DNA Binding by Dihydrofolate Reductase from *Lactobacillus casei*

Angela M. Gronenborn and R. Wayne Davies

From the Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom and the Department of Biochemistry, University of Manchester, Institute of Science and Technology, Manchester M60 1QD, United Kingdom

The protein-dependent retention of double-stranded DNA molecules on nitrocellulose filters has been used to show that pure dihydrofolate reductase from *Lactobacillus casei* has affinity for DNA. Dihydrofolate reductase will bind to end-labeled linear double-stranded DNA and to DNA in supercoiled form. Coenzymes and certain inhibitors do not affect the affinity of the protein to DNA, indicating that the DNA-binding region of the protein is distinct from the binding sites for these molecules. Comparison of the retention on filters by dihydrofolate reductase of two plasmid DNAs, differing only in a 3000-base pair insert containing the *L. casei* gene for dihydrofolate reductase, showed that in the presence of this DNA region lower concentrations of the protein were required to give significant retention; it is possible that a specific DNA-protein interaction underlies this effect. This presents the possibility of studying the interaction with DNA of a protein for which a crystal structure and considerable nuclear magnetic resonance data are already available.

Dihydrofolate reductase is the target of a group of drugs, including trimethoprim and methotrexate, which are widely used in the treatment of bacterial and protozoal infections, of neoplastic disease, and to suppress immune reactions (9). The enzyme is of central importance for cell metabolism. It catalyzes the reduction of dihydrofolic to tetrahydrofolic acid which is required for the *de novo* synthesis of thymidine, purines, and glycine (10), and is converted to a series of coenzymes which participate in a wide variety of 1-carbon group transfers. The metabolic and clinical importance of the enzyme has led to its biochemistry being studied in detail. Mechanisms by which cells become resistant to "anti-folate" drugs have received particular attention. In bacteria resistance can occur by acquisition of an R-factor carrying a gene coding for drug-resistant DHFR (11, 12), or by mutation leading either to a DHFR with reduced affinity for the inhibitor (13) or to increased synthesis of DHFR (13-15). In mammalian cells gene amplification has been shown to occur (16). In *E. coli* and *Diplococcus pneumoniae* the increased synthesis of DHFR (up to 30-fold and 120-fold, respectively) has been shown to depend upon increased levels of mRNA synthesis (17, 18). In *D. pneumoniae*, mutations leading to increased mRNA synthesis have been shown to map within the gene for DHFR (15, 18), and there is good evidence that this is the case for *E. coli* as well (13). One possible interpretation of this finding is that DHFR is in some way involved in regulating the expression of the gene that codes for it. If this involvement were direct, DHFR should show at least a nonspecific affinity for DNA, since this is characteristic of proteins that bind specifically, and in the case of *E. coli* lac-repressor the high nonspecific binding is an essential part of the mechanism of action (1).

The experiments described here show that bacterial DHFR from *L. casei* does bind DNA nonspecifically, and suggest a strong interaction with a particular DNA region.

**MATERIALS AND METHODS**

*Chemicals*—NADPH, methotrexate, folate, myoglobin type III from horse heart, and horse liver alcohol dehydrogenase were obtained from Sigma. Restriction endonucleases *Bam* H1, *Eco* R1, and *Bgl* II were purchased from Boehringer Corp. and [*-32P]*dATP (2000-3000 Ci/mmol) from Amersham. Other chemicals used were of the highest purity commercially available and were used without further purification.

*Protein Purification*—DHFR was purified as described by Dann et al. (19) and was more than 98% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, isoelectric focusing, and gel filtration. The enzyme concentration was determined from its absorbance at 280 nm and by assaying the catalytic activity (19). It was stored as a lyophlized powder at −15 °C.

