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A Fully Active Variant of Dihydrofolate Reductase with a Circularly Permuted Sequence†

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ABSTRACT: The amino acid sequence of mouse dihydrofolate reductase was permuted circularly at the level of the gene. By transposing the 3′-terminal half of the coding sequence to its 5′ terminus, the naturally adjacent amino and carboxyl termini of the native protein were fused, and one of the flexible peptide loops at the protein surface was cleaved. The steady-state kinetic constants, the dissociation constants of folate analogues, and the degree of activation by both mercurials and salt as well as the resistance toward digestion by trypsin were almost indistinguishable from those of a recombinant wild-type protein. Judged by these criteria, the circularly permuted variant has the same active site and overall structure as the wild-type enzyme. The only significant difference was the lower stability toward guanidinium chloride and the lower solubility of the circularly permuted variant. This behavior may be due to moving a mononucleotide binding fold from the interior of the sequence to the carboxyl terminus. Thus, dihydrofolate reductase requires neither the natural termini nor the cleaved loop for stability, for the conformational changes that accompany catalysis as well as the binding of inhibitors, and for the folding process.

The specific exchange of single amino acid residues by recombinant DNA technology is a powerful approach to identify residues that are crucial for the folding mechanism and stability of proteins (Matthews, 1991a,b). Circular permutation of the protein sequence is a more radical approach (Goldenberg & Creighton, 1983). It consists of connecting the natural amino and carboxyl termini covalently with a new peptide linker and cleaving the circular molecule at one of the surface loops to generate new termini. A given sequence can be permuted without compromising the interaction between secondary structural elements in the protein core in as many

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different ways as there are surface loops. These usually connect secondary structural elements that are also spatially adjacent (Finkelstein & Ptitsyn, 1987; Chothia & Finkelstein, 1990). Surface loops may be important in initiating protein folding (Skolnik & Kolinski, 1990), in stabilizing the folded state (Leszynsky & Rose, 1986; Urfer & Kirschner, 1992), or in mediating conformational changes that are important for catalysis (Lolis & Petsko, 1990).

The effect of circular permutation on the stability, function, and folding process of a protein can answer the following questions: (1) Does folding begin at the natural amino terminus? (2) Does the severed loop connect elements of secondary structure whose nearest-neighbor interaction is essential for the folding process? (3) Is the severed loop important for mediating conformational changes during catalysis and ligand binding?

Circular permutation at the level of the protein occurs in nature (Cunningham et al., 1979; Yamauchi & Minamikawa, 1990) and can be performed in vitro [e.g., bovine pancreatic trypsin inhibitor; Goldenberg & Creighton, 1983]. A more versatile approach is to permute the corresponding structural gene circularly [e.g., TRP1 that codes for yeast phosphoribosylanthranilate isomerase (Luger et al., 1989)]. Both the inhibitor and the isomerase are monomeric proteins, which apparently consist of single domains but of quite different structure. We wished to obtain further support for the notion that tolerance of the protein structure toward circular permutation of the sequence is not restricted to a particular protein fold.

Dihydrofolate reductase (DHFR)[1] is a particularly suitable candidate for successful circular permutation. It is a monomeric protein with only about 180 residues. High-resolution crystal structures have been reported for two bacterial DHFRs, *Escherichia coli* (eDHFR; Bolin et al., 1982; Bystroff & Kraut, 1991) and *Lactobacillus casei* (Filman et al., 1982), as well as for three vertebrate DHFRs, chicken (cDHFR; Voltz et al., 1982), mouse (mDHFR; Stammers et al., 1987), and human (hDHFR; Oefner et al., 1988; Davies et al., 1990). In each of these structures, the amino and carboxyl termini are located at the protein surface and in adjacent positions not expected on a purely random basis (Thornton & Sibanda, 1983).

DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate by NADPH and binds several synthetic antifolate drugs very strongly (Baccanari et al., 1982; Kraut & Matthews, 1987). The structures of several binary complexes [e.g., DHFR–$NADP^+$, DHFR–folate, DHFR–methotrexate (MTX)] and even of ternary complexes (e.g., DHFR–$NADP^+$–folate) have also been determined. It follows that the catalytic mechanism is known to considerable stereochemical detail.

Because each of the two substrates is large and carries two aromatic moieties, the active site occupies an unusually, large fraction of the protein's surface. Moreover, one bound ligand enhances the affinity of the other (depending on the particular source of DHFR) up to several thousandfold. These effects appear to be mediated by ligand-induced conformation changes, which involve loops that are distant from the active site (Tan et al., 1990; Bystroff & Kraut, 1991). Therefore, the possible changes of structural interactions brought about by circular permutation should affect the enzyme kinetic constants very sensitively.

We describe here the construction and expression of a circularly permuted variant of the gene of mDHFR by a strategy that differs from that of Luger et al. (1989). Judged by comparison of several properties of the purified proteins, this circularly permuted variant is almost indistinguishable from the wild-type DHFR.

Experimental Procedures

Materials. Dihydrofolate (DHF), trimethoprim (TMP), and MTX were purchased from Sigma, and NADPH was from Boehringer. The oligonucleotides for mutagenesis and sequencing were synthesized on an Applied Biosystem ABI 380 B oligonucleotide synthesizer and purified with use of disposable minicolumns from the same source. DNA was sequenced using the Sequenase DNA sequencing kit and procedures of the United States Biochemical Corp. Ultrapure guanidinium chloride (GuCl) was from Schwarz/Mann.

Plasmids and Strains. The plasmid pDS78/RBSII/DHFR carries a 5′-terminally extended sequence of mDHFR in expressible form under control of the T5 N25 promotor and the *lac* operator that can be regulated by the *lac* i^q gene product (D. Stüber, personal communication). The corresponding gene product was designated as reference DHFR (rfDHFR). The expression vector pDS56/RBSII/*Nco*I is a member of the pDS 5 family of plasmids (Bujard et al., 1987). The *Nco*I site adjacent to an efficient ribosome binding site encodes the starting methionine necessary for the expression of any coding sequence that is in the correct reading frame (D. Stüber, personal communication).

E. coli SG200-50 (strain collection D. Stüber) was used for the heterologous expression of both rfDHFR and the circularly permuted DHFR (cpDHFR). SG200-50 is a $lonA^-$ (Tn5) derivative of strain MC 4110 (F^-, Δ lacU169, araD139, rpsL, relA, thiA, flbB). It also lacks the lonA heatshock protease activity.

