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Primary Structure of Chicken Liver Dihydrofolate Reductase†

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ABSTRACT: The complete covalent structure of dihydrofolate reductase from chicken liver is described. The S-carboxymethylated protein was subjected to cleavage by cyanogen bromide which produced five fragments. Fragment CB2 contained an internal homoserine residue which was not cleaved by cyanogen bromide. Sequences and ordering of the cyanogen bromide fragments were established by means of automated sequencer analyses of the fragments and from smaller peptides generated by proteolysis with trypsin and staphylococcal protease. The covalent structure of the single polypeptide chain comprises 189 residues of molecular weight 21 651. The chicken liver enzyme is homologous to that from L1210 cells and shows regions of homology to dihydrofolate reductases from *Streptococcus faecium*, *Escherichia coli*, and *Lactobacillus casei*. These homologous regions in the chicken liver enzyme are primarily related to conserved amino acid residues implicated in the binding of NADPH and methotrexate by bacterial dihydrofolate reductases.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. The latter serves as a coenzyme for a number of one-carbon transfer reactions in purine and pyrimidine biosynthesis, including that of thymidylate (Blakely, 1969; Huennekens et al., 1971). The reductase appears to be the major intracellular receptor for the action of 4-amino analogues of folic acid, such as amethopterin (4-amino-10-methyl-4-deoxyfolate, methotrexate). Inhibition of the enzyme by methotrexate depletes the tetrahydrofolate pool, resulting in a decreased synthesis of thymidylate and, in turn, an inhibition of DNA synthesis. Thus, methotrexate has been employed extensively in the chemotherapeutic treatment of leukemias, lymphomas, psoriasis, and other clinical disorders (Bertino & Johns, 1972). In addition, a differential sensitivity to certain drugs, such as trimethoprim, by mammalian and bacterial reductases has lead to the development of a class of compounds with potent antibacterial activity (Burchall & Hitchings, 1965). The underlying molecular basis for these differential inhibitory effects must reside in differences in the three-dimensional architecture of the active centers of bacterial vs. animal dihydrofolate reductases. Thus, a knowledge of the complete primary and tertiary structure of dihydrofolate reductases from both bacterial and animal sources in the presence and absence of inhibitors, coenzymes, and substrates should aid in our understanding of these differential inhibitory effects. In addition, such knowledge may suggest approaches for the rational design of specific chemotherapeutic agents.

Previous results from this laboratory have lead to the elucidation of the complete amino acid sequence of dihydrofolate reductase from *Lactobacillus casei* (Freisheim et al., 1977, 1978; Bitar et al., 1977). This sequence information has aided in the resolution of the X-ray structure of the *L. casei* reductase–NADPH–methotrexate ternary complex at 2.5 Å as recently described by Matthews, Kraut, and co-workers (Matthews et al., 1978, 1979). Sequence homology alignments suggested by this laboratory (Freisheim et al., 1978; Bitar et al., 1977) have aided in implicating certain highly conserved

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amino acid residues in the three-dimensional binding of methotrexate and NADPH to dihydrofolate reductase (Matthews et al., 1978, 1979).

In attempting to account for the differential drug binding displayed by animal vs. bacterial enzymes, amino acid sequence studies have largely centered on bacterial reductases, primarily due to the large amounts of enzyme protein which can be isolated. Thus, in addition to the *L. casei* reductase (see above), the complete amino acid sequences of the enzymes from *Streptococcus faecium* (Gleisner et al., 1974), *Escherichia coli* strain RT500 (Stone et al., 1977), and *E. coli strain MB 1428* (Bennett et al., 1978) have been determined. Recently, Stone et al. (1979) have elucidated the amino acid sequence of the mouse L1210 cell dihydrofolate reductase. However, no indication is given by the authors that X-ray crystallographic studies are in progress. The X-ray structural studies on chicken liver dihydrofolate reductase are progressing well in the laboratory of Kraut, Matthews, and co-workers (Matthews et al., 1979; D. A. Matthews and J. Kraut, personal communication). Thus, the elucidation of the amino acid sequence of the chicken liver dihydrofolate reductase reported herein together with progressing structural results from X-ray diffraction studies should aid in a better understanding of the molecular basis for structural, functional, and drug binding differences between reductases from different species.

Materials and Methods

Chicken liver dihydrofolate reductase was purified by methotrexate affinity chromatography followed by isoelectric focusing as described by Kaufman & Kemerer (1977). The purified enzyme exhibited a single protein band on either 7.5 or 10% polyacrylamide gel electrophoresis in the presence or absence of $NaDodSO_4$[1] (Weber & Osborn, 1969). The enzyme was free from bound folates or pyridine nucleotides, following isoelectric focusing, as evidenced from its absorption spectrum. Trypsin treated with Tos-Phe-CH_2Cl and carboxypeptidases A and B treated with diisopropyl phosphorofluoridate were the most highly purified preparations available from Worthington. Carboxypeptidase Y was obtained from Pierce Chemicals. The neutral protease from *Staphylococcus aureus*, strain V8, was purchased from Miles Laboratories. Sephadexes and ion-exchange Sephadexes of various grades were obtained from Pharmacia Fine Chemicals. Bio-Gels of various porosities were supplied by Bio-Rad Laboratories. *N,N*-Dimethylbenzylamine, *N,N*-dimethylallylamine, phenyl isothiocyanate, heptafluorobutyric acid, ethanethiol, cyanogen bromide, *N,O*-bis(trimethylsilyl)acetamide, and phenylthiohydantoin amino acid standards were sequenal grade products from Pierce Chemicals. Some of these reagents were also obtained from Beckman Chemicals. Heptane, benzene, chlorobutane, ethyl acetate, and 1-propanol were the "glass-distilled" grade of Burdick and Jackson Laboratories. Acetyl chloride was obtained from Mallinckrodt Chemicals and polybrene from Aldrich Chemicals. Iodo[2-^{14}C]acetic acid was obtained from New England Nuclear Corp. Carboxymethylation of the single cysteine residue of the enzyme was accomplished by a modification of the method of Crestfield et al. (1963) using 6 M guanidine hydrochloride in place of urea. The S-carboxymethylated protein was then dialyzed against water to remove excess reagents and salts. In some instances, iodo[^{14}C]acetic acid was employed for the modification.

Cleavage by Cyanogen Bromide. Cleavage at methionyl bonds of dihydrofolate reductase was performed essentially as described by Gross (1967). Lyophilized, salt-free S-carboxymethylated protein was dissolved in 70% formic acid (10 mg/mL), and a threefold excess (w/w) of cyanogen bromide was added. The reaction vessel was flushed with nitrogen and tightly sealed, and the reaction was allowed to proceed at room temperature in the dark for 20 h. The reaction mixture was then diluted 10-fold with cold water and lyophilized twice from the same volume of cold water.

Enzymic Hydrolysis. Peptides or intact protein was generally stored at −20 °C in lyophilized, salt-free form or occasionally in 10% formic acid or water. Before cleavage with a particular proteolytic enzyme, an aliquot of the protein or peptide was dried, dissolved in an appropriate buffer, such as 0.1 M NH_4HCO_3 (pH 8), and treated with 2–5% by weight of the desired protease. Hydrolyses were usually carried out for 4 h at 37 °C and then terminated by adjustment to pH 3.0 with formic acid, followed by lyophilization.

Tryptic hydrolysis was limited to arginine residues by prior treatment of the enzyme with maleic anhydride (Butler et al., 1969). A 30-fold molar excess of maleic anhydride per lysine residue was added to 2.7 μmol of protein in 7 mL of 0.1 M $NaHCO_3$, pH 8.5. The pH was maintained between 8.5 and 10 with 5 N NaOH. The maleylated enzyme was then desalted on a 2.5 × 28 cm Sephadex G-25 column in 0.05 M NH_4HCO_3, pH 9.0, and lyophilized. The maleylated protein was dissolved in 0.2 M NH_4HCO_3, pH 8.0 (12 mg/mL), and treated with a total of 2% Tos-Phe-CH_2Cl-trypsin for 4 h at 37 °C. The pH was then lowered to 3.0 with acetic acid and the mixture lyophilized. Certain maleylated tryptic fragments were demaleylated in 30% acetic acid for 72 h at 37 °C and subjected to hydrolysis (at lysine residues) by Tos-Phe-CH_2Cl-treated trypsin (see above).

Fragment CB5 (1.2 μmol) was hydrolyzed with staphylococcal protease (24 nmol) in 4.0 mL of 0.05 M NH_4HCO_3, pH 7.8, for 4 h at 37 °C (Houmard & Drapeau, 1972). Carboxyl-terminal sequences were determined by using equimolar mixtures of carboxypeptidases A and B (Ambler, 1963). These exopeptidase digestions were carried out at 37 °C at enzyme/substrate molar ratios of from 1:30 to 1:50 for various time periods.

Peptide Purification. A variety of different conditions was employed for the purification of peptides generated by chemical and enzymic means. Sephadex G-75, G-50, and G-25 superfine resins in columns equilibrated with either 9% formic acid or 0.05 M NH_4HCO_3, pH 9.0, were used for peptide separations, as were Bio-Gels of various porosities. Chromatography of certain peptides on SP-Sephadex C-25 columns equilibrated with either 9% formic acid or 30% acetic acid using KCl gradients was also employed. Peptides were located in effluent fractions by absorbance at 230 or 280 nm or by reaction with ninhydrin following alkaline hydrolysis (Hirs et al., 1956). In general, peptides were desalted on columns of Sephadex G-10, Sephadex G-15, or Bio-Gel P-2 in 9% formic acid or dilute NH_4OH depending on the peptide. In some instances, small peptides were purified by preparative paper electrophoresis at pH 1.9 (90–100 kV min). The purity of small peptides was assessed by thin-layer electrophoresis at pH 1.9, followed by thin-layer chromatography in 1-butanol–pyridine–acetic acid–H_2O (15:10:3:12 v/v; Landon et al., 1971). In addition to thin-layer electrophoresis and chromatography of smaller peptides exhibiting a single ninhyd-

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Tos-Phe-CH_2Cl, L-1-chloro-3-(tosylamido)-7-amino-2-heptanone; CM-Cys, *S*-(carboxymethyl)cysteine; CB, cyanogen bromide; DMBA, *N,N*-dimethylbenzylamine; BSA, *N,O*-bis(trimethylsilyl)acetamide; DMAA, *N,N*-dimethylallylamine; PTH, phenylthiohydantoin; T, tryptic; MT, maleylated tryptic; SP, staphylococcal protease.

rin-positive spot, the presence of a single amino-terminal sequence was considered an adequate criterion of peptide purity. In the latter case 25–75 nmol of peptide was subjected to four to six turns of automated Edman degradation (see below).

