Biochimica et Biophysica Acta, 660 (1981) 214–218
Elsevier/North-Holland Biomedical Press

BBA 69329

CHARACTERIZATION OF THE CLONED *ESCHERICHIA COLI* DIHYDROFOLATE REDUCTASE

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(Received March 16th, 1981)

Key words *Cloning, Dihydrofolate reductase, Gene localization, (E coli)*

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate NADP$^+$ oxidoreductase, EC 1 5 1 3) was purified from *Escherichia coli* strains that carried derivatives of the multicopy recombinant plasmid, pJFM8 The results of enzyme kinetic and two-dimensional gel electrophoresis experiments showed that the cloned enzyme is indistinguishable from the chromosomal enzyme Therefore it can be concluded that these strains are ideal for use as a source of enzyme for further studies on the biochemistry and regulation of this important enzyme The plasmid derivatives were constructed by recloning experiments that utilized several restriction endonucleases From the analysis both of these plasmids and the purified dihydrofolate reductase enzymes it was possible to deduce the location and orientation of the dihydrofolate reductase structural gene on the parent plasmid, pJFM8

Introduction

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate NADP$^+$ oxidoreductase, EC 1 5 1 3) is an essential enzyme in cellular metabolism since it catalyses the NADPH-dependant reduction of dihydrofolate to tetrahydrofolate, an intermediate required for the biosynthesis of proteins and nucleic acids The enzyme is of significant clinical importance as it is the target enzyme for a number of antitumor and antibacterial agents, such as methotrexate or trimethoprim [1] Dihydrofolate reductase has therefore been the subject of intensive research interest in the last few years In this laboratory we have been most interested in the mechanism of the inhibition of the enzyme by folate analogues [2,3] Since many of these experiments require relatively large amounts of enzyme, we cloned the *Escherichia coli* K-12 dihydrofolate reductase structural gene, *folA*, so as to obtain a strain that overproduces this enzyme [4] Strains carrying the resultant multicopy plasmid, pJFM8, produce 20–30-times more dihydrofolate reductase than control strains carrying the vector plasmid, pBR322

The aims of the current study were to map *folA* on pJFM8 and to purify and analyse the enzymes obtained from strains carrying this plasmid or its derivatives We therefore isolated deletion derivatives of pJFM8 and examined dihydrofolate reductase production and trimethoprim resistance in strains carrying these recombinant plasmids From these experiments it was possible to determine the location and orientation of *folA* on pJFM8 In addition we were able to demonstrate that under our experimental conditions the enzyme coded for by the recombinant plasmids is physically and kinetically indistinguishable from the parent chromosomally-determined enzyme

Materials and Methods

Bacterial strains, media and chemicals All strains were derivatives of JFM43 (i e , C-600 rk$^-$ mk$^+$)

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Minimal medium [5] was supplemented with the appropriate amino acids and unless otherwise stated the rich medium was brain heart infusion (Oxoid Ltd , U K) Chemicals were from sources previously described [4]

Preparation and analysis of plasmid DNA Plasmid DNA was purified, digested with restriction endonucleases, used for transformation, ligated, or subjected to agarose or polyacrylamide gel electrophoresis as previously described [4,6] Strains carrying the deletion plasmids were selected on rich medium containing ampicillin (100 μg/ml) with the one exception that minimal medium containing trimethoprim (2 μg/ml) was used in the selection of the *Pst*I-derived derivative

Gel electrophoresis of proteins Two-dimensional gel electrophoresis was essentially as described by O'Farrell et al [7,8] Proteins were stained using Coomassie brillant blue R250 Non-denatured 7% acrylamide gels were run in a vertical slab gel apparatus at 3 mA/gel for 2 5 h using Tris-glycine buffer, pH 8 3 Activity staining for dihydrofolate reductase was as per Gunlack et al [9] Gels were incubated in a solution of 1 mM NADPH/1 mM dihydrofolate/50 mM potassium phosphate buffer, pH 6 9/0 4 mg/ml 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) for 30 min The product of the dihydrofolate reductase reaction, tetrahydrofolate, reduces MTT directly, yielding an insoluble coloured formazan