Site-Directed Mutagenesis. The gapped-duplex DNA method of site-directed mutagenesis (Stanssens et al., 1989) was used to generate the necessary restriction sites in the rfDHFR gene. This method makes use of vectors with alternating resistance toward either chloramphenicol or ampicillin as well as of the recipient strains WK6, WK6 mutS, and K_{12}. The vector specifying resistance to one antibiotic has an amber mutation in the other antibiotic resistance gene, and vice versa. The mutagenic oligonucleotides were about 30 nucleotides long, and the gap covered the whole rfDHFR gene. All other recombinant DNA methods followed standard protocols (Sambrook et al., 1989).

Expression and Purification of rfDHFR and cpDHFR. The plasmids bearing the various DHFR coding sequences were transformed into the recipient strain, together with a compatible plasmid overproducing the lac repressor. The cells were grown in LB medium. The production of the DHFR proteins was induced in mid-log phase by addition of 1 mM isopropyl thiogalactoside, and the bacteria were harvested 3 h later. In the following purification protocol, the temperature was 0–4 °C and all buffers were supplemented with 0.5 mM phenylmethanesulfonyl fluoride and 0.1 mM *N*-tosyl-L-phenylalanyl chloromethyl ketone. The harvested bacteria were resuspended to a concentration of 0.1 g of wet pellet/mL in a buffer containing 100 mM potassium phosphate, pH 7.9, 1 mM EDTA, and 1 mM dithioerythritol (DTE; Thillet et al., 1988).

[1] Abbreviations: BSA, bovine serum albumin; DHFR, dihydrofolate reductase; cDHFR, chicken DHFR; hDHFR, human DHFR; mDHFR, mouse DHFR; rfDHFR, reference mDHFR; cpDHFR, circularly permuted mDHFR; eDHFR, DHFR from *Escherichia coli*; DHF, 7,8-dihydrofolate; MTX, methotrexate; TMP, trimethoprim; NADPH, nicotinamide adenine dinucleotide phosphate, reduced; GuCl, guanidinium chloride; DTE, dithioerythritol; pHMB, *p*-(hydroxymercuri)benzoate; SDS, sodium dodecyl sulfate.

Cells were disrupted by sonication (3 × 2 min). After removal of the cell debris by centrifugation (20 min at 30000*g*), DNA was eliminated from the supernatant by precipitation with protamine sulfate. A solution of 5% protamine sulfate in water was added dropwise up to a ratio of protamine sulfate/DNA = 0.5 (w/w), and stirred for 30 min at 0 °C. The precipitate was removed by centrifugation at 30000*g* for 10 min. The supernatant was then fractionated with ammonium sulfate. The 55–80% ammonium sulfate pellet (Thillet et al., 1990) was dissolved in a minimal volume of 50 mM Tris-HCl buffer at pH 8.3, containing 1 mM EDTA and 1 mM DTE, and dialyzed against the same buffer. It was then applied to a column (2.5 × 21 cm) of DEAE-Sephacel equilibrated with the same buffer. The load was 2 mg of protein/mL bed volume. The DHFR activity was eluted with the same buffer and appeared after the dead volume. The protein was generally sufficiently pure to be stored after this step. The fractions containing the highest DHFR activity were pooled, concentrated with Centricon 10 membranes, supplemented with 10% glycerol, and stored at −70 °C.

The protein content of partially purified fractions was determined according to Bradford (1976). The concentration of homogeneous DHFR preparations was estimated by the absorbance at 280 nm using ϵ = 29 200 M^{-1} cm^{-1} (Duffy et al., 1987). Polyacrylamide gel electrophoresis in the presence of SDS followed the procedure of Laemmli (1970). Analytical electrofocusing employed the LKB ampholine kit and followed the manufacturer's instructions.

Amino-Terminal Sequencing. The amino termini of both purified rfDHFR and cpDHFR were checked by pulsed-liquid-phase sequencing on an Applied Biosystems 477 A sequencer according to the manufacturer's specifications. Identification of the corresponding PTH-amino acids was carried out on-line on an 120 A amino acid analyzer from Applied Biosystems.

Enzyme Assay. DHFR activity was monitored spectrophotometrically at 25 °C with 0.05 M imidazole hydrochloride buffer at pH 7.0, containing 1 mM EDTA and 1 mM DTE, modified from the method of Baccanari et al. (1975). DHF and NADPH concentrations were 50 and 100 μM, respectively, and the reaction was initiated by adding the enzyme. DHF concentration was determined spectrophotometrically at pH 7.0 and 282 nm with ϵ = 28 000 M^{-1} cm^{-1} (Blakley, 1960), and NADPH at pH 7.0 and 340 nm with ϵ = 6200 M^{-1} cm^{-1} (Horecker & Kornberg, 1948). One unit of enzyme reduces 1 μmol of DHF/min under these conditions, corresponding to $\Delta\epsilon$ = 12.3 mM^{-1} cm^{-1} at 340 nm (Hillcoat et al., 1967).

Steady-State Kinetics. Initial estimates of K_M^{DHF} and K_M^{NADPH} values were obtained from direct linear plots (Cornish-Bowden & Eisenthal, 1978) as input for the analysis of entire progress curves (Stone & Morrison, 1982). Different progress curves were recorded with one substrate constant at a saturating concentration and varying the other substrate with initial concentrations of 2, 5, and 10 μM. Data were analyzed using the computer program COSY (Eberhard, 1990).

The concentration of active enzyme in purified preparations was determined by methotrexate titration (Sheldon & Brenner, 1976). The concentration of MTX was determined spectrophotometrically at 302 nm (ϵ = 22.1 mM^{-1} cm^{-1} in 0.1 N KOH; Seeger et al., 1949).

Determination of Methotrexate and Trimethoprim Inhibition Constants. The inhibition constant of trimethoprim (TMP) was determined according to Dixon (1953), varying the concentrations of inhibitor and DHF while NADPH was kept constant at saturating concentration (Baccanari et al., 1982). The concentration of TMP was determined spectrophotometrically at 271 nm (ϵ = 6.06 mM^{-1} cm^{-1}; Roth & Strelitz, 1969). The enzyme was preincubated with the inhibitor and NADPH and the reaction started with DHF. The upper limit of the inhibition constant of methotrexate was estimated roughly from the methotrexate titration curves.

Effects of DHFR Activating Agents. Activation by *p*-(hydroxymercuri)benzoate (pHMB; Duffy et al., 1987) was monitored by the time-dependent increase of catalytic activity. Enzyme was incubated at 4 °C with 1000-fold excess of pHMB in Tris-HCl buffer at pH 7.5. Aliquots were removed at various times after addition of pHMB and assayed for activity as described above at 25 °C in standard imidazole buffer lacking both EDTA and DTE. The activation effect of 0.8 M KCl in the presence of 10 μM BSA was measured in the same way, but incubating and assaying both at 25 °C.