Amino Acid Analysis. Samples were hydrolyzed in constant-boiling HCl in evacuated tubes flushed with nitrogen at 110 °C for 20–120 h. Analyses were performed on a Durrum D-500 amino acid analyzer according to instructions supplied with the instrument, and all results are expressed as molar ratios. Unless specified otherwise, values given in the tables are uncorrected either for partial destruction of certain amino acids or for incomplete liberation of valine and isoleucine after 24 h of hydrolysis. Cysteine was determined as cysteic acid and methionine as methionine sulfone (Hirs, 1967). In some cases, cysteine was determined as CM-Cys (Crestfield et al., 1963). Tryptophan was determined by hydrolysis with 4 M methanesulfonic acid (Simpson et al., 1976). A time course of hydrolysis was done on the purified enzyme with time points of 24, 48, 72, 96, and 120 h. The protein values for serine and threonine were determined by extrapolating to zero time while valine and isoleucine were obtained from 120-h values.

Edman Degradation. Automated Edman degradations were performed according to the method of Edman & Begg (1967) on a Beckman Model 890C sequencer equipped with a cold trap (California Lab Equipment Co. no. 5007) cooled by a CC-100 cryocool unit (Neslab) and placed in line before the low vacuum pump to reduce chemical contamination of the pump oil. In later sequence runs, a Model P-6 autoconverter (Sequemat) was installed in-line between the waste-collect valve and the fraction collector. The autoconverter functions by drying with nitrogen the anilinothiazolinone amino acid extracted from the spinning cup with butyl chloride and then converting this amino acid derivative to the phenylthiohydantoin form with 1.5 N methanolic HCl (Horn & Bonner, 1977). The reagent is then removed by drying under nitrogen and the phenylthiohydantoin amino acid is delivered to the fraction collector with dichloroethane–methanol (7:3) (v/v).

The intact protein and large fragments were sequenced by using a single cleavage protein program designated DMBA II (Hermodson et al., 1972). Smaller fragments were sequenced by using one of the following peptide programs with or without polybrene as an inert carrier (Capra et al., 1977): DMBA I double cleavage "peptide program" (Koide et al., 1978); DMBA IV (Hermodson, 1977); DMAA I (Beckman no. 102974); Quadrol III, a 0.1 M quadrol peptide program (Brauer et al., 1975). All the peptide programs were modified to end the Edman cycle with the peptide in the coupled state (Hermodson et al., 1972), to incorporate the techniques of precipitating the peptide before solvent extractions, and to remove the heptafluorobutyric acid as an azeotropic mixture with chlorobutane before product extraction (Crewther & Inglis, 1975). In several instances, severe problems were encountered with regard to retention of protein or peptide material in the spinning cup of the sequenator. This was especially true for very hydrophobic peptides. In some cases, polybrene lessened the retention problem, but in others it did not.

The phenylthiohydantoin amino acids were identified mainly by the gas chromatography procedure of Pisano & Bronzert (1969) as modified by Hermodson et al. (1972). In later experiments, high-performance liquid chromatography was used in conjunction with the P-6 autoconverter for identification of the phenylthiohydantoin amino acids.

Samples of phenylthiohydantoin amino acids were recovered from the sequencer in dichloroethane–methanol (7:3 v/v) and dried under a stream of nitrogen with gentle heating. The dried phenylthiohydantoin amino acids were redissolved in 50 μL of methanol, and suitable aliquots were injected into a Waters Model ALC/GPC 204 liquid chromatograph equipped with a Model 710 WISP autosampler. Models 450 and 440 fixed and variable wavelength detectors were used in conjunction with a Shimadzu Chromatopac-E1A integrator. The procedure used was an adaptation of the method of Bridgen et al. (1976). The phenylthiohydantoin amino acids were separated on a Waters C_{18}-μBondapack column by a 31-min linear gradient of methanol (14–56%) in aqueous buffer (0.01 sodium acetate, pH 4.22) at a flow rate of 2.2 mL/min controlled by a Waters Model 660 solvent programer. The total running time, including column equilibration, was 46 min. Most phenylthiohydantoin amino acids were identified by their absorbance at 254 nm. PTH-δ-Thr was also detected at 313 nm. The P-6 autoconverter on the sequencer eliminates the ethyl acetate extraction from the sample workup so that all the phenylthiohydantoin amino acids are in a single phase. The P-6 autoconverter also converts PTH-Asp, PTH-Glu, and PTH-CM-Cys to the corresponding methyl esters. These methyl ester derivatives elute in unique positions between PTH-Ala and PTH-Tyr, between PTH-Tyr and PTH-Pro, and between PTH-Pro and PTH-Val, respectively. With this system, PTH-Met and PTH-Val coelute as does PTH-Phe with PTH-Ile and PTH-Ala with PTH-Arg. However, these residues can easily be identified from compositional considerations, gas chromatographic analysis, or in the case of PTH-arginine by the spot test (Hermodson et al., 1972).

Results

Amino Acid Composition and Sequence Analysis of the Intact Enzyme. Chicken liver dihydrofolate reductase contains five methionine residues and a single cysteine residue based on amino acid analysis (Table I), in agreement with previous results (Kaufman & Kemerer, 1977). Automated sequence analysis of the intact S-[^{14}C]carboxymethylated enzyme placed the first 27 residues and located the single cysteine residue at position 11 (Table II). The protein subjected to sequence analysis contained 145 060 cpm, corrected for background. At steps 11 and 12 a total of 35 280 cpm was released; this amounts to 139 000 cpm, based on an *absolute* sequence yield of 25% at step 11. Thus, of the total counts per minute applied, 96% was released in steps 11 and 12. Step 11 clearly contains the radiolabel and step 12 represents a 20% overlap of the [^{14}C]CM-Cys from the previous step. The observed trimethylsilylated PTH-δ-Ser (6 nmol) is the major breakdown product of trimethylsilylated CM-Cys from gas chromatographic analysis (Table II, step 11). In addition, the first methionine residue was located at position 14. The general strategy employed to generate peptides of suitable length for sequence analysis of the protein is illustrated in Figure 1.

Separation of Fragments of Dihydrofolate Reductase Generated by Cleavage with Cyanogen Bromide. The intact [^{14}C]carboxymethylated protein (52 mg, ~2.3 μmol) was cleaved with cyanogen bromide as described under Materials and Methods. The products were dissolved in 5 mL of 9% formic acid and applied to a column of Sephadex G-50 superfine (SF). The effluent profile is indicated in Figure 2. The first fraction in the profile appears as an unresolved mixture of minor cyanogen bromide cleavage products. The second and third fractions contained fragments CB3 and CB5, respectively; each of these fragments was further purified on a 1.5 × 83 cm Sephadex G-75 SF column in 9% formic acid (not shown). The fourth fraction (CB2) was homogeneous, based on sequencer analysis. The fifth fraction contained [^{14}C]CM-cysteine, fragment CB1, and ~5% of the sequence

Table I: Amino Acid Compositions of Dihydrofolate Reductase[a] and Derived Cyanogen Bromide Fragments[b]

amino acid	reductase	CB1 (1–14)[c]	CB2 (15–52)	CB3 (53–111)	CB4 (112–125)	CB5 (126–189)
Asx	18.8 (19)[d]	1.8 (2)	3.9 (4)	6.8 (7)		6.2 (6)
Thr	6.9 (7)		1.9 (2)	1.1 (1)	0.9 (1)	2.9 (3)
Ser	11.6 (12)	1.8 (2)	1.7 (2)	5.3 (6)	0.1	2.0 (2)
Glx	20.2 (21)	0.9 (1)	3.6 (4)	4.1 (4)	0.1	11.0 (12)
Pro	11.3 (11)		2.5 (3)	3.9 (4)		3.8 (4)
Gly	10.3 (10)		3.9 (4)	2.1 (2)	2.2 (2)	2.2 (2)
Ala	11.0 (11)	1.1 (1)	1.1 (1)	4.0 (4)	3.2 (3)	2.0 (2)
Cys[e]	1.1 (1)	1.1 (1)				
Val	14.1 (14)	3.1 (3)	2.0 (2)	1.6 (2)	3.1 (3)	3.8 (4)
Met[f]	4.9 (5)	0.8 (1)	1.9 (2)	1.1 (1)	1.0 (1)	
Ile	11.7 (12)	1.0 (1)	1.8 (2)	2.5 (3)	1.0 (1)	4.9 (5)
Leu	17.2 (17)	1.4 (1)	2.1 (2)	8.5 (9)	0.1	5.2 (5)
Tyr	8.0 (8)		1.8 (2)	1.2 (1)	1.0 (1)	3.8 (4)
Phe	8.0 (8)		1.4 (1)	1.1 (1)		6.1 (6)
His	4.2 (4)		1.1 (1)	1.2 (1)		2.2 (2)
Lys	17.9 (18)	0.1	2.8 (3)	8.7 (9)	1.0 (1)	5.2 (5)
Arg	8.2 (8)	1.2 (1)	1.9 (2)	3.0 (3)		2.1 (2)
Trp[g]	3.1 (3)		(1)[h]	(1)[h]	(1)[h]	
% yield		30	62	86	70	84
total residues	(189)	(14)	(38)	(59)	(14)	(64)

[a] Based on hydrolysis times of 20, 48, 72, 96, and 120 h in constant-boiling HCl. [b] Based on 20- and 72-h hydrolysis times. [c] In this table and those following, inclusive residue numbers are based on the complete amino acid sequence of the protein. [d] In this table and the ones to follow, the numbers in parentheses indicate the numbers of residues found by sequencing. [e] Cysteine was determined as cysteic acid (Hirs, 1967) or as *S*-(carboxylmethyl)cysteine (Crestfield et al., 1963). [f] Methionine was determined as methionine sulfone (Hirs, 1967) or, in CB fragments, as homoserine + homoserine lactone. [g] Tryptophan was determined by hydrolysis with 4 M methanesulfonic acid (Simpson et al., 1976). [h] From sequence.