*DNA Purification*—DNA was prepared from *L. casei* cells that were harvested in the logarithmic phase of growth. Washed cells were frozen in liquid nitrogen, then thawed directly in 2% Sarkosyl, 50 mM Tris·HCl, pH 8.0, and 50 mM EDTA at 55 °C. After 5 h of incubation at this temperature, cell debris was centrifuged off and the solution...
treated with proteinase K (Sigma) at 200 μg/ml and 37 °C for 12 h. DNA was concentrated by precipitation with polyethylene glycol and purified by equilibrium density gradient centrifugation in CsCl containing 150 μg/ml of ethidium bromide. DNA prepared in this way usually has a molecular weight of greater than $3 \times 10^6$. DNA was cut and end labeled as described by Davies et al. (20). It was kept frozen in this solution until used.

Plasmid Preparation—The molecular cloning experiments by means of which pWDLcBl was constructed was described elsewhere. The L. casei DNA was inserted into pBR322. The supercoiled plasmid DNA was purified by this and all other plasmids by a modification of the procedure of Clewell and Helinski (21). Plasmid DNA was cut with restriction endonucleases Bam HI or Bgl II, 3' end labeled, and purified from unincorporated nucleotides as before (20).

Filter Binding—In the filter binding experiments 40 ng of aliquots of labeled DNA were incubated with various amounts of DHFR, horse liver alcohol dehydrogenase, or horse heart myoglobin in 100 μl of 100 mM Tris-HCl and 100 mM KCl, pH 7.5 (binding buffer) at 22 °C for 10 min. Millipore HAWP 02,500 filters, 0.45-μm pore size, were soaked in this buffer for at least 2 h before use. The filter apparatus was flushed through with buffer, and 1 ml of fresh binding buffer was passed through each filter before the sample was added. The samples were individually passed through filters at a flow rate of 1 ml/min, and the filters were washed twice with 1 ml of binding buffer each time. Individual experiments were carried out in triplicate.

Background values were obtained by passing through filters labeled DNA that had been incubated for 10 min in binding buffer without added protein. Filters were then dried, and bound radioactivity was determined by scintillation counting.

RESULTS AND DISCUSSION

The binding of DHFR from L. casei was first studied with random DNA fragments. As an assay we have used the filter binding method, which depends on the fact that intact double-stranded DNA will pass through a nitrocellulose filter, while proteins bind to it. Thus if a protein is incubated with DNA and the mixture then passed through a filter, any retention of DNA on such a filter above normal background levels can be regarded as being due to the binding of a DNA molecule or at least one molecule of protein (22). We have routinely labeled DNA by filling in the recessed 3' -ends left by some restriction endonucleases, using the large fragment of DNA polymerase (20). This simple procedure leaves DNA molecules with flush ends largely intact and gives lower background than nick translation.

We compared the retention of 3'-end labeled DNA on nitrocellulose filters after incubation with either DHFR or one of the two control proteins, horse liver alcohol dehydrogenase and horse heart myoglobin. Alcohol dehydrogenase was chosen because it contains a coenzyme-binding site very similar to DHFR, and if binding to DNA would be due to binding to this site, one would expect a similar amount of retention of radioactivity on the filter for both proteins. Myoglobin is similar in size to DHFR, and if retention of radioactivity on the filter were due to physical blockage of the filter pores, this again should be similar for both proteins. The results of a typical experiment aimed simply at showing a difference between these proteins are shown in Fig. 1. At the protein concentrations used in Fig. 1, DHFR binds 25 to 30% of the input DNA, while the same concentration of alcohol dehydrogenase or myoglobin shows no DNA retention above the background level. The actual plateau level of DNA binding is probably determined by factors other than protein-DNA interaction; Riggins et al. (22) found that the lactose repressor of E. coli, a well characterized DNA-binding protein, also retained less than 50% of DNA containing the lac operator on nitrocellulose filters, which was ascribed to loss of protein-DNA complexes during the filtration and washing procedure. The level of the plateau of retention of DNA on the filter is not related to the strength of binding between protein and DNA, which must be determined from equilibrium binding curves at subsaturating enzyme concentrations of the kind shown in Fig. 3. Nevertheless, it is qualitatively clear that under the conditions of the experiment shown in Fig. 1, DHFR binds DNA much more strongly than the other proteins.