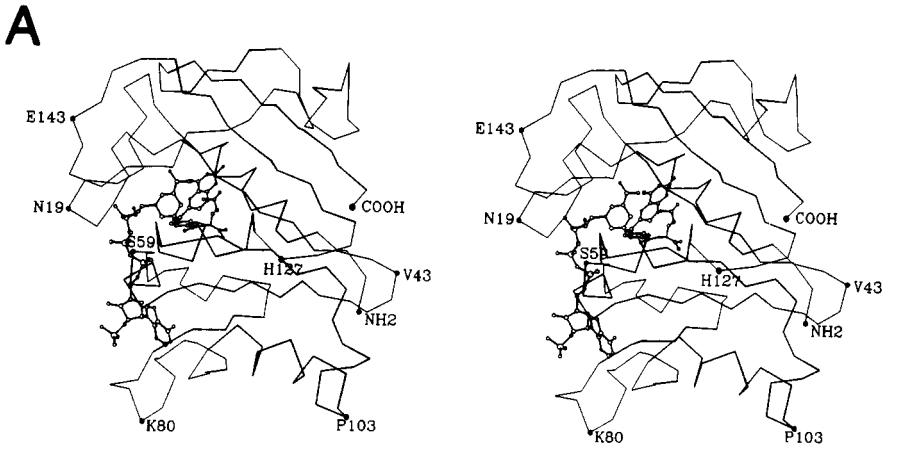
Folding Studies. Unfolding as a function of guanidinium chloride (GuCl) was monitored in 10 mM potassium phosphate buffer, pH 7.5, 0.2 mM EDTA, and 0.1 mM DTE by circular dichroism at 220 nm on a Cary 60 spectrometer. All samples were allowed to equilibrate for 60 min at the appropriate final GuCl concentration at room temperature before the spectra were measured. The protein concentration was about 0.3 mg/L. The reversibility of unfolding was checked by means of CD spectra upon appropriate dilution of the protein denatured in 4 M GuCl.

RESULTS

Design and Construction of a Circularly Permuted Gene of mDHFR. The coordinates of mDHFR were not available, but we had access to those of hDHFR (courtesy of F. Winkler). The structure of hDHFR (Figure 1A) is a good model of mDHFR because 169 out of 186 amino acid residues are identical (Masters & Attardi, 1983). The amino and carboxyl termini (V1 and D186) of hDHFR are 18.5 Å apart (Oefner et al., 1988; Davies et al., 1990), so that an artificial linker comprising seven amino acids should be sufficient to connect the two termini without undue strain (Bird et al., 1988; Huston et al., 1988).

Volz et al. (1982) have shown that vertebrate and bacterial DHFRs can be superimposed remarkably well although the sequences have only about 30% identical positions. The two structures differ mainly with regard to three surface loops, where the vertebrate species have insertions of four to eight residues. These insertions comprise T39–Q47 (insert II between αI and βB, Figure 1B), Q102–V109 (insert VI, which includes αIV, between αIII and βE), and P160–S167 (insert VIII between βG1 and βG2). Inserts II and VI are relatively flexible in hDHFR as judged by temperature factors that are above average. The active site that is identified in Figure 1A by bound substrates (NAPDH and folate) is on the opposite side of these three surface loops. We reasoned that covalent linkage of the natural termini and cleavage of any one of these loops (Goldenberg & Creighton, 1983) should not destabilize the interactions between the secondary structural elements in the core of mouse DHFR. We decided to cleave the molecule at insert VI, because it is in the middle of the sequence of mDHFR where transposition of the carboxyl-terminal region to the amino terminus would be the most drastic change of sequence.

The vector pDS78/RBSII/DHFR is suitable for expressing mDHFR to high levels (Stueber et al., 1984; Bujard et al., 1987). Figure 2A shows the gene of mDHFR schematically divided into the 5′-terminal region I and the 3′-terminal region II by insert VI. Figure 3A shows the sequences of the short



B

1	4	27	47	53	71	88	92	102	109	117	130	157	168	175	
N	βA	αI	βB	αII	βC	βD	αIII	αIV	βE	αV	βF	βG1	βG2	βH	C
	10	40	53	59	76	90	102	108	116	126	139	159	172	185	186

FIGURE 1: Model of the structure of mouse dihydrofolate reductase. Same numbering for human and mouse sequences (Masters & Attardi, 1973). (A) Stereo drawing of the α-carbon backbone of hDHFR with bound molecules of NADPH (left) and folate (middle). The model depicts a hypothetical binding mode of NAPDH to the known binary hDHFR–folate complex (courtesy of Dr. F. K. Winkler). Heavy lines emphasize secondary structural elements. The loop that was cleaved in the circularly permuted variant of mDHFR lies between Q102 and V109. (B) Sequence of secondary structural elements of hDHFR. Nomenclature of Oefner et al. (1988); assignments according to Davies et al. (1990). Residue numbers give the amino-terminal (top) and carboxyl-terminal (bottom) limits of each element. The target loop that is cleaved in the circularly permuted variant comprises αIV.