Preparation and assay of dihydrofolate reductase Cell extracts were prepared routinely as before [4] except that when the extract was to be used in purification of dihydrofolate reductase the cells were grown in medium (10 l) containing tryptone (100 g)/yeast extract (50 g)/NaCl (100 g)/Na_2HPO_4 (25 g)/NaH_2PO_4 (23 g)/sodium ampicillin (1 g) The enzyme was purified by affinity chromatography using a Sepharose-methotrexate column [3] after which it was desalted on Sephadex G-75, concentrated by ultra-filtration through a Dia-flow UM-10 membrane and then stored at –20°C until further use

Dihydrofolate reductase activity was determined as previously described [3,4] 1 unit enzyme is defined as that amount of enzyme that will catalyze the transformation of 1 μmol dihydrofolate/min at 30°C Protein was estimated by the biuret method using bovine serum albumin as a standard [10] The concentration of dihydrofolate reductase was determined by fluorescence titration with methotrexate [3] and trimethoprim inhibition studies were performed as described previously [4]

Results

Construction of deletion derivatives of pJFM8

Previous results [4] showed that the 9 3 kb cloned *BamH*I fragment of pJFM8 contains single *Pst*I and *Eco*RI sites and two *Sal*I sites After double digestion of pJFM8 with *BamH*I + *Eco*RI two plasmids (pJFM12 and pJFM13) were constructed [4], each of these plasmids has a different *BamH*I – *Eco*RI segment from the original plasmid (Fig 1) Additional deletion derivatives were isolated following digestion of pJFM8 DNA with either *Sal*I or *Pst*I, ligation and use for the subsequent transformation of strain JFM43 to ampicillin resistance The 8 0 kb *Sal*I-derived plasmid pJFM20 was shown by restriction analysis to be identical to pJFM8 except that both the 5 1 and 0 5 kb *Sal*I fragments were absent (Fig 1) The plasmid pJFM29 was derived similarly and, except for the presence of the 0 5 kb *Sal*I fragment, was identical to pJFM20 Further comparative restriction analysis, whereby the plasmids pBR322, pJFM8, pJFM20 and pJFM29 were digested individually with either *Alu*I, or *Alu*I + *Sal*I, clearly demonstrated that the orientation of the 0 5 kb *Sal*I fragment with respect to the 8 0 kb *Sal*I fragment was the same in both pJFM8 and pJFM29 The 9 7 kb *Pst*I-

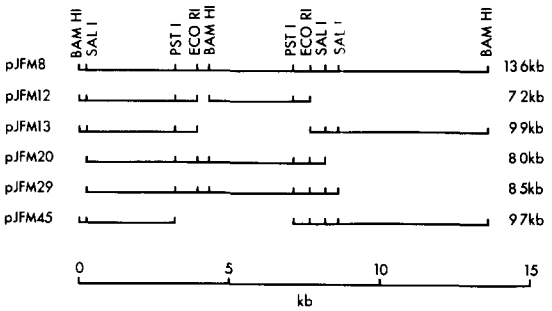

Fig 1 Restriction maps of pJFM8 and its deletion derivatives The restriction map of each plasmid is represented, to scale, in linear form and is aligned in accordance with its relationship to pJFM8 All fragments are shown in their correct orientation except that in pJFM12 the 3 4 kb *BamH*I – *Eco*RI fragment is in the opposite orientation

TABLE I

DIHYDROFOLATE REDUCTASE PRODUCTION AND TRIMETHOPRIM RESISTANCE OF STRAINS CARRYING pJFM8 DERIVATIVES

Cells extracts were prepared as described [4] and assayed for dihydrofolate reductase Trimethoprim resistance (R) or sensitivity (S) was determined by plating to minimal medium containing trimethoprim (2 μg/ml) All strains were derivatives of JFM 43 and carried the plasmid indicated in the table