Table II: Automated Sequence Analysis of [^{14}C]Carboxymethylated Dihydrofolate Reductase[a]

step	amino acid identified	yield (nmol)	cpm[b]	step	amino acid identified	yield (nmol)	cpm[b]
1	Val	91	51	15	Gly	49	119
2	Arg	*c*	78	16	Ile	44	95
3	Ser	5	51	17	Gly	31	91
4	Leu	58	54	18	Lys	3	89
5	Asn	45	78	19	Asp	13	85
6	Ser	4	85	20	Gly	19	72
7	Ile	81	69	21	Asn	4	78
8	Val	58	71	22	Leu	16	68
9	Ala	30	90	23	Pro	6	74
10	Val	50	185	24	Trp	3	72
11	Cys	*d*	3091	25	Pro	10	67
12	Gln	8	618	26	Pro	10	63
13	Asn	35	195	27	Leu	12	64
14	Met	26	177				

[a] Sequence analysis of 440 nmol of [^{14}C]carboxymethylated protein. The repetitive yield was 93%. [b] Sample aliquots of 10% were taken for the determination of [^{14}C]carboxymethylated groups. The numbers shown are uncorrected for background counts per minute. [c] Determined from spot test. [d] Determined as [^{14}C]CM-Cys from counts per minute released.

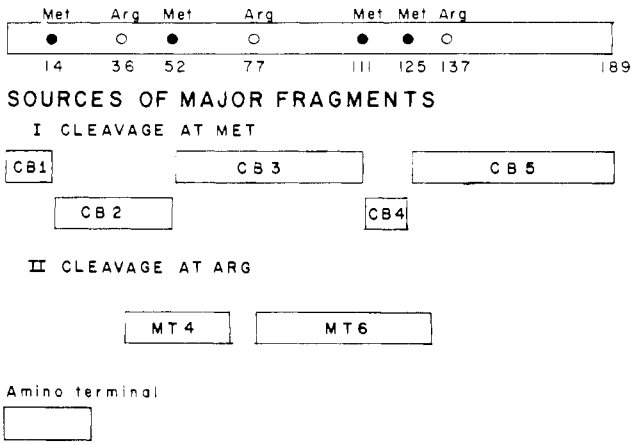

FIGURE 1: Diagrammatic summary of the major fragments generated for sequence analysis of chicken liver dihydrofolate reductase. The top bar represents the residues, numbered below the bar, that are important for its cleavage. The prefix CB indicates a fragment generated by cleavage with cyanogen bromide. The prefix MT denotes a peptide which has been generated by tryptic cleavage at arginine of a maleylated fragment. Fragment MT6 was not isolated, but fragments derived therefrom were isolated as described in the text.

of residues 15–37 based on sequenator and amino acid analyses. Fragment CB1 gradually precipitated in 9% formic acid and was isolated by centrifugation. The sixth fraction contained homogeneous fragment CB4. The amino acid compositions of the five cyanogen bromide fragments are given in Table I. Fragment CB3 (59 residues) forms an aggregate in 9% formic acid which accounts for its separation from fragment CB5 (Figure 2) which contains 64 residues. Fragment CB1 (14 residues) is well separated from fragment CB4 which also contains 14 residues. The latter fragment is relatively hydrophobic and probably interacts with the Sephadex beads, resulting in a greater retention on the column.

Cleavage of Dihydrofolate Reductase at Arginyl Bonds with Trypsin. Maleylated dihydrofolate reductase (60 mg, ~2.7 μmol) was digested at pH 8 for 4 h at 37 °C with Tos-Phe-CH_2Cl-trypsin (enzyme/substrate, 2% w/w). Maleylated tryptic (MT) peptides were separated on a Sephadex G-50 SF column (Figure 3). Each of the first three fractions was rechromatographed on Sephadex G-50 SF and the fourth fraction, a mixture of peptides MT1, MT3, and MT7, was purified by thin-layer electrophoresis at pH 1.9 (400 V, 90 min) and chromatography. The first fraction was a mixture of equimolar amounts of peptides MT6 and MT8 as revealed by sequencer analysis following demaleylation. The amino acid compositions of five of the eight MT peptides are shown in Table III.

Sequence Determination of the Cyanogen Bromide and Maleylated Tryptic Fragments. The strategy and results used in the determination of the sequence of residues 1–111 (CB1 through CB3) are summarized in Figure 4. Fragment CB1 (residues 1–14) contained the radioactivity associated with

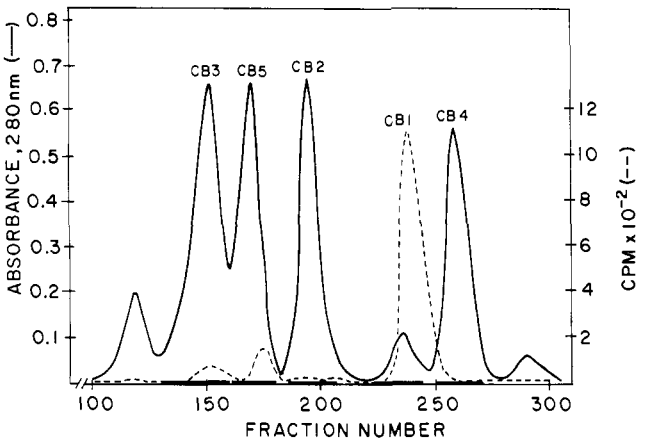

FIGURE 2: Separation of the cyanogen bromide fragments of S-[^{14}C]carboxymethylated dihydrofolate reductase on Sephadex G-50 superfine (2.5 × 95 cm) equilibrated with 9% formic acid. Fractions of 1.6 mL were collected at a flow rate of 24 mL/h. In this figure and those following, pooled fractions are indicated by the horizontal bars.

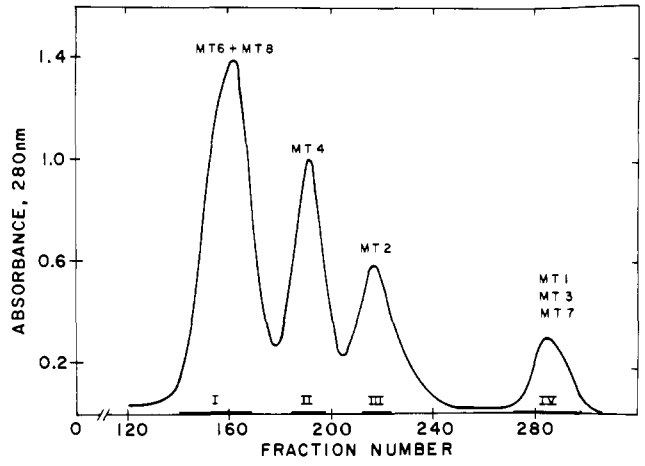

FIGURE 3: Fractionation of the maleylated tryptic (MT) peptides of dihydrofolate reductase on a 2.5 × 95 cm Sephadex G-50 superfine column equilibrated with 0.05 M ammonium bicarbonate, pH 9.0. Fractions (1.6 mL) were collected at a flow rate of 20 mL/h.

[^{14}C]CM-Cys-11 in the intact protein (Table II) and its amino-terminal 14 residues. Sequenator analysis of CB1 (165 nmol) yielded Val_1 through Val_{10}, except for residues 5 and 6 which were shown to be Asn and Ser, respectively, in the intact protein (Table II). Sequence analysis of each of the other four CB fragments was performed as described below. (See paragraph at end of paper regarding supplementary material.)

Table III: Amino Acid Analysis of Tryptic Peptides from Maleylated Dihydrofolate Reductase

amino acid	MT1[a] (1–2)	MT2 (3–28)	MT3[a] (3–28)	MT4 (37–70)	MT7[a] (133–137)
Asx		4.8 (5)	0.7 (1)	3.0 (3)	0.2
Thr				2.8 (3)	0.9 (1)
Ser	0.1	0.6 (1)	0.1	1.9 (3)	0.1
Glx	0.2	0.8 (1)	2.2 (2)	2.6 (3)	
Pro		2.9 (3)		2.0 (2)	
Gly	0.1	3.2 (3)	0.1	2.1 (2)	0.1
Ala		1.0 (1)		1.0 (1)	
Cys		(1)			
Val	0.70 (1)	1.9 (2)		2.2 (2)	1.1 (1)
Met		1.1 (1)		2.1 (2)	
Ile		1.7 (2)		2.0 (2)	0.2
Leu		2.8 (3)		1.2 (1)	0.6 (1)
Tyr		0.1	2.0 (2)		
Phe			1.0 (1)	0.9 (1)	0.9 (1)
His				0.9 (1)	
Lys		1.0 (1)	0.4 (1)	4.1 (5)	
Arg	1.00 (1)	0.9 (1)	1.0 (1)	2.0 (2)	1.0 (1)
Trp		(1)[b]		(1)[b]	
total residues	(2)	(26)	(8)	(34)	(5)
% yield	58	73	58	74	58

[a] Peptides MT1, MT3, and MT7 were isolated by thin-layer electrophoresis and chromatography (see Materials and Methods) of fraction IV (Figure 3). Peptide MT5 (71–77) was not isolated here but was isolated as CB3-MT2 (cf. Table IV). Fraction I (Figure 3) is an equimolar mixture of peptides MT6 (78–132) and MT8 (138–189). [b] From sequence.