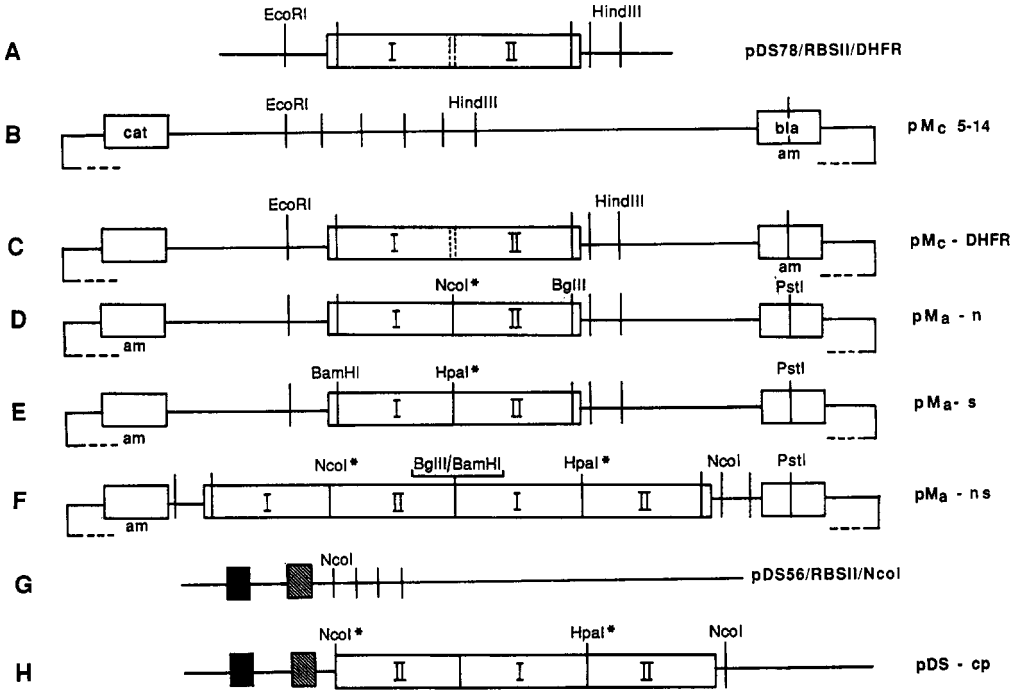


FIGURE 2: Schematic description of the plasmids used for mutagenesis and expression. See text for details. (Open boxes) Coding sequences. (I) 5′-Terminal and (II) 3′-terminal regions of *rfdhfr*. (Vertical dotted lines) Insert VI. (*cat*) Gene of chloramphenicol acetyltransferase. (*bla*) Gene of β-lactamase. (am) Amber stop codon used for selecting mutated DNA strand (Stanssens et al., 1989). (Black boxes) Promoter/operator. (Hatched boxes) Ribosome binding site. (Vertical bars) Restriction site. (A) Vector for expressing *rfdhfr*. See Figure 3A for sequences. (B) Vector for mutagenesis; *cat* and *bla* are marker genes for selection of the mutated DNA strand. (C) Product of transfer of the *Eco*RI–*Hin*dIII fragment of (A) into the multiple cloning site of (B). (D) Mutant of (C) carrying a new *Nco*I* site and start codon. See Figure 3B for sequences. (E) Mutant of (C) carrying a new *Hpa*I* site and stop codon. See Figure 3C for sequences. (F) Tandem duplication and fusion of *rfdhfr* carrying the mutations of (D) and (E). (G) Vector for cloning coding sequences bounded by *Nco*I sites. (F) Product of transfer of the *Nco*I fragment of (F) containing *cpdhfr* into the *Nco*I site of (G).

extensions at both termini of the modified mDHFR gene in pDS78/RBSII/DHFR. These were useful for the manipulations, which are described later. We designate this extended form of the enzyme as the "reference DHFR" (rfDHFR), because we expect its stability and catalytic activity to be only marginally different from those of mDHFR (Vestweber & Schatz, 1988).

For the construction of cpDHFR, the *Eco*RI/*Hin*dIII fragment from pDS78/RBSII/DHFR that carries the entire coding sequence of rfDHFR was first subcloned into the

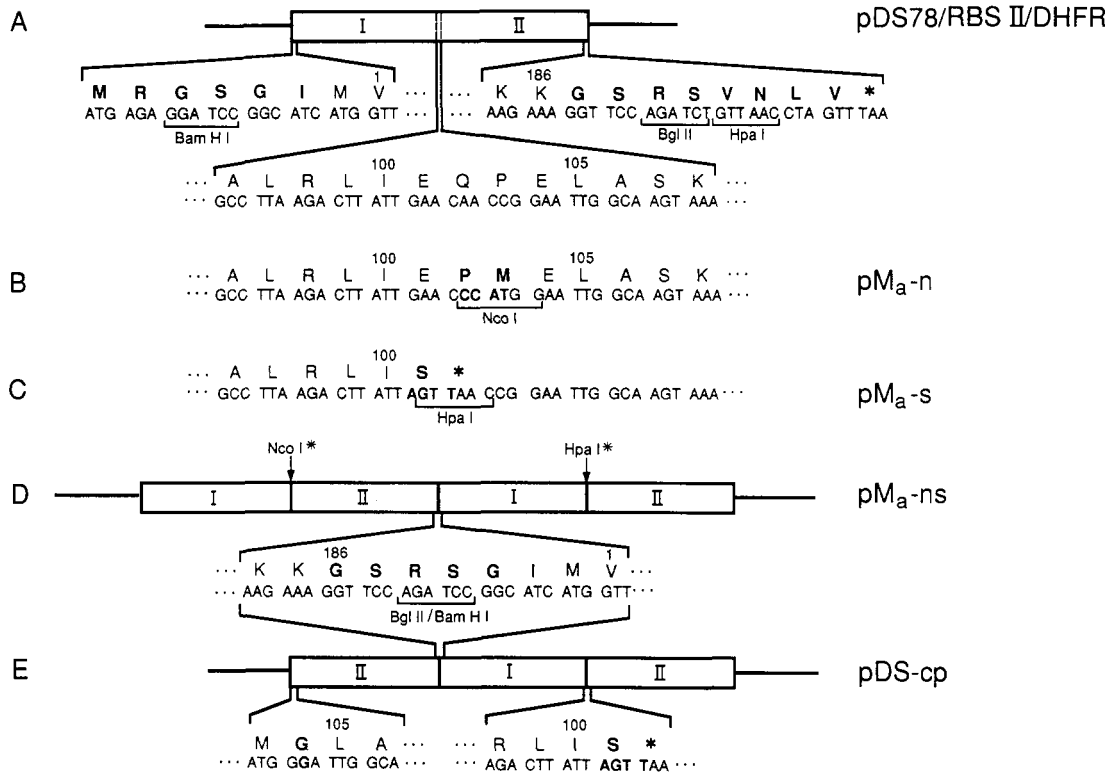


FIGURE 3: Construction of the gene encoding a circularly permuted variant of mouse dihydrofolate reductase. Partial nucleotide and amino acid sequences. Numbering as in Masters and Attardi (1983). Added or replaced amino acids in boldface type. (I) 5′-Terminal and (II) 3′-terminal regions of *rfdhfr*. (A) Reference DHFR (rfDHFR). Sequences of the terminal extensions and the wild-type insert VI of mDHFR (cf. Figure 2A,C). (B) Mutation within insert VI leads to the vector pMa-n (cf. Figure 2D). (C) Mutation within insert VI leads to the vector pMa-s (cf. Figure 2E). (D) Ligation of the *Pst*I/*Bgl*II fragment of pMa-n to the *Bam*HI/*Pst*I fragment of pMa-s leads to pMa-ns (cf. Figure 2F). (E) Sequence of cpDHFR carried by pDS-cp (Figure 2H).

