme there is 1 basic residue in excess. The negative charges of h-DHFR are spread throughout all the primary structure. Unlike h-DHFR, in the 4Fe-4S ferredoxins of *H. marismortui* (12) and *H. halobium* (11) a large fraction of the excess negative charges is concentrated in the NH_2 terminus of the protein as an extra 22-amino acid long polypeptide.

The Effect of Salt Concentration on h-DHFR—In spite of the resemblance of the structure of h-DHFR to the structures of DHFRs from non-halophilic sources, its function is fully adapted to the intracellular high salt concentration of the halobacterium. When analyzing the effect of salt concentration on halophilic enzymes, a distinction should be made between its effect on the stability of the enzyme and its effect on the individual rate constants of the catalytic reaction. In general, the stability of the enzymes increases with increase in salt concentration.

The effect of salt concentration on the enzymatic activity was determined by measuring the activity at various salt concentrations immediately after diluting the enzyme 2500-fold into solutions containing the substrates and the desired concentrations of salts at pH 6. Under these conditions the enzyme remains stable throughout the measurements. As shown in Fig. 7, the enzymatic activity increases with increasing the salt concentration. Also, KCl which is the predominant intracellular salt is more effective than NaCl.

The effect of salt concentration on the stability of h-DHFR was determined by incubating aliquots of the enzyme at various salt concentrations for 24 h at 25 °C and then assaying the residual activity at standard salt concentration. As shown in Fig. 8, the enzyme is stable at concentrations above 1.5 M NaCl. Similar results are obtained in KCl (data not shown).

Conclusion—The data presented, clearly show that *H. volcanii* DHFR is halophilic, as it is unstable at low salt concentrations and its catalytic activity is facilitated by salt. When the amino acid sequence of this enzyme is compared with those of DHFRs of non-halophilic organisms the only distinctive characteristic is its excess negative charge. It seems, therefore, that it is the distribution of these charges that has a role in the adaptation of this enzyme to function at high salt concentrations. Since amino acid sequences of other soluble enzymes of extreme halophilic organisms are so far unavailable, it is yet unknown to what extent this feature of the halophilic DHFR can be generalized to other halophilic enzymes. However, the availability of the gene coding for *H. volcanii* DHFR and the methodology of its purification will enable a systematic search for structural factors that participate in the halophilic adaptation of this enzyme.

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Supplementary Material

Dihydrofolate reductase of the extremely halophilic archaebacterium *Halobacterium volcanii*: The enzyme and its coding gene.

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Experimental Procedures

Bacterial strains: *E. coli* 71/18 (Δ[lac-pro] F'[lac I^q lac ZΔM15 pro AB] sup E) (29).*Halobacterium volcanii* WR215 (*trp* 3) (22).

Bacterial growth: *E. coli*: For plasmid work LB medium {[5 g yeast extract (Difco), 10 g trypton (Difco), 10 g NaCl] per liter} was used, for M13 bacteriophage work YT medium {[8 g pepton (Difco), 5 g yeast extract (Difco), 5 g NaCl] per liter}.
H. volcanii was grown in medium containing 2.14 M NaCl, 0.25 M $MgCl_2$, 0.029 M K_2SO_4 , 5 g yeast extract (Difco) and 5 g trypton (Difco) per liter. After autoclaving, sterile $CaCl_2$ is added to a final concentration of 1 mM and the pH is adjusted to 6.8. Trimethoprim (dissolved in DMSO) is added to a final concentration of 1 μg/ml.

Chemicals: Dihydrofolic acid and NADPH were products of Sigma. All salts employed were of analytical grade. Sepharose 4B (Pharmacia) and DEAE-cellulose DE52 (Whatman) were used.

Enzymatic assay: Enzymatic activity was measured in 1ml volume containing (final concentration): 2M KCl, 0.1M K phosphate pH 7, 0.05mM dihydrofolic acid , 0.08mM NADPH. The oxidation of NADPH and the reduction of dihydrofolate were determined at 340 nm and 25 °C using a molar absorption change of 12.3 (3). One enzyme unit is defined as the amount of enzyme that, under the above conditions, oxidizes 1μmole of NADH per minute.