CB2 (residues 15–52). Sequencer analysis of this peptide (410 nmol) yielded the sequence Gly_{15}-Ile-Gly-Lys- through Thr_{40}, except for residue 37, which was determined to be a methionine in peptide MT4 (see below). This sequence corresponds to that beginning at step 15 of the intact protein (Table II). CB2 contains two homoserine residues (Table I); the internal homoserine must be located at position 37 followed by Thr-38, indicating the presence of a Met-Thr bond in the intact protein. Such a peptide bond is reported to resist cleavage by cyanogen bromide (Schroeder et al., 1969; Stone et al., 1979). This finding explains the generation of five rather

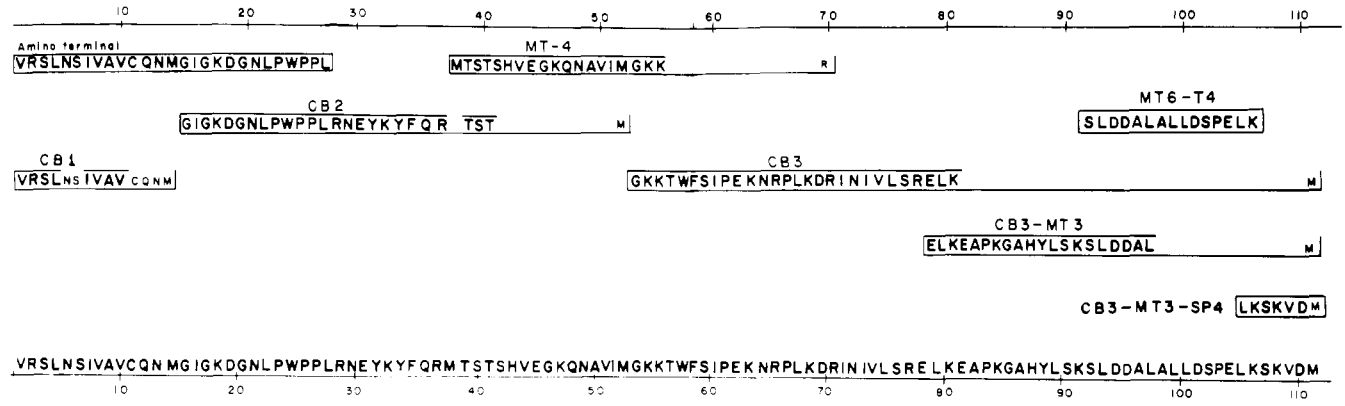

FIGURE 4: Summary proof of the amino acid sequence of residues 1–111 of chicken liver dihydrofolate reductase. The one-letter code within the bars designates amino acid residues in that peptide. Large capital letters indicate amino acid residues identified after Edman degradation or by carboxypeptidase digestion. Smaller capital letters indicate placement by composition and cleavage specificity. The length of each bar indicates the length of the peptide; enclosure at the top of the bar indicates that portion of the sequence which has been proven; gaps in the upper enclosure indicate portions of the sequence not identified. In CB fragments, methionine was identified as homoserine. The one-letter amino acid abbreviations are the following: A (alanine), C (cysteine), D (aspartic acid), E (glutamic acid), F (phenylalanine), G (glycine), H (histidine), I (isoleucine), K (lysine), L (leucine), M (methionine), N (asparagine), P (proline), Q (glutamine), R (arginine), S (serine), T (threonine), V (valine), W (tryptophan), and Y (tyrosine).

Table IV: Amino Acid Compositions of Tryptic and Staphylococcal Protease Peptides Derived from Fragment CB3 and Tryptic Peptides from Fragment MT6[a]

parent fragment:	CB3			CB3-MT3	MT6		
derived peptide:	MT1	MT2	MT3	SP4	T4	T5	T6
residue no.:	53–70	71–77	78–111	105–111	92–106	107–122	123–132
figure (fraction):	5A (II)	5A (III)	5A (I)	5C	6C (I-2)		6A (II)
Asx	2.0 (2)	1.3 (1)	4.2 (4)	1.0 (1)	3.0 (3)	0.9 (1)	0.6 (1)
Thr	0.7 (1)	0.1	0.2		0.1	0.9 (1)	
Ser	1.0 (1)	1.1 (1)	4.1 (4)	1.0 (1)	1.7 (2)	0.8 (1)	
Glx	1.4 (1)	0.2	3.2 (3)	0.1	1.4 (1)		1.3 (1)
Pro	2.2 (2)		2.2 (2)		1.1 (1)		0.9 (1)
Gly	1.2 (1)	0.2	1.4 (1)	0.1	0.2	2.2 (2)	
Ala			4.1 (4)		2.0 (2)	1.2 (1)	1.5 (2)
Val		0.5 (1)	1.0 (1)	1.0 (1)		3.9 (4)	
Met[b]			1.1 (1)	0.7 (1)		1.4 (1)	0.9 (1)
Ile	1.1 (1)	2.0 (2)	0.3		0.3	0.9 (1)	0.7 (1)
Leu	1.4 (1)	1.4 (1)	7.0 (7)	0.8 (1)	4.6 (5)	0.2	
Tyr			1.2 (1)		0.1	1.2 (1)	
Phe	0.6 (1)				0.2	0.2	
His			0.9 (1)				0.7 (1)
Lys	3.5 (4)	0.3	5.0 (5)	1.6 (2)	1.1 (1)	1.8 (2)	0.9 (1)
Arg	1.6 (2)	0.5 (1)					0.7 (1)
Trp	(1)[c]					(1)[c]	
% yield	30	13	26	24	46	13	18

[a] Residues/mole by amino acid analysis or (in parentheses) from the sequence. [b] Determined as homoserine + homoserine lactone. [c] From sequence.

than six CB fragments from the five methionine residues in the protein (Table I).

MT4 (residues 37–70). Following demaleylation in 30% acetic acid, sequencer analysis of this peptide (400 nmol) provided the methionine overlap between CB2 and CB3 (-Ile_{51}-Met-Gly-Lys-Lys_{55}) and proved the presence of a Met-Thr bond at positions 37 and 38. The N-terminal 19 residues of peptide MT4 were identified.

CB3 (residues 53–111). Similar analysis of CB3 (420 nmol) yielded the sequence Gly_{53}-Lys-Lys- through Glu-Leu-Lys_{80}. The remainder of the sequence of CB3 was determined from tryptic and staphylococcal protease (SP) peptides derived from either fragment CB3 or maleylated tryptic peptides from the intact protein.

Maleylated CB3 (825 nmol) was digested with Tos-Phe-CH_2Cl-trypsin for 4 h, applied to a column of Sephadex G-50 SF, and separated into three fractions: I (CB3-MT3), II (CB3-MT1), and III (CB3-MT2) as indicated in Figure 5A. The amino acid compositions of each fraction are shown in Table IV. The sequences of peptides CB3-MT1 (residues 53–70) and CB3-MT2 (residues 71–77) were determined in CB3 and placed by amino acid composition. Peptide CB3-MT3 was found to be the carboxyl-terminal portion (residues 78–111) of the CB3 fragment. Sequencer analysis of 200 nmol of this peptide yielded the sequence Glu_{78}-Leu-Lys- through Asp-Ala-Leu_{97}. The results of digestion of CB3-MT3 (700 nmol) with staphylococcal protease, following separation on Bio-Gel P-2, are shown in parts B and C of Figure 5. The amino acid composition of CB3-MT3-SP4 (105–111) is indicated in Table IV. Sequencer analysis of CB3-MT3-SP4 (150 nmol) yielded the sequence Leu-Lys-Ser-Lys-Val-Asp_{110}-. The position of Hse-111 was inferred from the composition.

Maleylated tryptic peptides from the whole protein were utilized in order to complete the sequence of residues 97–105 in CB3 and to generate overlapping peptides containing methionine residues. Fraction I (Figure 3) was found to be a mixture of peptides MT6 (residues 78–132) and MT8 (residues 138–189) based upon subsequent analyses. Cation-exchange resins employing a variety of conditions failed to separate the two peptides. Use of anion exchangers (e.g., DEAE-Sephadex A-25) resulted in extremely poor yields of these two peptides.

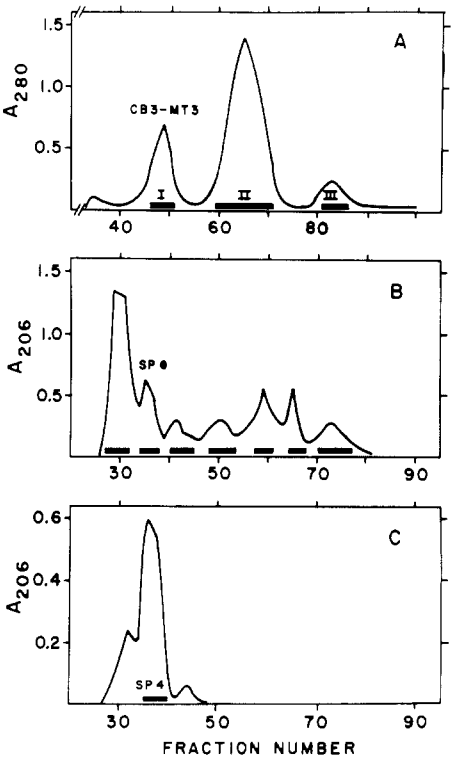

FIGURE 5: (A) Separation of the tryptic digest of maleylated CB3 on a Sephadex G-50 superfine column (1.5 × 93 cm) in 0.05 M ammonium bicarbonate, pH 9.0. Fractions (1.6 mL) were collected at a flow rate of 18 mL/h. (B) Fractionation of the staphylococcal protease (SP) digest of CB3-MT3 on Bio-Gel P-2 (1.5 × 85 cm) equilibrated with 9% formic acid. Fractions of 1.5 mL, collected at a flow rate of 20 mL/h, were pooled as indicated. (C) Further fractionation of CB3-MT3-SP4 on Bio-Gel P-2. Conditions were as in (B).