In the experiment shown in Fig. 1, DNA from L. casei itself was used, but qualitatively the same results were obtained with DNA fragments from E. coli or Aspergillus nidulans. All these experiments were carried out in the absence of Mg2+ ions and in the presence of 100 mM KCl. Fig. 2 shows that at low DHFR concentrations (9 × 10^-7 m) the degree of retention of DNA on filters by DHFR is weak in the absence of KCl but already maximal at KCl concentrations above 25 mM. At KCl concentrations such as 300 mM and greater, significant retention of DNA by alcohol dehydrogenase and myoglobin was observed, presumably because aggregation of these proteins leads to unspecific trapping of DNA in the complexes. The addition of Mg2+ ions to a concentration of 10 mM had no effect on the maximal level of DNA retention by DHFR. A strong candidate for the site of DNA binding is the coenzyme binding site of DHFR. However, at a concentration of DHFR (2 × 10^-7 m) that is just sufficient to give maximal retention of DNA, addition of excess NADPH, NADP, methotrexate, or trimethoprim seems to have no effect on the level of DNA retention as can be judged by the unchanged amount of labeled DNA retained on the filter (Table I). The dissociation constant of the protein-NADPH complex, for instance, is 1.0 × 10^-6 m (23). A preliminary estimate of the binding affinity of the enzyme for DNA can be made by taking the midpoint of the curve for binding to pBR322 DNA given in Fig. 3; a value of the order of 1 × 10^-7 m is obtained, so that at the concentrations of protein and ligand used in these experiments the coenzyme would have displaced the more weakly bound DNA if the same part of the protein were involved. These experiments show, therefore, that the DNA-binding activity of DHFR is located in a different part of the protein molecule than the sites of interaction with its coenzyme.

All known mutations affecting the expression of DHFR map either in or very near to the structural gene for the enzyme. If the nonspecific DNA binding shown by DHFR has any relation to a possible regulatory function, the most likely site for DHFR to exert a specific effect is in the region of the structural gene itself, and particularly in the region required for transcription of the gene. We decided to investigate whether DHFR would bind more strongly to this region of DNA than to random DNA pieces.
DNA Binding of Dihydrofolate Reductase

FIG. 2. The effect of salt concentration and Mg\textsuperscript{2+} ions on filter binding of DNA by DHFR. Binding assays were conducted as described under “Materials and Methods,” and reactions were carried out in 10 mM Tris, pH 7.4. \textbullet, \(9 \times 10^{-5} \text{ M}\) DHFR, \(\bigcirc, +10 \text{ mM MgCl}_2; \bigcirc, \text{ myoglobin}, \bigtriangleup, +10 \text{ mM MgCl}_2; \bigtriangleup, \(9 \times 10^{-4} \text{ M}\) alcohol dehydrogenase.

FIG. 3. Filter binding curves of \textit{L. casei} DHFR binding to plasmid DNA with and without the \textit{L. casei} gene. Aliquots of pWDLcB1 or pBR322 DNA were incubated with various concentrations of DHFR, and filter binding was measured as described under “Materials and Methods.” All experiments were performed within 2 days of labeling the DNA. The restriction enzymes used were \textit{Bam} H1 and \textit{Bgl} II, which linearize (i.e., have single cuts in) pBR322 and pWDLcB1, respectively, and leave identical molecular ends. \textbullet, DHFR binding to DNA from pWDLcB1; \bigtriangledown, DHFR binding to DNA from pBR322; \square, \textit{myoglobin}; \bigtriangleup, alcohol dehydrogenase binding to both pBR322 DNA and pWDLcB1 DNA.