Table I: Purification of Mouse Dihydrofolate Reductase (rfDHFR) and a Circularly Permuted Variant (cpDHFR)[a]

protein	purification step	total act. (units)	total protein (mg)	sp act. (units/mg)	yield (%)
rfDHFR	crude extracts	900	706	1.2	100
	protamine sulfate supernatant	980	583	1.7	109
	ammonium sulfate (55%–80% ppt)	745	212	3.5	77
	DEAE-Sephacel eluate	270	40	7.5	30
cpDHFR	crude extract	340	775	0.4	100
	protamine sulfate supernatant	362	715	0.5	107
	ammonium sulfate (55%–80% ppt)	205	250	0.8	57
	DEAE-Sephacel eluate	35	10	3.5	7

[a] See Experimental Procedures for details. Starting material was 7 g of wet cell paste in each case.

multiple cloning site of the mutagenesis vector pMc 5-14 (Figure 2B), giving pMc-DHFR (Figure 2C). Figure 3B shows how a new restriction site (*Nco*I*) was introduced subsequently by site-directed mutagenesis into insert VI of pMc-DHFR, leading to pMa-n (Figure 2D). Simultaneously, the codons for Q102 and P103 were replaced by those for P and M, respectively. Figure 3C shows how a new restriction site (*Hpa*I*) was introduced subsequently into insert VI of another sample of pMc-DHFR, leading to pMa-s (Figure 2E). At the same time, the codon of E101 was replaced by one of the codons for S. The presence of these mutations was first checked by appropriate restriction analysis and then confirmed by DNA sequencing. Two silent mutations had occurred inadvertently, namely, K108 (AAA → AAG; cf. Figure 3A) and L131 (CTT → CTC; data not shown).

The *Pst*I/*Bgl*II fragment of pMa-n that carries the new *Nco*I* site and start codon and also the *Bam*HI/*Pst*I fragment that carries the new *Hpa*I* site and stop codon were purified and ligated to generate the plasmid pMa-ns (Figure 2F). It carried the sequences of the two separately mutated coding regions of rfDHFR fused in tandem. This genetic fusion added six new codons in the linker between the original 5′- and 3′-termini of the rfDHFR gene (Figure 3D). Four of the six newly encoded residues (2S, 1G) are relatively flexible according to the scale of Karplus and Schultz (1985).

Finally, the *Nco*I fragment of pMa-ns (Figure 2F) that contains the circularly permuted gene of DHFR (separated from the coding sequence of the redundant carboxyl-terminal segment II by a stop codon; Figure 3E) was cloned in the correct orientation into the unique *Nco*I site of the expression vector pDS56/RBSII/*Nco*I (Figure 2G) to give the new expression vector pDS-cp (Figure 2H).

Sequencing the segment containing the new start codon revealed that an A → G transition had occurred next to the new start codon of cpDHFR during the last subcloning step, changing the codon of E104 (GAA) into a codon for glycine (GGA). As this replacement should not compromise the stability of the circularly permuted DHFR (cpDHFR) a priori, we decided to characterize this mutant of the originally planned cpDHFR.

Purification and Characterization of cpDHFR. The same purification scheme was used for both rfDHFR and cpDHFR

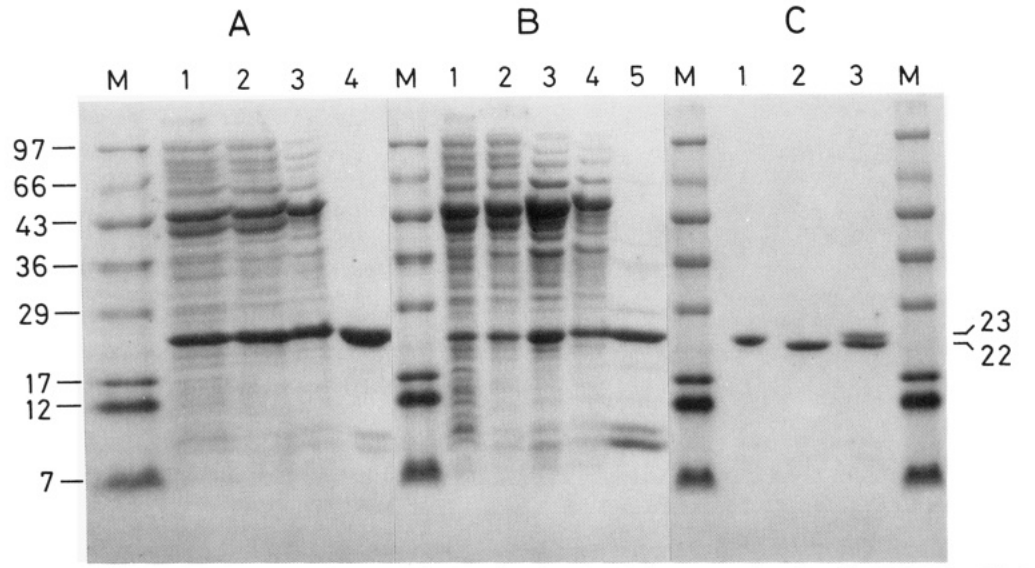


FIGURE 4: Purification of mouse dihydrofolate reductase (rfDHFR) and a circularly permuted variant (cpDHFR). Gel electrophoresis in the presence of SDS. See Table I for quantitative data. (M) Marker proteins with indicated 10^{-3} M_r values. (A) rfDHFR: 1, crude extract; 2, protamine sulfate supernatant; 3, ammonium sulfate fraction at 55–80% saturation; 4, DEAE-Sephacel eluate. (B) cpDHFR: 1, crude extract; 2, protamine sulfate supernatant; 3, 55% saturation ammonium sulfate supernatant; 4, ammonium sulfate fraction at 55–80% saturation; 5, DEAE-Sephacel eluate. (C) Proteins purified by gel chromatography on columns of Sephacryl-S200: 1, rfDHFR; 2, cpDHFR; 3, rfDHFR + cpDHFR.

(Table I). Two different methods were tested for preparing the crude extract. The method using cell membrane permeabilization by Triton X100 (Miozzari et al., 1978) was rapid and reproducible but had the important drawback that it was difficult to get rid of the detergent during the subsequent purification steps. We therefore used sonication to disrupt the cells (Thillet et al., 1988) followed by removal of cell debris and precipitation of DNA with protamine sulfate. We also used phosphate buffer containing a thiol and a chelating agent to stabilize the proteins. rfDHFR represented about 15% of the protein content of the crude extract, but the production of the circularly permuted variant (cpDHFR) was 3 times less as judged both by polyacrylamide gel electrophoresis in the presence of SDS and by Western blots. Only relatively small amounts of both rfDHFR and cpDHFR were found in the pellets (not shown) so that the differences cannot be due to different partitioning into insoluble aggregates. The lower yield of cpDHFR may be due to less efficient transcription or translation, or both, of the permuted gene. It may also be due to the lower stability of the protein, which would render it more susceptible toward intracellular proteolysis (Thillet et al., 1988).

The subsequent fractionation of the supernatant with ammonium sulfate and the isocratic filtration through an anion exchanger followed the procedure of Thillet et al. (1988). In the latter step, eDHFR is charged negatively at pH 8.2 (p*I* = 4.6; Gupta et al., 1977) and is therefore retained whereas both rfDHFR and cpDHFR (p*I* = 9.2 and 9.4, respectively, see below) pass through unhindered. The major difference between rfDHFR and cpDHFR was the strong tendency of the latter to precipitate, increasingly so with increasing purity. This property led to large and uncontrollable losses of protein (cf. Table I) that could not be prevented by the addition of glycerol to 5% (v/v).