Sequencer analysis of this demaleylated fraction yielded an equimolar mixture of two sequences. The first six degradations indicated the presence of (1) Glu-Leu-Lys-Glu-Ala-Pro- and (2) Ile-Leu-His-Glu-Phe-Glu. Peptide CB3-MT3 yielded the sequence Glu-Leu-Lys-Glu-Ala-Pro-, suggesting that fraction I (Figure 3) contained a fragment beginning with this sequence

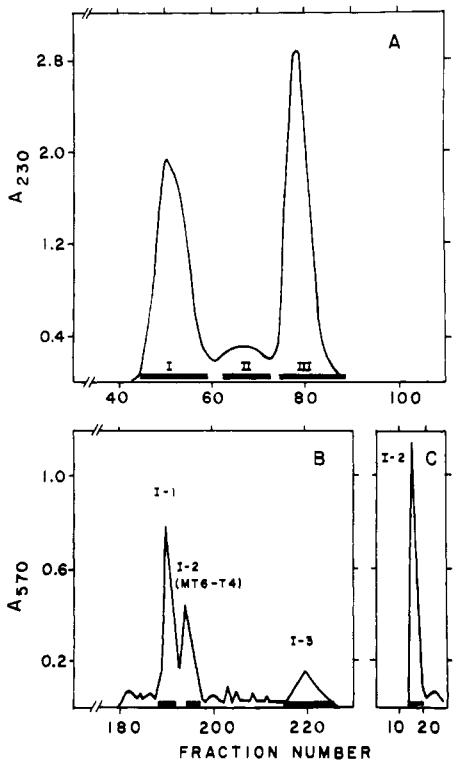

FIGURE 6: (A) Fractionation of the tryptic digest of demaleylated peptides MT6 and MT8 on a Sephadex G-25 superfine column (1.5 × 93 cm) in 0.05 M ammonium bicarbonate, pH 9.0. Fractions (1.6 mL) were collected at a flow rate of 20 mL/h. (B) Separation of fraction I peptides (A) on SP-Sephadex C-25 (0.9 × 9 cm) equilibrated with 30% acetic acid. A gradient of 0–0.2 M NaCl (200 mL each) containing 30% acetic acid was employed, beginning at fraction no. 80. Fractions of 1.6 mL were collected at a flow rate of 10 mL/h and monitored by ninhydrin after alkaline hydrolysis. (C) Further purification of fraction I-1 on a 1.5 × 85 cm Bio-Gel P-2 column in 30% acetic acid. Fractions (1.5 mL) were collected at a flow rate of 20 mL/h and monitored by ninhydrin after alkaline hydrolysis.

and an equal amount of one beginning with Ile-Leu-His-Glu-Phe-Glu-. Subsequent analysis of CB5 revealed the latter sequence following Arg-137. Since the two fragments (MT6 and MT8) were inseparable, the mixture (20 mg) was demaleylated and digested with Tos-Phe-CH_2Cl–trypsin for 4 h. During digestion at pH 8.0, a precipitate formed. The reaction mixture was then lyophilized and suspended in 2 mL of 0.05 M NH_4HCO_3, pH 9.0, and the insoluble material was centrifuged and washed twice with 0.5 mL of the same buffer. The soluble portion of the digest was fractionated on Sephadex G-25 SF as shown in Figure 6A. Fraction I contained three peptides, including MT6-T4 (92–106). This peptide was further purified on an SP-Sephadex C-25 column (Figure 6B) and on Bio-Gel P-2 (Figure 6C). The second fraction (Figure 6A) containing peptide MT6-T6 (123–132) was purified by high-voltage electrophoresis at pH 1.9. The insoluble peptide (MT6-T5) generated during trypsin hydrolysis was washed twice at pH 8.0, solubilized in 30% acetic acid, and subjected to amino acid analysis. The amino acid compositions of these tryptic peptides are shown in Table IV.

Sequencer analysis of 580 nmol of peptide MT6-T4 (Table IV; residues 92–106) established the entire sequence Ser_{92}-Leu-Asp-Asp- through -Glu-Leu-Lys_{106}, which, together with a similar analysis of CB3-MT3-SP4 (see above), established the sequence of CB3.

The strategy and analyses used to determine residues 112–189 are summarized in Figure 7. A total of 180 nmol of the neutral or alkaline pH insoluble peptide MT6-T5 (Table IV; residues 107–122) yielded the sequence Ser_{107}-Lys-Val-

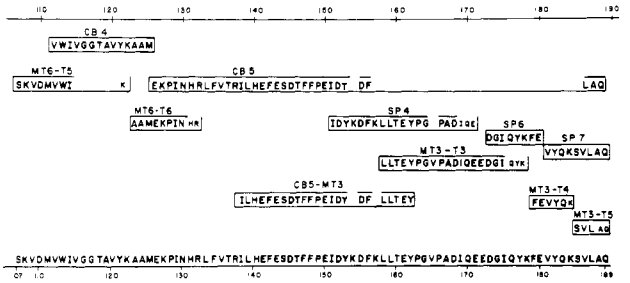

FIGURE 7: Summary proof of the sequence of residues 112–189 of chicken liver dihydrofolate reductase, using the same annotations as in Figure 4.

Asp-Met-Val-Trp-Ile_{114}, providing the methionine overlap between fragments CB3 and CB4. The sequence of this peptide overlaps with CB3-MT3-SP4 (105–111) and, therefore, must be assigned to the position given, demonstrating that it was derived from MT6.

CB4 (residues 112–125). This peptide (450 nmol) was analyzed by automated sequencing which yielded 12 of the 14 residues, i.e., Val_{112}-Trp-Ile-Val-Gly-Gly-Thr-Ala-Val-Tyr-Lys-Ala_{123}. The remainder of the sequence was inferred from the composition and proven by sequence analysis of peptide MT6-T6 (see below). Carboxypeptidase Y digestion of CB4 confirmed the C-terminal sequence -Ala-Ala-Hse. Fragment CB4 was insoluble at pH values of ~7–9 but was soluble at pH values of ~2–4.5. The same hydrophobic sequence present in peptide MT6-T5 (Table IV; residues 107–122) is the most probable explanation for the precipitation of this peptide during tryptic hydrolysis at pH 8 (see above). The generation of this insoluble peptide by cleavage of the Lys_{106}-Ser_{107} bond may explain, in part, the absence of detectable cleavage by trypsin of the Lys_{108}-Val_{109} bond in peptide MT6-T5.

Peptide MT6-T6 (Table IV; residues 123–132). Sequencer analysis of this peptide demonstrated 8 of the 10 residues, Ala_{123}-Ala-Met-Glu-Lys-Pro-Ile-Asn_{130}, and provided the methionine overlap between fragments CB4 and CB5. The latter lacks homoserine and is the carboxyl-terminal cyanogen bromide fragment.

CB5 (residues 126–189). Automated sequencing of this fragment yielded the sequence Glu_{126}-Lys-Pro- through Phe_{156}, except for residue 154. The remainder of the sequence of this fragment was established from peptides generated by tryptic and staphylococcal protease digestions.

Maleylated CB5 (530 nmol) was treated with Tos-Phe-CH_2Cl–trypsin for 4 h, and the lyophilized digest was fractionated on a Sephadex G-50 SF column (Figure 8A). The amino acid compositions of peptides CB5-MT1, CB5-MT2, and CB5-MT3 are shown in Table V. Peptides CB5-MT1 (residues 126–132) and CB5-MT2 (residues 133–137) were placed by amino acid composition in the sequence of fragment CB5. Sequencer analysis of 400 nmol of demaleylated peptide CB5-MT3 (residues 138–189) yielded the sequence Ile_{138}-Leu-His through Tyr_{162} except for residues 154 and 157. Demaleylated peptide CB5-MT3 (800 nmol) dissolved in 3.0 mL of 0.2 M NH_4HCO_3, pH 8.0, was digested with 2% trypsin at 37 °C for 4 h, the pH adjusted to 3.0, and the mixture lyophilized. The digest was separated on Sephadex G-50 SF (Figure 8B), and peptide CB5-MT3-T3 was purified on Sephadex G-25 SF (Figure 8C). Fraction II, CB5-MT3-T1 (Figure 8B), was purified on Sephadex G-25 and gave a composition consistent with its placement in the sequence (residues 138–154). Fraction III (Figure 8B), which contained peptides CB5-MT3-T2, -T4, and -T5, was further fractionated on Sephadex G-10 (not shown), and the peptides were sepa-