The source of purified DNA fragments containing the region around the \textit{L. casei} structural gene for DHFR is the plasmid pWDLcB1, which we made by cloning \textit{Bam} H1-cut DNA from a methotrexate-resistant strain of \textit{L. casei} into the \textit{E. coli} multicopy vector pBR322 (24). The plasmid pWDLcB1 confers methotrexate and trimethoprim resistance on the \textit{E. coli} host, and we have shown this to be due to the plasmid-dependent synthesis of \textit{L. casei} DHFR. The DHFR gene makes up 20% of the cloned \textit{L. casei} DNA, We have studied the binding of DHFR to this region of \textit{L. casei} DNA by comparing filter binding of pWDLcB1 and pBR322 DNA, which differ only in the presence or absence of the \textit{L. casei} insertion.

We showed first that we could essentially repeat the results obtained with random DNA fragments as the binding sub-

strate using this time pure plasmid DNA that had been cut with a restriction enzyme and end labeled as before. Fig. 3 shows that as with random DNA pieces, there is a clear large difference in the capacity of DHFR, alcohol dehydrogenase, and myoglobin to bind pWDLcB1 or pBR322 DNA. In order to find out whether the presence of the \textit{L. casei} DNA fragment containing the DHFR gene in pWDLcB1 leads to a different affinity of DHFR for this DNA compared to its affinity for pBR322 DNA without the inserted fragment, we measured the retention of these DNAs on nitrocellulose filters by concentrations of DHFR below those giving maximal retention. The filter binding curves given in Fig. 3 show that between DHFR concentrations of \(5 \times 10^{-5} \text{ M}\) and \(5 \times 10^{-6} \text{ M}\) the level of retention of pWDLcB1 DNA changes from a level indistinguishable from the background binding in the absence of DHFR up to the maximum level of retention. Detectable binding of pBR322 DNA is not found until \(2 \times 10^{-5} \text{ M}\) DHFR, maximal binding being attained by \(2 \times 10^{-5} \text{ M}\) DHFR. There is, therefore, a clear difference in the retention on filters of these two DNAs, differing only in a small (3.0 kilobase pairs) \textit{L. casei} insert. We tested whether these results were due to differences between the states of pWDLcB1 and pBR322 DNA preparations, e.g., the frequency of nicks in the duplex. For this we made use of the fact that with care it is possible to obtain preparations of these plasmids that consist of over 95% covalently closed circular (supercoiled) molecules.

Supercoiled molecules must of necessity be intact double-stranded nick-free DNA duplexes. Even a single nick leads to relaxation of the supercoil to an open circle from which it is easily distinguished by its mobility in agarose gel electrophoresis. In these experiments we incubated intact supercoiled plasmid DNA with DHFR under the conditions used to obtain the binding curves in Fig. 3. After filter binding and washing as before, the DNA was eluted from the filter by 3 h of incubation at 65 °C, ethanol precipitated, cut with the restriction endonuclease \textit{Bam} HI, and the resulting fragments 5'-end labeled and run into an agarose gel. Gel sections containing the labeled DNA were cut out, after autoradiography had allowed them to be located precisely, and the amount of radioactivity in them was determined (25). Using such preparations, we have in this way obtained binding curves identical with those shown in Fig. 3 for pWDLcB1 and pBR322 DNA, except that the amounts of DNA bound were lower throughout the curves, which is due to poor release of DNA from the protein-filter complex. The maximum levels of retention of pWDLcB1 DNA (compared to similar input amounts of DNA that were ethanol precipitated and labeled without the filter binding step) were typically 16-20%, well above the level of nonsupercoiled DNA (usually no more than 1%) present in these preparations. The DNA was shown to remain intact.

TABLE I

Effect of important ligands on filter binding of DNA by DHFR

The experiments were carried out exactly as described in the legend to Fig. 1, except that all concentrations of protein and ligands are \(2 \times 10^{-5} \text{ M}\), and the ligands were added just prior to the addition of protein.