The amino-terminal sequence of the purified proteins was determined to provide an independent identity check. The observed sequence for cpDHFR (GLASKVDMVW...) agreed with the independently determined coding sequence, including the adventitious extra point mutation E104G that follows the first methionine residue (cf. Figure 3E). The amino-terminal sequence of rfDHFR (MRGSGIMV...) also agreed with expectations (cf. Figure 3A). Thus, rfDHFR synthesized in *E. coli* retains the translation initiating methionine at its amino terminus but cpDHFR does not.

Table II: Kinetic Constants of Mouse Dihydrofolate Reductase (rfDFR) and a Circularly Permuted Variant (cpDHFR)

protein	k_{cat} (s^{-1})	K_m^{NADPH} (μM)	K_m^{DHF} (μM)	K_i^{TMP} (μM)	K_i^{MTX} (nM)
rfDHFR[a]	3.7	0.8	1.1	4.0	≤0.1
cpDHFR[a]	3.5	0.9	1.2	3.0	≤0.1

[a] 0.05 M imidazole hydrochloride buffer, pH 7.0, 1 mM EDTA, and 1 mM DTE, at 25 °C.

Analysis by gel electrophoresis in the presence of SDS indicated that the preparations of rfDHFR were routinely more than 95% pure after chromatography on DEAE-Sephacel (Figure 4A) but cpDHFR was less pure (Figure 4B). Further purification of the proteins was achieved by gel filtration through columns of Sephacryl S200 (Figure 4C), but losses of cpDHFR were severe. Figure 4C also shows that the small difference in molecular weight values (rfDHFR, 22 878, 200 residues; cpDHFR, 21 912, 191 residues) can lead to the electrophoretic separation of the bands. Both rfDHFR and cpDHFR bands were stained with the same relative intensity when anti-mDHFR antibodies were used in Western blots of these gels (data not shown).

The proteins migrated as sharp bands in nondenaturing polyacrylamide gels in the presence as well as in the absence of methotrexate (data not shown). The isoelectric points of rfDHFR and cpDHFR were 9.2 and 9.4, respectively, as determined at 10 °C using a pH gradient from 3 to 10. Authentic mDHFR has a p*I* of 8.2 (Gupta et al., 1977). The p*I* values of rfDHFR and cpDHFR were decreased to 8.6 and 9.3, respectively, upon incubation with methotrexate (data not shown).

Because methotrexate binds to mDHFR stoichiometrically under the conditions used, the smallest concentration of methotrexate that causes complete inhibition corresponds to the concentration of active sites. Figure 5 shows that both rfDHFR and cpDHFR bind methotrexate strongly leading to

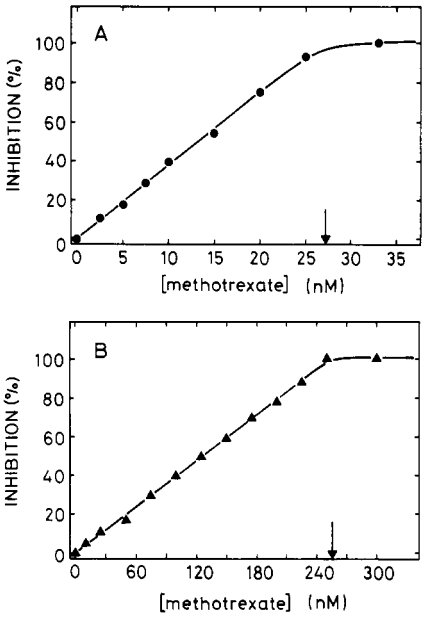


FIGURE 5: Titration of mouse dihydrofolate reductase (rfDHFR) and a circularly permuted variant (cpDHFR) with methotrexate. Plots of the percent inhibition of DHFR activity. DEAE-Sephacel fractions were incubated with the indicated concentrations of methotrexate as described (Sheldon & Brenner, 1976) and assayed. (A) 42 nM rfDHFR; (B) 600 nM cpDHFR. (—) Best-fit curves indicating that $K_d^{MTX} \leq 0.1$ nM. Arrows indicate the MTX concentration equivalent to the active-site concentration.

complete inhibition of enzymic activity. The upper limit of the inhibition constant was the same for both proteins ($K_i^{MTX} \leq 0.1$ nM, Table II) and only 4-fold larger than that of mDHFR ($K_i^{MTX} = 0.025$ nM; Thillet et al., 1988). However, the stoichiometry of binding of MTX (rfDHFR at 67%, cpDHFR at 43%) indicates that the preparations are either not pure (cf. Figure 4A,4B) or partially denatured, or both.

Enzyme Kinetics. Steady-state kinetic measurements were used to compare indirectly the structures of the active sites of rfDHFR and cpDHFR. The k_{cat} values were corrected for the proportion of active enzyme in the different preparations as judged by titration with methotrexate (cf. Figure 5A,B). The results are summarized in Table II and show that both the k_{cat} and the K_M values of rfDHFR and cpDHFR are practically the same, and in good agreement with some of the published values (Eilers & Schatz, 1986; Cha et al., 1981) for mouse L1210 lymphoma cell DHFR and authentic mDHFR (Thillet et al., 1988, 1990), but quite different from mDHFR from fibroblasts (Haber et al., 1981). It is reasonable to attribute discrepancies to the different assay conditions used by the other investigators. The competitive inhibition constants of trimethoprim (TMP) versus DHF were also very similar for rfDHFR, cpDHFR (Table II), and authentic mDHFR (3.5 μM; Thillet et al., 1988), albeit the latter value refers to somewhat different conditions.

Effects of DHFR Activating Agents. A remarkable kinetic property of the DHFRs from some vertebrate species is their activation by agents such as mercurials, salts, and urea (Delcamp et al., 1983; Duffy et al., 1987; Huang et al., 1989; Endo & Schatz, 1988; Prendergast et al., 1988; Thillet et al., 1990; Tan et al., 1990), whereas DHFRs from other species are either unaffected or actually inhibited (Blakley, 1984).

As seen in Figure 6, the treatment with *p*-(hydroxymercuri)benzoate (pHMB) in great molar excess produced for both rfDHFR and cpDHFR a transient increase in catalytic activity that reached a maximal value of about 200% after 10 min. Thereafter it decreased slowly over the next hour to

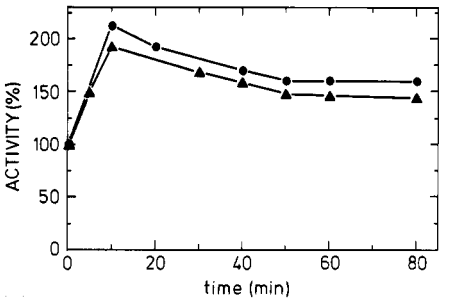


FIGURE 6: Activation of mouse dihydrofolate reductase (rfDHFR) and a circularly permuted variant (cpDHFR) by *p*-(hydroxymercuri)benzoate. Enzymic activity was normalized with respect to the control lacking pHMB. (●) rfDHFR. (▲) cpDHFR.