Table V: Amino Acid Compositions of Tryptic and Staphylococcal Protease Peptides Derived from Fragment CB5[a]

parent fragment:	CB5			CB5-MT3			CB5		
derived peptide:	MT1	MT2	MT3	T3	T4	T5	SP4	SP6	SP7
residue no.:	126–132	133–137	138–189	158–178	179–184	185–189	151–171	173–180	181–189
figure (fraction):	8A (II)	8A (III)	8A (I)	8C	8B	8B	9B (I-2)	9C (II-1)	9C (II-2)
Asx	1.2 (1)	0.2	5.1 (5)	2.1 (2)	0.1		2.7 (3)	1.2 (1)	0.1
Thr	0.1	1.3 (1)	1.9 (2)	0.9 (1)	0.1	0.1	0.9 (1)	0.2	
Ser	0.3	0.1	1.8 (2)	0.1	0.2	0.9 (1)	0.2	0.2	1.0 (1)
Glx	1.3 (1)	0.3	9.6 (11)	4.7 (5)	1.9 (2)	0.7 (1)	3.2 (3)	2.0 (2)	1.7 (2)
Pro	1.2 (1)	0.2	3.2 (3)	1.9 (2)			1.9 (2)	0.1	
Gly	0.3	0.2	2.2 (2)	1.9 (2)	0.1	0.1	1.1 (1)	1.1 (1)	0.2
Ala	0.2		1.7 (2)	1.1 (1)		0.6 (1)	1.0 (1)	0.1	1.1 (1)
Val	0.2	1.0 (1)	2.6 (3)	0.9 (1)	0.9 (1)	1.0 (1)	1.0 (1)		2.0 (2)
Ile	1.2 (1)		3.8 (4)	1.8 (2)	0.1		2.1 (2)	0.9 (1)	
Leu	0.3	1.1 (1)	4.0 (4)	2.0 (2)	0.2	1.0 (1)	2.3 (2)	0.1	1.1 (1)
Tyr			3.8 (4)	1.9 (2)	0.8 (1)		2.1 (2)	0.9 (1)	0.9 (1)
Phe		0.8 (1)	4.3 (4)	0.3	0.9 (1)	0.1	1.0 (1)	1.1 (1)	
His	1.1 (1)		1.0 (1)	0.1					
Lys	1.2 (1)	0.2	4.2 (4)	1.0 (1)	1.0 (1)		2.1 (2)	1.0 (1)	0.9 (1)
Arg	1.0 (1)	1.4 (1)							
% yield	70	52	81	76	32	19	28	37	48

[a] Residues/mole by amino acid analysis or (in parentheses) from the sequence.

rated by high-voltage electrophoresis at pH 1.9. The composition of T2 placed it at residues 155–157. The amino acid compositions of peptides CB5-MT3-T3, -T4, and -T5 are indicated in Table V. Automated sequence analysis of 500 nmol of CB5-MT3-T3 (residues 158–178) revealed 18 of the 21 residues in the sequence Leu_{158}-Leu-Thr- through Asp-Gly-Ile_{175}. A similar analysis of 160 nmol of peptide CB5-MT3-T4 (residues 179–184) yielded the sequence Phe_{179}-Glu-Val-Tyr-Gln_{183}-. Sequenator analysis of 90 nmol of CB5-MT3-T5 (residues 185–189) gave the sequence Ser_{185}-Val-Leu.

Most of the remaining sequence of CB5 was determined from staphylococcal protease peptides. Fragment CB5 (1.2 μmol) in 4.0 mL of 0.05 M NH_4HCO_3, pH 7.8, was digested with 0.024 μmol of staphylococcal protease for 4 h at 37 °C. The lyophilized product was fractionated on a Sephadex G-25 SF column as indicated in Figure 9A. Fraction I was separated into three major peptides on SP-Sephadex C-25 as shown in Figure 9B. Fraction I-2 was determined to be peptide CB5-SP4 (residues 151–171). Sequencer analysis of 200 nmol of this peptide yielded Ile_{151}-Asp-Tyr- through -Pro-Ala-Asp_{168}, except for residue 165. Residues 154 and 157, not determined in CB5 or CB5-MT3, were determined to be lysines in CB5-SP4. Residue 165 was shown to be a valine from sequence analysis of CB5-MT3-T3. Fraction II (Figure 9A) was further separated on a Bio-Gel P-2 column (Figure 9C), and peptide CB5-SP6 (residues 173–180) was purified from fraction II-1 by high-voltage electrophoresis at pH 1.9. Fraction II-2 contained only peptide CB5-SP7 (residues 181–189). Sequencer analysis of CB5-SP6 yielded Asp_{173}-Gly-Ile-Gln-Tyr-Lys-Phe_{179}. Carboxypeptidase Y digestion of this peptide yielded Lys-Phe-Glu_{180}. The amino acid compositions of the CB5-SP peptides are shown in Table V. Carboxypeptidase A and B digestion of fragment CB5 yielded Leu-Ala-Gln_{189}. Sequencer analysis of CB5-SP7 (residues 181–189) yielded the complete sequence Val_{181}-Tyr-Gln-Lys-Ser-Val-Leu-Ala-Gln_{189}. Carboxypeptidase Y digestion of this peptide confirmed the sequence Val-Leu-Ala-Gln_{189}, and sequenator analysis of CB5-MT3-T5 (185–189) confirmed the sequence Ser_{185}-Val-Leu. The complete amino acid sequence of chicken liver dihydrofolate reductase is shown in Figure 10.

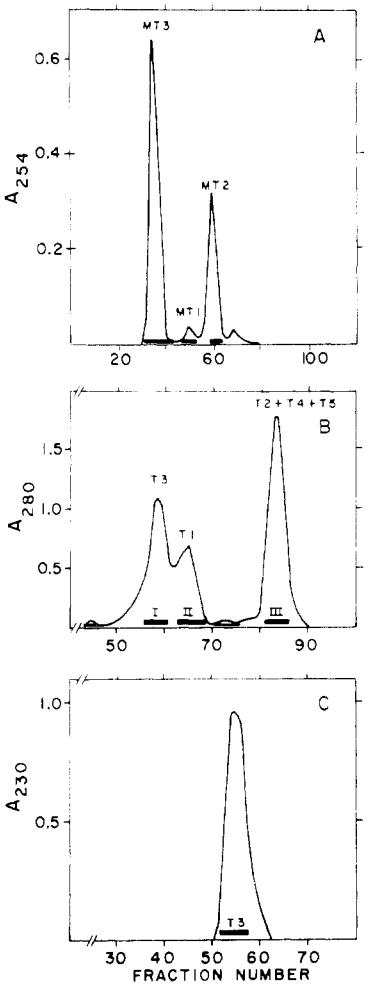

FIGURE 8: (A) Separation of maleylated tryptic peptides of CB5 on a 1.5 × 95 cm Sephadex G-50 superfine column equilibrated with 0.05 M ammonium bicarbonate, pH 9.0. Fractions of 1.5 mL were collected at a flow rate of 20 mL/h and pooled as indicated. (B) Fractionation of the tryptic digest of demaleylated peptide CB5-MT3 on Sephadex G-50 superfine (1.5 × 95 cm). Other conditions were as in (A). (C) Further separation of CB5-MT3-T3 on Sephadex G-25 superfine (1.5 × 93 cm) in ammonium bicarbonate, pH 9.0. Fractions (1.4 mL) were collected at a flow rate of 20 mL/h.

Discussion

The amino acid sequence of chicken liver dihydrofolate reductase contains 189 residues, which corresponds to a molecular weight of 21 651. This value is in good agreement with the molecular weight of 22 500 ± 650 as determined by sedimentation equilibrium studies (Kaufman & Kemerer, 1977).

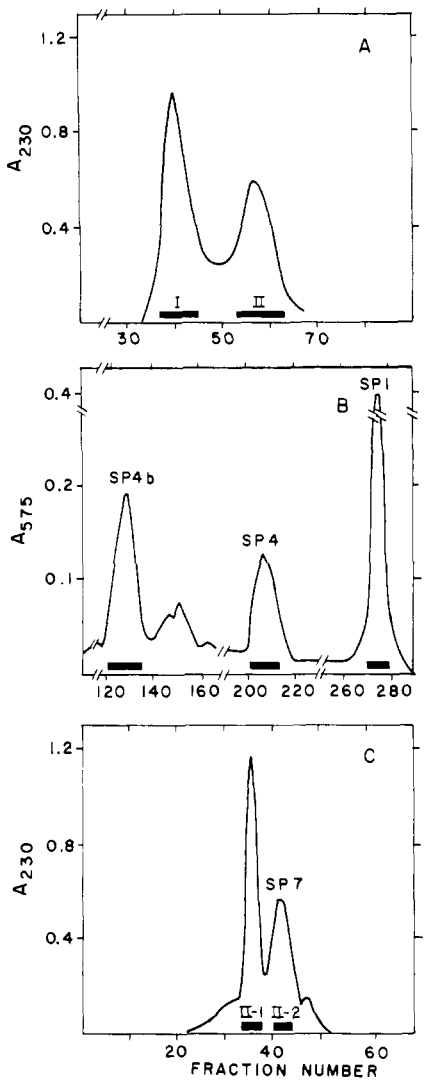

FIGURE 9: (A) Fractionation of the staphylococcal protease (SP) digest of fragment CB5 on Sephadex G-25 superfine (1.5 × 93 cm) in 0.05 M ammonium bicarbonate, pH 9.0. Fractions (1.8 mL) were collected at a flow rate of 24 mL/h and pooled as indicated. (B) Chromatography of fraction I, from (A), on an SP-Sephadex C-25 column (0.9 × 9.5 cm) equilibrated with 9% formic acid followed by a gradient of 0–0.2 M KCl (200 mL each) in 9% formic acid. A second gradient consisted of 200 mL of 0.2 M KCl in 9% formic acid vs. 200 mL of 0.2 M KCl–0.05 M potassium formate, pH 6.0. Fractions (1.5 mL) were collected at a flow rate of 10 mL/h and monitored with ninhydrin following alkaline hydrolysis. (C) Separation of fraction II peptides [from (A)] on a 1.5 × 82 cm Bio-Gel P-2 column in 0.05 M ammonium bicarbonate, pH 9.0. Fractions of 1.6 mL were collected at a flow of 15 mL/h.