Protein determination: Protein content was determined using a modified biuret reaction according to (15) with BSA as standard. This method is not sensitive to ammonium ions and, thus, enables the direct measurement of the protein content at every step of the purification process.

Protein sequence determination: Protein sequence determination was performed by Protein Sequencer model 470A (Applied Biosystems) using program 03RPTH. This instrument was coupled, on line, with PTH Analyzer model 120A (Applied Biosystems), equipped with C-18 type column (220x2.1 mm) and using the standard manufacturer gradient conditions.

SDS-polyacrylamide gel electrophoresis: The analysis of protein samples by SDS-polyacrylamide gel electrophoresis was performed according to (26). In order to desalt the samples 1/10 volume of 100% trichloroacetic acid was added and the samples were left on ice for 15 minutes; after centrifuging the samples in a minifuge (Eppendorf) the supernatant was removed and the pellet was washed several times with ether, dried and then dissolved in water.

Solvents: Solution A: 2 M KCl, 50 mM K-phosphate pH 6; Solution B: 1.5 M $(NH_4)_2SO_4$, 25 mM TrisHCl pH 8; Solution C: 2.5 M $(NH_4)_2SO_4$, 25 mM TrisHCl pH 8; Solution D: 3.5 M $(NH_4)_2SO_4$, 25 mM TrisHCl pH 8; Solution E: 3.5 M NaCl, 25 mM TrisHCl pH 7.5.

Purification of the enzyme:
1. Preparation of crude extract: Two liters culture were grown in four 500 ml flasks to a late logarithmic phase. The cells were harvested in a Sorvall GSA rotor spun at 6000 rpm for 10 minutes at 4°C. The cells were washed three times in a solution of 2.14 M NaCl, 250 mM $MgCl_2$ and were then suspended with the addition of 20 ml of 'solution A'. The cells were broken by sonication until the solution was not viscous any more (throughout the sonication the extract was kept ice cold). The extract was then centrifuged in a Sorvall SS34 rotor at 15,000 rpm for 15 minutes at 4°C. The supernatant (S1) was collected.

2. Batch DEAE-cellulose fractionation: The crude extract S1 was dialyzed against 'solution B' (two times against 1 liter). The volume was measured and an equal volume of a slurry of DEAE-cellulose DE52 in 'solution B' was added. 'Solution D' was added slowly, while stirring, to bring the final concentration of ammonium sulfate to 2.5 M. The suspension was centrifuged and the gel was suspended in 'solution C' and packed into a column. The column was washed with 'solution C' until the eluent was clear. The enzyme was, then, eluted with 'solution B' and 6 ml fractions were collected. The chromatogram is given in Figure 1. The active fractions were pooled (S2).

3. DEAE-cellulose column: The ammonium sulfate concentration of S2 was brought to 2.5 M by adding an equal volume of 'solution D'. The solution was, then, loaded on a DEAE-cellulose DE52 column that had been preequilibrated with 'solution C'. The enzyme was eluted with 'solution B' and 5ml fractions were collected. The chromatogram is given in Figure 2. The active fractions were pooled (S3).

4. Sepharose 4B column: The ammonium sulfate concentration of S3 was brought to 2.5 M by adding an equal volume of 'solution D' and the solution was loaded on a column of Sepharose 4B that had been preequilibrated with 'solution C'. The enzyme was eluted with a decreasing ammonium sulfate concentration gradient from 2.5 M to 1 M in 25 mM TrisHCl pH8 and 5 ml fractions were collected. The chromatogram and the corresponding SDS-gel electrophoresis are shown in Figures 3a and 3b. Only the pure fractions were pooled (S4). In case that the SDS gel shows impurities, gel-filtration on Sephacryl S-100 HR (in solvent B) was applied (S5).

5. Final concentration: The ammonium sulfate concentration of S4 or S5 was adjusted to 2.5 M and the solution was loaded on 1 ml DEAE-cellulose column that had been preequilibrated with 'solution C'. The enzyme was eluted with 'solution E'.

The purification degrees and the recoveries of each step are summarized in Table I.