150% of the control. In contrast to the observation of Duffy et al. (1987), the presence of 10 μM bovine serum albumin did not stabilize the activated form of DHFR. The activation effect of 0.8 M KCl was also examined. A maximum of 5-fold increase in the activity was observed for both rfDHFR and cpDHFR in the presence of 10 μM BSA (data not shown). Activation was rapid, followed by a slow return to an intermediate level of activity, again in contrast to the report of Duffy et al. (1987) on authentic mDHFR, where activation was long-lived.

Folding Studies. The effect of the circular permutation of the amino acid sequence on the intrinsic stability of rfDHFR was analyzed by monitoring the unfolding of both rfDHFR and cpDHFR at equilibrium. Because mDHFR is activated by urea (Duffy et al., 1987; Endo & Schatz, 1988) and also by GuCl (A. Buchwalder, unpublished work), we decided to use a spectroscopic method rather than enzyme activity to monitor the unfolding transitions of the two proteins.

Figure 7A shows that the CD spectra of rfDHFR and cpDHFR in the peptide region are not identical but comparable to that of DHFR from a MTX-resistant subline of a murine lymphoma ($\theta_{210} \sim 8000$ deg cm^2 dmol^{-1}; Gupta et al., 1977). However, in view of the inhomogeneity of our preparations (Figure 4), it is not clear whether the difference is significant. For the same reason, the difference between the CD spectra of rfDHFR and cpDHFR was not analyzed with respect to the computed content of secondary structure, although the difference may be due in part to loss of the amino-terminal helix αIV of cpDHFR (cf. Figure 1B).

Increasing concentrations of GuCl induce the unfolding of eDHFR between 1 and 2 M GuCl (Villafranca et al., 1987). We have measured the normalized unfolding transitions of both rfDHFR and cpDHFR by monitoring θ_{222} as a function of GuCl concentration (Figure 7B). The data scatter considerably but show nevertheless that the unfolding of both proteins is reversible within the error limits. The most important point is that the transition of cpDHFR is shifted by about 0.2 M toward lower concentrations of GuCl by comparison to that of rfDHFR. It did not appear to be fruitful to analyze the data quantitatively because it is not clear whether these proteins unfold via a simple two-state equilibrium process as does eDHFR (Ahrweiler & Frieden, 1991; Kuwajima et al., 1991). The initially low values of θ_{222} were not reproducible in the renaturation experiments because we used dilution (to minimally 0.2 M GuCl) rather than dialysis. Moreover, the starting material was inhomogeneous (cf. Figure 4) and contained aggregated material that is formed during storage.

mDHFR is relatively stable toward trypsin (Vestweber & Schatz, 1988) when monitored by polyacrylamide gel electrophoresis in the presence of SDS. We have confirmed this

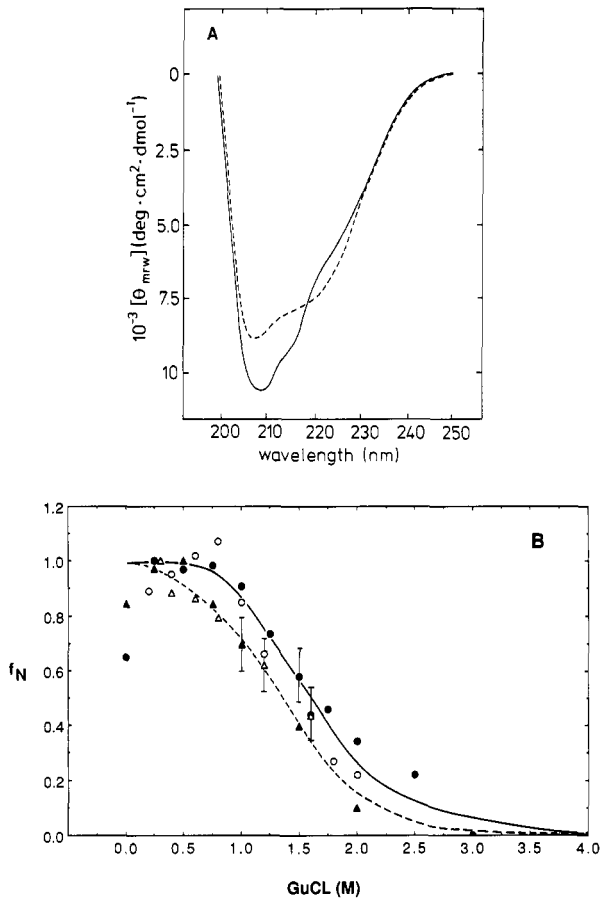


FIGURE 7: Circular dichroism spectra and unfolding transitions of mouse dihydrofolate reductase (rfDHFR) and a circularly permuted variant (cpDHFR). 10 mM potassium phosphate buffer at pH 7.5, containing 0.2 mM EDTA and 0.1 mM DTE. Temperature: 20 °C. (A) Normalized far-UV CD spectra of rfDHFR (—) and cpDHFR (---). (B) Unfolding by guanidinium chloride monitored at 220 nm. The linear change of θ_{220} above 3 M was extrapolated to 0 M GuCl concentration. f_N is the apparent fraction of native protein and represents the deviation of θ_{222} from the base line and is normalized by the value at 0 M GuCl concentration. (●) Unfolding of rfDHFR; (○) refolding of rfDHFR; (▲) unfolding of cpDHFR; (△) refolding of cpDHFR.

report for both rfDHFR and cpDHFR (data not shown). However, as shown by Duffy et al. (1987) for mDHFR, the enzymic activity of both rfDHFR and cpDHFR decreased significantly upon treatment with trypsin.

Discussion

The goal of this work was to use circular permutation of the amino acid sequence of rfDHFR to probe the roles of the natural amino and carboxyl termini and of the loop between αIII and βE in the stability, activity, and refolding process of the protein. We have compared several enzymological and physicochemical properties of cpDHFR with those of rfDHFR (the recombinant wild-type standard) to arrive at the following general conclusions.

Most of the functional properties of cpDHFR examined in this work were very similar to those of rfDHFR. In view of the complex catalytic mechanism of mDHFR (Thillet et al., 1990), which involves slow isomerization between two conformers, it is striking that the values of k_{cat} and K_m for NADPH and DHF are identical within the error limits (Table II). Even the transient activation in response to pHMB (Figure 6), which seems to be associated with the conformational flexibility of the loop between αI and βB (Tan et al., 1990; cf. Figure 1A,B) is indistinguishable.