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  1 VAL-ARG-SER-LEU-ASN-SER-ILE-VAL-ALA-VAL-CYS-GLN-ASN-MET-GLY-ILE-GLY-LYS-ASP-GLY-ASN-LEU-PRO-TRP-PRO-
 26 PRO-LEU-ARG-ASN-GLU-TYR-LYS-TYR-PHE-GLN-ARG-MET-THR-SER-THR-SER-HIS-VAL-GLU-GLY-LYS-GLN-ASN-ALA-VAL-
 51 ILE-MET-GLY-LYS-LYS-THR-TRP-PHE-SER-ILE-PRO-GLU-LYS-ASN-ARG-PRO-LEU-LYS-ASP-ARG-ILE-ASN-ILE-VAL-LEU-
 76 SER-ARG-GLU-LEU-LYS-GLU-ALA-PRO-LYS-GLY-ALA-HIS-TYR-LEU-SER-LYS-SER-LEU-ASP-ASP-ALA-LEU-ALA-LEU-LEU-
101 ASP-SER-PRO-GLU-LEU-LYS-SER-LYS-VAL-ASP-MET-VAL-TRP-ILE-VAL-GLY-GLY-THR-ALA-VAL-TYR-LYS-ALA-ALA-MET-
126 GLU-LYS-PRO-ILE-ASN-HIS-ARG-LEU-PHE-VAL-THR-ARG-ILE-LEU-HIS-GLU-PHE-GLU-SER-ASP-THR-PHE-PHE-PRO-GLU-
151 ILE-ASP-TYR-LYS-ASP-PHE-LYS-LEU-LEU-THR-GLU-TYR-PRO-GLY-VAL-PRU-ALA-ASP-ILE-GLN-GLU-GLU-ASP-GLY-ILE-
176 GLN-TYR-LYS-PHE-GLU-VAL-TYR-GLN-LYS-SER-VAL-LEU-ALA-GLN
```

FIGURE 10: The complete amino acid sequence of chicken liver dihydrofolate reductase.

Chicken liver dihydrofolate reductase contains five Met residues. One of these occurs in a Met-Thr linkage at positions 37 and 38, respectively, in the sequence. This linkage is cleaved by cyanogen bromide to the extent of ~5%, and thus five, rather than six, major cyanogen bromide fragments are produced.

Attempts to digest the S-carboxymethylated intact enzyme with carboxypeptidases A, B, or Y under a variety of conditions were unsuccessful. The same observation is reported by Stone et al. (1979) for the L1210 enzyme. However, the C-terminal sequence of the chicken liver protein was determined by sequenator analyses and by carboxypeptidase digestions of fragment CB5 and peptides derived therefrom (see Results). The alignment of the five cyanogen bromide peptides using overlapping tryptic peptides is unequivocal, and CB5 is clearly the C-terminal cyanogen bromide fragment. Other problems encountered in the sequence determination include the cleavage of certain Asp-X bonds with staphylococcal protease. For example, CB5-SP4 (151–171) was isolated in a 28% yield (Table V, Results). However, two other peptides from this region of the sequence were also isolated. Peptides CB5-SP4A (151–155) and CB5-SP4B (156–171) were isolated in yields of 14 and 12%, respectively, indicating that the Asp_{155}-Phe_{156} bond was cleaved in addition to the Glu_{150}-Ile_{151} and Glu_{171}-Glu_{172} bonds. The staphylococcal protease did not cleave CB5 between residues Asp_{145}-Thr_{146} or between Asp_{152}-Tyr_{153}. More surprisingly, the Glu_{161}-Tyr_{162} was resistant to cleavage since both peptides CB5-SP4 and -SP4B were isolated with this peptide linkage intact. A similar digestion of CB3-MT3 (78–111) indicated that some cleavage of the Asp_{94}-Asp_{95} bond had occurred; however, the corresponding peptides were not completely purified. Austen & Smith (1976) studied the specificity of the staphylococcal protease with a series of peptides from the NAD-specific glutamate dehydrogenase from *Neurospora crassa*. These investigators found cleavage of the Glu-Tyr bond in the peptide Leu-Gly-Thr-Glu-Tyr-Thr-Ser-Leu, whereas no cleavage of the Glu-Tyr bond occurred in the chicken liver reductase sequence Leu-Leu-Thr-Glu_{161}-Tyr-Pro-Gly-Val-Pro-. The lack of cleavage in the latter case may be due to the inaccessibility of this bond in CB5 due to secondary and/or tertiary structural considerations or to the presence of residues in the sequence which prevent productive binding to the protease (e.g., Pro-163 and/or Pro-166). Austen & Smith (1976) have shown that -Glu-Pro- bonds are not hydrolyzed but that Pro-Glu-X bonds are susceptible to hydrolysis. These workers also suggest that susceptible Glu-X bonds near the N terminus or C terminus of a peptide are not cleaved. Thus, the peptides Lys-Ala-Glu-Thr-*Pro*-Gly-Ile- and Val-Glu-Tyr-Pro-Lys-*Pro*- were not hydrolyzed. However, longer peptides containing the -Glu-Tyr-Pro- sequence in the middle were not investigated and the effect of the presence of Pro on the hydrolysis of -Glu-X-Pro- bonds in peptides remains to be determined. Austen & Smith (1976) also indicate that -Glu-Glu- and -Asp-Asp- linkages are not hydrolyzed by this protease, whereas our results indicate otherwise (see above).

Chicken liver dihydrofolate reductase contains a preponderance of basic amino acid residues (30 basic and 25 acidic) and an isoelectric point at pH 8.4 (Kaufman & Kemerer, 1977). The bovine liver enzyme has a p*I* of 6.8 (Kaufman & Kemerer, 1976). From nearly complete sequence data on the bovine liver reductase (Lai et al., 1979) certain positional changes in amino acid residues may serve to partially explain the observed differences in p*I* between the avian and bovine enzymes. At positions 32, 106, and 154 Lys is present in the chicken liver enzyme, as indicated in Figure 10. At the same positions Gln, Thr, and Glu occur, respectively, in the bovine liver enzyme. Similarly, the avian enzyme contains His at positions 42, 131, and 140, whereas these residue positions are Ser, Val, and Gln, respectively, in the bovine reductase. In addition, an Ala → Glu occurs at position 98 and a Ser →

Asp at position 102 in comparing the avian → bovine enzyme amino acid replacements.

Chicken liver dihydrofolate reductase is activated 12–13-fold by a stoichiometric amount of methylmercuric hydroxide (MeHgOH) within 1 min at 0 °C, and a 2-fold molar excess of *p*-(hydroxymercuri)benzoate (pHMB) results in an 8-fold maximum stimulation of enzymic activity (Freisheim et al., 1979). By contrast, the bovine enzyme is inhibited by MeHgOH (~20% after 35 h at 0 °C) and no enzymic activation is observed. A 1.8-fold activation of the bovine reductase by a 2-fold excess of pHMB is observed after 20 h at 0 °C, followed by inhibition thereafter (Freisheim et al., 1979). The L1210 reductase is also stimulated ca. 2.5-fold by low concentrations of pHMB (Perkins et al., 1967).

Such differences in reactivities toward organic mercurials between these enzymes would appear to reside in the location and microenvironment surrounding their respective Cys residues. Cys-6 in the bovine liver enzyme may be less accessible to these reagents, and the enzyme appears to be more unstable when modified. By contrast, the organic mercurials react with Cys-11 in the chicken liver reductase and the stimulation at 0 °C is virtually instantaneous and stable. The data suggest that a conformational change[2] occurs following mercurial modification of Cys-11 which affects the overlapping binding regions for NADPH and dihydrofolate.

By contrast, bacterial reductases which contain one or more cysteine residues are inhibited by mercurials. The *S. faecium* reductase (isoenzyme I) contains a single cysteine residue which is not located in the N-terminal 25 amino acid residues (Figure 10). Nearly stoichiometric amounts of pHMB lead to complete inactivation of this enzyme (Warwick & Freisheim, 1975). *E. coli* dihydrofolate reductase can be modified and inactivated with certain thiol reagents (Williams & Bennett, 1977). The two reactive cysteine residues are located at positions 85 and 152. Thus, it is concluded that, for animal dihydrofolate reductases stable to such treatment, enzyme activation by organic mercurials is directly related to the presence of a cysteine residue in the amino-terminal region of their amino acid sequence.

Recent primary structural studies of bacterial dihydrofolate reductases have shown them to contain from 159 to 167 amino acid residues, depending on the source. Thus, the bacterial reductases are 22–30 residues shorter in length than the chicken liver enzyme. Therefore, gaps in the bacterial amino acid sequences must be introduced in order to compare these to reductase sequences from animal sources. The amino acid sequence of chicken liver dihydrofolate reductase is compared with those obtained for enzymes from bovine liver, L1210 cells, *L. casei*, *S. faecium*, and *E. coli* as indicated in Figure 11. The avian liver enzyme sequence shows 75% and 76% identities with the bovine liver and L1210 reductases, respectively. However, the chicken liver enzyme shows only a 24% identity with either the *L. casei* or the *E. coli* reductase and only a 22% identity with the *S. faecium* isozyme II enzyme, based on the proposed amino acid sequence alignments (Figure 11). The residues common to both bacterial and animal reductases are distributed throughout the length of the molecules, which suggests a similarity in their overall structures.

In certain regions the degree of sequence homology is greater than in other areas, particularly between residues 16 and 36, 51 and 77, 112 and 128, and 133 and 149. In these regions there are a number of sequence identities or isosterically conserved residues, many of which arise from single base