Plasmid	Trimethoprim resistance (2 μg/ml)	Dihydrofolate reductase (μmol/min per mg protein)
pBR322	S	0 006
pJFM8	R	0 128
pJFM12	S	0 007
pJFM13	S	0 006
pJFM20	S	0 020
pJFM29	R	0 129
pJFM45	R	0 107

derived plasmid, pJFM45, was isolated in a similar fashion as above and shown to have an identical restriction map to pJFM8 except that the 3 9 kb *Pst*I fragment of the latter plasmid had been deleted (Fig 1)

Dihydrofolate reductase levels of derivatives

Dihydrofolate reductase assays were carried out using cell extracts prepared from JFM43 derivatives carrying pJFM8 or one of the deletion derivatives The results (Table I) showed that strains with pJFM29 or pJFM45 produced as much dihydrofolate reductase as the original cloned pJFM8 derivative In addition these strains were resistant to trimethoprim By contrast, cells containing pJFM12 and pJFM13 produced only wild-type chromosomal levels of this enzyme and were sensitive to trimethoprim The pJFM20-derivative grew very slowly on minimal medium containing trimethoprim and had enzyme levels that were significantly higher than those of the control pBR322-derivative but much lower than strains carrying plasmids that had the complete 1 6 kb *Pst*I – *Eco*RI – *Sal*I – *Sal*I region from pJFM8 (Fig 1)

Enzyme kinetics and gel electrophoresis of purified enzymes

To determine if the decreased dihydrofolate reductase level detected in extracts of the pJFM20-derivatives was due to reduced enzyme production or to the synthesis of a less active enzyme, dihydrofolate reductase from this strain and from three of the other derivatives was purified by affinity chromatography The results of kinetic analyses showed that the enzymes derived from strains carrying pJFM8, pJFM20 or pJFM29 had K_m values for dihydrofolate, K_i values for trimethoprim, and turnover numbers that were not significantly different from those values obtained from the purified chromosomal enzyme that was derived from the control strain carrying the vector plasmid pBR322 (Table II)

Electrophoresis of the purified enzymes on 7% acrylamide followed by subsequent staining for dihydrofolate reductase activity showed that the major protein band in each preparation was dihydrofolate reductase When the pJFM8- and pJFM20-coded dihydrofolate reductases were mixed together and then subjected to two-dimensional gel electrophoresis (isoelectric focusing in one direction and SDS polyacrylamide gradient gel electrophoresis in the second dimension) only a single band, corresponding to dihydrofolate reductase, was observed (Fig 2) Similar results were obtained when the pJFM8-determined enzyme was subjected to coelectrophoresis with either the chromosomal or pJFM29-coded enzymes In neither experiment were the latter enzymes separated from pJFM8-coded dihydrofolate reductase

TABLE II

KINETIC CONSTANTS OF PURIFIED ENZYMES

Dihydrotolate reductase was purified from JFM43 derivatives carrying the specified plasmid Kinetic parameters of the purified enzymes were determined as described [3,4]

Plasmid	K_m (dihydrotolate) (μM)	K_i (trimethoprim) (nM)	Turnover number (min^{-1})
pBR322	1 4 ± 0 6	0 82 ± 0 11	1 210 ± 120
pJFM8	0 9 ± 0 1	0 66 ± 0 09	960 ± 20
pJFM20	1 2 ± 0 1	0 63 ± 0 09	1 120 ± 40
pJFM29	0 9 ± 0 1	1 09 ± 0 21	810 ± 40

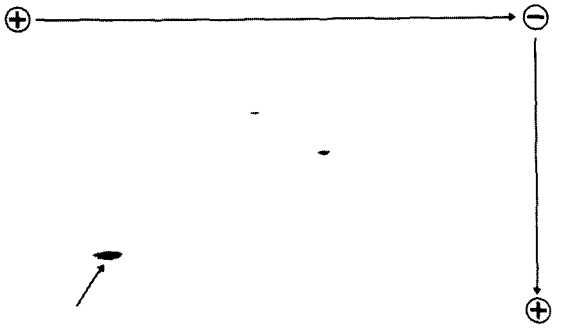

Fig 2 Two-dimensional gel electrophoresis of purified dihydrofolate reductases 20 μg of each of the purified pJFM8- and pJFM20-coded dihydrofolate reductases were mixed and subjected to two-dimensional gel electrophoresis The direction of migration of the isoelectric focusing and SDS-polyacrylamide gradient dimensions are indicated by the horizontal and vertical arrows, respectively Dihydrodolate reductase is indicated on the electropherogram by an arrow