Cloning of the gene coding for DHFR: DNA was prepared from *H. volcanii* WR215 cells as described in (22). The DNA, made from a 1.5 ml culture, was digested with the restriction endonuclease PstI and the amplified 1.9 kb fragment was purified by centrifugation through a 5-20 % sucrose gradient (preformed in 0.15 M NaCl, 10 mM TrisHCl pH 8). The centrifugation was performed in a Beckman SW40 rotor run at 30,000 rpm and 4 ^{0}C for 16 hours. Fractions of 0.4 ml were collected and the DNA was precipitated by adding 1 ml of ethanol. The DNA was dissolved in 30 µl of TE (10 mM TrisHCl , 1 mM EDTA pH 8) and 10 µl were taken for analysis. The purified fragment was cloned into the PstI site of the plasmid pUC19.

DNA manipulation and sequencing: All restriction reactions were performed according to the instruction of the manufacturers of the restriction endonucleases. The ligations were performed with the enzyme T4 DNA ligase (Bethesda Research Laboratories) using the ligation buffer supplied. After subcloning into M13mp18 and M13mp19 vectors the DNA was sequenced according to the method of Sanger (24) using the sequencing kit and protocol of International Biotechnological Inc. (New Haven, CT, U.S.A.). In addition to use of the universal 17mer primer supplied with the sequencing kit, one region of the gene was sequenced using a synthetic oligonucleotide corresponding to nucleotides 1-17 of the coding region of DHFR. DNA restriction fragments were analyzed by agarose gel electrophoresis using 1 % agarose in buffer containing 1 mM EDTA and 40 mM Tris acetate pH 8.

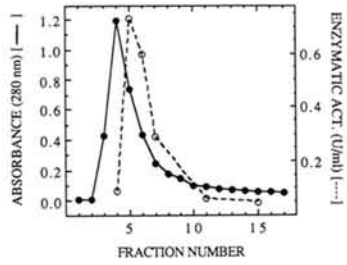


Figure 1: The chromatogram of the first DEAE cellulose column. The column dimensions are 2.5x9 cm. The flow rate was 40 ml/hour and the fraction volume was 6ml.

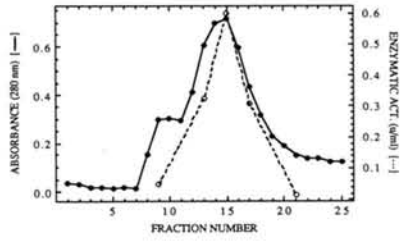
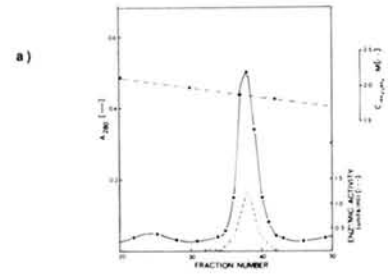


Figure 2: The chromatogram of the second DEAE celulose column. The column dimensions are 1.1x55 cm. The flow rate was 40 ml/hour and the fraction volume was 5 ml.



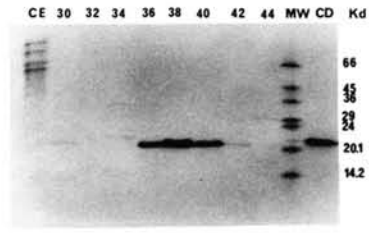


Figure 3: a) The chromatogram of the Sepharose 4b column. The enzyme was loaded in 2.5M ammonium sulfate and eluted by a decreasing gradient of ammonium sulfate. Column dimensions: 1.1x55 cm. Flow rate: 25 ml/hour. Fraction volume: 5 ml. The salt gradient profile is indicated. b) The SDS-gel electrophoresis profile of the different fractions. (CE-crude extract; CD- Chicken liver DHFR; MW- Molecular weight markers)

Table I: Summary of the Purification of h-DHFR

Step	Vol (ml)	Total amount of protein (mg)	Total activity (Enzyme units)	Yield (%)	Purification factor
Crude extruct	26	702	12.3	100	1
First DEAE	42	92.4	10.2	83.5	6.3
Second DEAE	21	17.6	7.2	58.4	23
Sepharose 4B	25	15	6	48.7	23