<table>
<thead>
<tr>
<th>Counts per min</th>
<th>Retained radioactivity</th>
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<tbody>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>\textit{L. casei} DHFR alone</td>
<td>11,200</td>
</tr>
<tr>
<td>+ NADPH</td>
<td>13,800</td>
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<tr>
<td>+ NADP</td>
<td>10,900</td>
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<tr>
<td>+ MTX</td>
<td>12,700</td>
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<tr>
<td>+ TMP</td>
<td>13,000</td>
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<tr>
<td>+ NADPH/MTX</td>
<td>12,300</td>
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<tr>
<td>+ NADPH/TMP</td>
<td>11,400</td>
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\*MTX, methotrexate; TMP, trimethoprim.
during incubation with DHFR by running DNA from the incubation mixtures alongside untreated DNA in agarose gels.

These experiments demonstrate that DHFR has a higher affinity for pWDLcBl DNA than for pBR322 DNA when they are both in the form of intact supercoiled duplexes. If the greater affinity of DHFR for pWDLcBl were due to differences in base composition of the L. casei insert rather than to the presence of a particular DNA sequence, then some plasmid DNAs other than pWDLcBl should also be bound with a higher affinity than pBR322 DNA. In particular, it is common for DNA-binding proteins to show preference for AT-rich regions of DNA. We, therefore, used the filter binding test to obtain binding curves for DHFR with two other plasmids. One, CV13, is part pBR322, part yeast 2μ plasmid, and part yeast nuclear DNA including the leu 2 gene. The other, PAMHd 3/5, is pBR322 with an HindIII fragment of A. nidulans mitochondrial DNA cloned into the HindIII site. This section of Aspergillus mitochondrial DNA is approximately 70% AT in base composition. These DNAs are retained on filters less well than pWDLcBl DNA but slightly better than pBR322 DNA. Since both are bigger molecules than pBR322 (CV 13, 10.7 kilobase pairs PAMHd 3/5, 9.3 kilobase pairs; pBR233, 4.5 kilobase pairs), the shift in the binding curves reflects wholly or in part the increase in nonspecific binding that must go with increasing size of the DNA molecule.

The results presented here show that DHFR from L. casei is a protein which in addition to its well characterized enzymatic activity shows a strong affinity for intact double-stranded DNA molecules. This affinity for DNA is not affected by the presence of high concentrations of coenzyme or some inhibitors of the enzymatic activity, so that a part of the protein molecule different and spatially separated from the normal active site must be involved. Moreover, the addition of pBR322 DNA of a 3.0-kilobase pairs containing the gene coding for DHFR increases the affinity of L. casei DHFR for this DNA. Evidence has been presented showing that this effect is due neither to differences in the state of the DNA nor in overall base composition. This suggests strongly that specific DNA binding by DHFR is being detected. The difference between the binding curves for pWDLcBl and pBR322 DNA is, however, only small compared with the large difference observed with other DNA-binding proteins like E. coli lac repressor, which shows a difference between specific and nonspecific binding of at least 2 orders of magnitude.

However, the cloned L. casei DNA fragment derives from a methotrexate-resistant strain and itself confers high level trimethoprim and methotrexate resistance on E. coli, presumably because of increased DHFR gene expression. It is, therefore, likely that any specific recognition site which might be present next to the DHFR structural gene may well have been mutated to reduce the efficiency of the binding. Thus the equilibrium binding constant for the interaction between DHFR and the wild type DNA sequence might be much larger, and we are investigating this at present. We, therefore, consider it possible that this DHFR-DNA complex formation occurs in vivo and plays a physiological role; most likely DHFR reduces the level of expression of its own gene. This conclusion is consistent with the genetic evidence from various bacterial species mentioned previously (17, 18).

These results are clearly important to our understanding of the regulation of bacterial dihydrofolate reductase levels. Even more interesting is the fact that for the first time a protein for which a crystal structure is available as well as considerable nuclear magnetic resonance data, has been shown to interact with DNA. It represents a unique opportunity to study DNA-protein interaction at the atomic level.

Acknowledgments—We are most grateful to Gill Ostler and John McCormich for invaluable technical assistance. We would also like to thank O. C. K. Roberts for helpful discussions and A. S. V. Burgen for encouraging and stimulating support.

REFERENCES


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4. R. W. Davies, unpublished work.