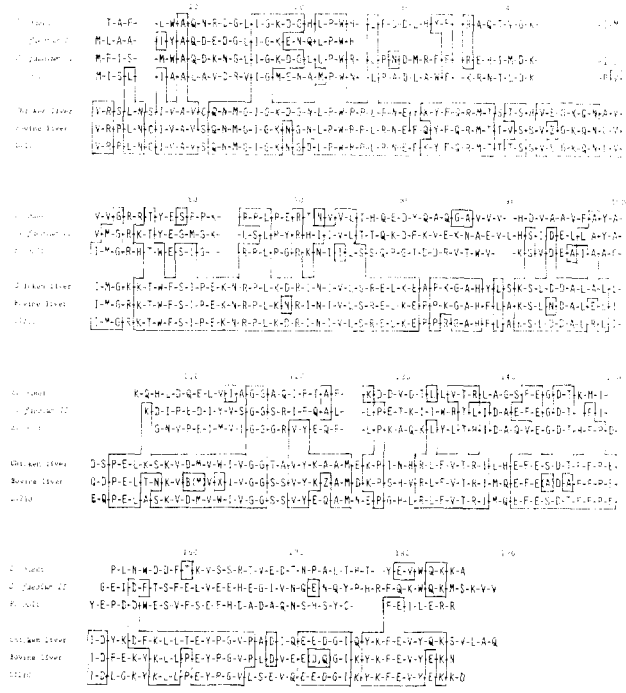

FIGURE 11: Comparison of the amino acid sequence of dihydrofolate reductase from chicken liver with those from bovine liver (Lai et al., 1979), L1210 cells (Stone et al., 1979), *L. casei* (Freisheim et al., 1978), *S. faecium* isozyme I (Freisheim et al., 1977), *S. faecium* isozyme II (Gleisner et al., 1974), and *E. coli* (Stone et al., 1977). Position numbers are based on the chicken liver enzyme sequence, and identical residues from other species are enclosed. Gaps are introduced to achieve maximum homology, and single-letter amino acid residue designations are used (cf. Figure 4).

changes in the respective codons. It is instructive to consider these four sequence regions in terms of the X-ray crystallographic structure of the ternary complex of the *L. casei* dihydrofolate reductase with methotrexate and NADPH, which has recently been solved at 2.5-Å resolution (Matthews et al., 1978, 1979). The backbone structure is dominated by an eight-stranded β sheet with seven parallel strands and one antiparallel strand. Four helices and six extended loops provide connections within the β sheet. The structure consists of two lobes separated by a deep cleft (~15 Å wide). Methotrexate binds within this cavity draped over helix αB in an open conformation with its pteridine ring nearly perpendicular to the aromatic ring of its *p*-aminobenzoyl group. The other side of this deep cleft provides a binding site for the nicotinamide ring of NADPH while the remaining portions of the coenzyme molecule occupy a shallow groove that winds back over β strands B, C, D, and E. The pyrazine ring of methotrexate provides one side of the cavity in which the nicotinamide moiety is bound. The NADPH molecule is bound to the reductase in an extended conformation, ~17 Å between the adenine and nicotinamide bases. Two extended loops connecting βA to αB (residues 10–24) and βF to βG (residues 117–135) move 2 to 3 Å when NADPH binds to dihydrofolate reductase (Matthews et al., 1979).

Selected regions of the amino acid sequences of *L. casei* and *E. coli* dihydrofolate reductases involved in the binding of both methotrexate and NADPH are compared to similar sequences of other reductases from various sources as summarized in Figure 12. The sequence alignments in these binding regions are the same as those indicated in their entireties in Figure 11. The binding of methotrexate involves a large number of hydrophobic interactions. By use of the chicken liver reductase sequence numbering, 7 of the 13 residues known to be involved

[2] Unpublished results of circular dichroic, fluorescence, and ultraviolet difference spectroscopic studies from this laboratory.

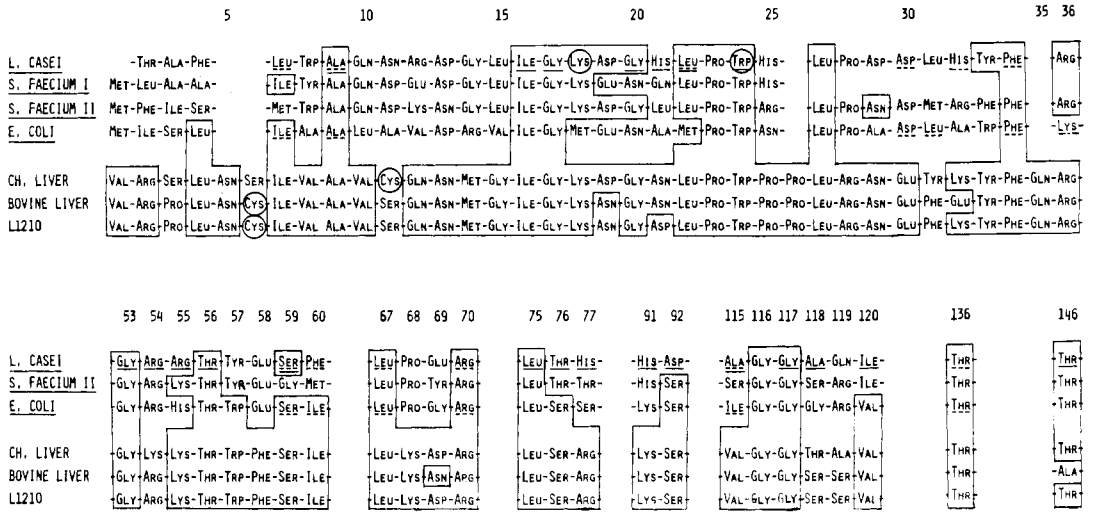

FIGURE 12: Comparison of the amino acid sequences of dihydrofolate reductases from various species in selected regions. Position numbers are based on the chicken liver reductase sequence, and identical residues from other species are enclosed. Circled residues indicate those which have been chemically modified. Residues implicated in methotrexate (---) or NADPH (—) binding are indicated. Other descriptions are as in Figure 11.

in methotrexate binding in the *L. casei* enzyme are identical in the chicken liver reductase, based on the sequence alignments shown (Figure 12). Of these seven identical residues, five are invariant in all enzymes sequenced to date; these include Ala-9, Phe-34, Leu-67, Arg-70, and Thr-136. At position 22 Leu is replaced by Met only in the *E. coli* enzyme; similarly, Ser-59 is Gly only for the *S. faecium* II reductase. Other replacements in proposed methotrexate binding residues are analogous or isosteric but may perform the same structural function in the methotrexate binding pocket. For example, in comparing the *L. casei* sequence to that of the avian at position 7, Leu is replaced by Ile and at position 30 an Asp → Glu replacement occurs.

The overlapping binding domain for NADPH in the *L. casei* enzyme consists of a large network of hydrogen bonds as well as a number of charge and hydrophobic interactions (Matthews et al., 1979). Of the 24 residues involved in NADPH binding in the *L. casei* enzyme, 12 are identical in the chicken liver reductase (i.e., positions 9, 16, 17, 20, 22, 24, 53, 56, 59, 75, 117, and 146). A total of 8 of these 12 residues are invariant in all species whose dihydrofolate reductase sequences have been established. These include Ala-9, Ile-16, Gly-17, Trp-24, Gly-53, Thr-56, Leu-75, and Gly-117. In addition, four residues of the *L. casei* enzyme are in contact with both NADPH and methotrexate molecules (Ala-9, Leu-22, Ser-59, and Ala-115). In the homologous cluster of residues between positions 16 and 36 (cf. Figures 11 and 12), 10 residues are involved in either methotrexate binding, NADPH binding, or both. Between residues 51 and 77, eight residues are involved in dinucleotide and/or inhibitor binding interactions. Beyond residue 120 only Thr-136 and Thr-146 are involved in methotrexate and NADPH binding, respectively. Thr-136 is homologous in all species of reductases sequenced, whereas Thr-146 is replaced by Ala in the bovine liver enzyme (Figure 12).

The side-chain carboxyl of Asp-30 (Figure 12) has been implicated by Matthews et al. (1978) to involve a charge interaction with $N_{(1)}$ of the pteridine ring of methotrexate. The Asp-30 is conserved in all bacterial reductases examined, but is replaced by Glu in all animal enzymes sequenced thus far. A similar role for Glu-30 in the chicken liver reductase for the binding of methotrexate is highly probable. The Arg-54 and Arg-55 residues implicated in the binding of the phosphoryl moieties of NADPH in the *L. casei* enzyme through charge interactions are Lys-54 and Lys-55 in the avian reductase (Figure 12). The two arginines in the *L. casei* enzyme probably correspond to the two reactive arginines which, in the presence of NADPH, are protected from chemical modification with phenylglyoxal (Vehar & Freisheim, 1976). The other reductases contain either Arg-Lys or Arg-His at these positions. The Arg-70, involved in the binding of the α-carboxyl group of the glutamate moiety of methotrexate, is identical in all species. Previous chemical modification studies have implicated Trp-24 in the function of the *L. casei* reductase (Freisheim et al., 1977). The position of this tryptophan residue is conserved in all reductases examined (cf. Figure 12). The presence of neighboring proline residues creates a small loop containing the Trp-24 which, in the presence of bound NADPH, forms hydrophobic contact between its indole ring and the carboxamide group of NADPH. In addition, this invariant Trp-24 moves ~3 Å more deeply into the active-site region in the process. The binding of the γ-carboxyl group of the glutamate moiety of methotrexate is not as specific as that for the α-carboxyl group (see above). Either His-32 (*L. casei*) or Lys-36 (*E. coli*) fulfills the γ-carboxyl binding function. In the chicken liver enzyme this charge interaction might occur with either Lys-32 or Arg-36. Certainly Glu-32 could not play a similar role in the bovine reductase. In this connection, the guanido group of Arg-36 is approximately equidistant between the α- and γ-carboxyl groups of methotrexate (~4 Å) and could also serve to neutralize the negative charges of these carboxyl groups in the binding process. Arginine occurs at position 36 in five of the six species examined (Lys in *E. coli*).

The observations that the presumed substrate (or inhibitor) and dinucleotide binding pockets of chicken liver dihydrofolate reductase are highly homologous to those of the *L. casei* enzymes are consistent with the finding that methotrexate is equally effective as an inhibitor of both enzymes. Subsequent X-ray analyses of the chicken liver reductase should aid in clarifying the exact interactions involved in methotrexate and NADPH binding in animal vs. bacterial dihydrofolate reductases.

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Supplementary Material Available

Tables VI–XXIX giving (a) the details of automated sequence analysis of fragments generated from chicken liver dihydrofolate reductase, including repetitive yields and the yield in nanomoles at each step, (b) the results of hydrolysis of peptides by carboxypeptidases, (c) amino acid analyses of certain confirmatory peptides, (d) electrophoretic mobilities of certain confirmatory peptides, and (e) electrophoretic mobilities of certain peptides isolated by high-voltage electrophoresis (12 pages). Ordering information is given on any current masthead page.

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