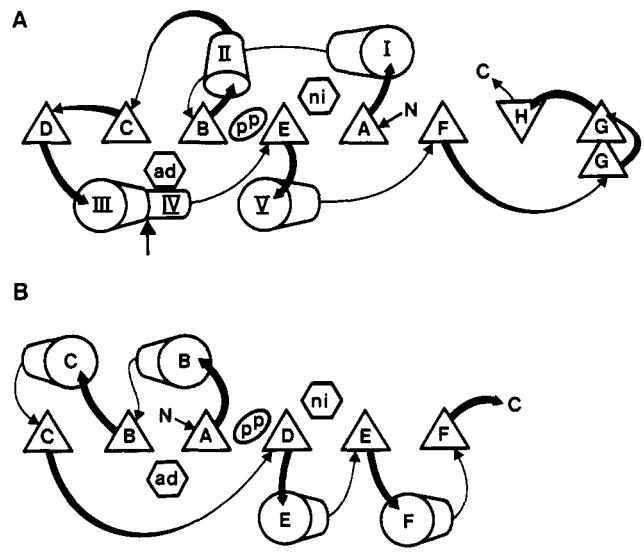


FIGURE 8: Putative mononucleotide binding fold of mouse dihydrofolate reductase (Kraut & Matthews, 1987). (N) Amino terminus; (C) carboxyl terminus; (△) β strands rising toward the observer; (▽) β strands descending; (○) α helices; (ad) adenosine moiety; (ni) nicotinamide nucleoside moiety; (pp) pyrophosphate moiety of NADPH or NADP$^+$. (A) Mouse DHFR, cf. Figure 1A,B. (Arrow) Loop cleaved in cpDHFR. (B) Idealized NAD$^+$ binding domain of dehydrogenases.

The similarity of the dissociation constants for the folate analogues TMP and MTX is even more remarkable for the following reasons. First, Thillet et al. (1988) have shown that various point mutants of mDHFR involved in direct interaction with DHF suffer up to 10^3-fold increases of K_i^{MTX} with only minor changes in k_{cat} and K_M^{NADPH}. Second, these residues are located mainly on βA, αI, and βF, which are directly or indirectly affected by the new linker between βA and βH in cpDHFR (Figure 8A). These observations indicate that the active sites of rfDHFR and cpDHFR must be almost identical. Moreover, the resistance of both proteins toward trypsin is the same, so that also their surface loops are equally inaccessible, although the new linker between βH and βA of cpDHFR contains an arginine residue (Figure 3D). Therefore, the three-dimensional structures of the two proteins must also be essentially the same. The major difference between rfDHFR and cpDHFR is only qualitative, namely, the decreased stability of the latter, which is reflected by the relative shift of the GuCl-induced unfolding transition (Figure 7B) as well as by its increased tendency to aggregate.

These results prove that the severed loop is unimportant for catalysis of the DHFR reaction. Although this loop is distant from the active site (cf. Figure 1A), mDHFR is thought to undergo synergistic conformational changes upon binding substrates (Appleman et al., 1989). These involve distant regions of the molecule and also appear to be responsible for the activating effects exerted by mercurials, salts, and denaturants (Tan et al., 1990) as well as the solubilizing effect of salt (Williams & Morrison, 1991).

Our data also prove that the natural amino and carboxyl termini do not play a mandatory role during the refolding process. More importantly, the loop between αIII and βE of mDHFR is also dispensable. That is, if a short-range interaction between αIII and βE were necessary in early folding intermediates, it is not essential for these secondary structural elements to be connected directly (Creighton, 1991).

Transient folding intermediates have been detected in the case of eDHFR (Garvey et al., 1989; Frieden, 1990; Kuwajima et al., 1991). Moreover, Ahrweiler and Frieden (1991) have

suggested from the effect of a deletion in the equivalent αIII–loop–βE region of eDHFR, that this loop may act as a hinge in the folding process. Although it remains to be shown whether the overall refolding mechanisms of mDHFR and eDHFR differ in this important respect, it is clear that circular permutation of the sequence provides for an alternative and more radical approach toward probing surface loops than replacements, insertions, and deletions. It is also more versatile than fragment complementation (Wetlaufer et al., 1981; Taniuchi et al., 1986) because the importance of sequence segments can be assessed without converting the first-order folding into a second-order assembly process. Additionally, the expression and purification of a single gene product are simpler.

The backbone fold of DHFR resembles that of NAD^+ binding domains of several dehydrogenases (Rossmann et al., 1975; Bolin et al., 1982; Wierenga et al., 1985; Kraut & Matthews, 1987). The schematic drawing of the structure of mDHFR (Figure 8A) shows that the region comprising βB·αII·βC·βD·αIII is similar to the mononucleotide binding subdomain of dehydrogenases (Figure 8B) to the extent that it interacts mainly with the adenosine 2′,5′-diphosphate moiety of NADPH (Oefner et al., 1988; Davies et al., 1990). The major difference is the distribution of external α helices αIII and αC. This subdivision of the structure of DHFR into two subdomains is supported by the thermal mobility of the loop between αI and βB of hDHFR (Oefner et al., 1988). Moreover, Bystroff and Kraut (1991) have shown that the same region (designated the adenosine binding domain) in eDHFR moves as a rigid body during the binding of $NADP^+$ and MTX.

From the structural point of view, circular permutation of the sequence of rfDHFR has cleaved one of the flexible hinges connecting the adenosine binding domain to the remainder of the protein molecule. It has also connected the amino and carboxyl termini that otherwise interrupt the second major domain. This domain comprises the region between βE and αI (Figure 8A; Bystroff & Kraut, 1991) and is responsible mainly for binding the nicotinamide mononucleotide moiety of NADPH and the pteridine ring of DHF. Therefore, circular permutation has altered the topological connectivity of the polypeptide chain from an insert of the adenosine binding domain into the second domain to that of a new position at its carboxyl terminus. Perhaps the strong tendency of cpDHFR to aggregate may be due to an intermolecular interaction between these putative subdomains, which would lead to linear polymers.

To our knowledge rfDHFR is the third monomeric protein for which an active variant with a circularly permuted sequence has been constructed. The approach used here differs from that used by Luger et al. (1989), in that the entire coding sequence of the protein was duplicated and fused in tandem, accompanied by elimination or inactivation of the amino- and carboxyl-terminal coding regions. A similar mechanism for transposing coding regions is likely to have occurred during evolution (Li & Graur, 1991).

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Registry No. DHFR, 9002-03-3; MTX, 59-05-2; NADPH, 53-57-6; DHF, 4033-27-6; TMP, 738-70-5.

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