Discussion

Recloning experiments that utilized various restriction enodnucleases were used to construct five deletion plasmids that were derived from the recombinant plasmid, pJFM8 Strains carrying either pJFM29 or pJFM45, two of these deletion plasmids, were trimethoprim resistant and gave elevated yields of dihydrofolate reductase, as did strains carrying the parent plasmid pJFM8 Since restriction endonuclease analysis of these plasmids showed that the only portion of the cloned 9 3 kb *BamH*I fragment of pJFM8 that was common to pJFM8, pJFM29 and pJFM45 was the 1 6 kb *Pst*I – *Eco*RI – *Sal*I – *Sal*I segment it is concluded that *folA* is located within this region This inference is supported by the observation that the remaining three deletion plasmids (pJFM12, pJFM13 and pJFM20) all had deletions extending through part of this 1 6 kb region and all failed to confer either trimethoprim resistance or elevated dihydrofolate reductase production

It was previously suggested that the *Eco*RI site that is within the 1 6 kb region is located either in *folA* or in the regulatory genes essential for *folA* expression [4] Based on the results presented here similar conclusions can be made regarding the location of the internal *Sal*I site of this fragment If this *Sal*I site was within *folA* then, irrespective of whether the *Eco*RI site was also within *folA* or was in a regulatory region proximal to *folA*, the enzyme coded for by pJFM20 must be smaller than the normal enzyme The lower levels of dihydrofolate reductase activity observed in extracts of strains with pJFM20 would have been due to production of an enzyme with altered kinetic parameters The results of kinetic analyses on the purified enzymes failed to reveal any differences between the pJFM20-derived and wild-type enzymes Furthermore, these enzymes could not be separated by two-dimensional gel electrophoresis These findings suggest that this internal *Sal*I site is not located within *folA* but within the *folA* regulatory region Deletion of the internal 0 5 kb *Sal*I fragment of the 1 6 kb region therefore results in the disruption of the integrity of this regulatory region and leads to decreased enzyme production It then follows from the results that the internal *Eco*RI site must be within *folA* itself and that transcription is proceeding from right to left (Fig 1)

Smith and Calvo [11] have also cloned the *E coli* dihydrofolate reductase structural gene but utilized a different procedure Although the plasmid obtained was not identical to pJFM8 it did have a similar *Pst*I – *Eco*RI – *Sal*I – *Sal*I region and the authors showed that *folA* is within the 1 0 kb *Pst*I – *Sal*I portion of this region These results together with those of DNA sequence analysis of their plasmids [12] are in good agreement with the conclusions presented in this paper

Dihydrofolate reductase has been extensively studied in recent years because it is the target enzyme for the antitumor agent, methotrexate The majority of these studies [3,13,14] have been carried out using enzymes purified from bacterial strains, that were previously subjected to mutagenesis so as to increase the yield of dihydrofolate reductase It is possible that the enzymes used in these experiments may have been altered such that they had different kinetic properties compared to the original wild-type enzymes The results presented here show that purified cloned *E coli* dihydrofolate reductase could not be distinguished from the purified chromosomal enzyme by either two-dimensional gel electrophoresis or kinetic analysis These observations taken together with the previously reported findings regarding the elevated enzyme yields [4] show that *E coli* strains carrying pJFM8, pJFM29 or pJFM45 are ideal for use

as a source of chromosomal *E coli* dihydrofolate reductase Current studies are aimed at further increasing the yield of dihydrofolate reductase and obtaining an insight into the regulation of dihydrodolate reductase production in *E coli*

Acknowledgements

We acknowledge the most capable technical assistance of Leah Matthews and Margaret Sneddon Dr G Cox is thanked for the two-dimensional gel electrophoresis experiments J W W was supported by a grant from the American Cancer Society

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