DIHYDROFOLATE REDUCTASE FROM LACTOBACILLUS CASEI: N-TERMINAL SEQUENCE AND COMPARISON WITH THE SUBSTRATE BINDING REGION OF OTHER REDUCTASES

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SUMMARY

We report the N-terminal amino acid sequence of dihydrofolate reductase from a methotrexate-resistant strain of Lactobacillus casei. The data is correlated with a nuclear magnetic resonance study of enzyme-substrate interaction, and sequence comparison with two other reductases reveals fourteen positions of sequence identity. The sequence given is based upon mass spectrometric evidence, and represents part of a study involving the first major use of mass spectrometry in sequencing a protein of unknown structure in the absence of a concurrent classical strategy.

Dihydrofolate reductase catalyzes the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, the coenzyme involved in a number of important one-carbon transfer reactions. The enzyme is also the target of a clinically useful group of folate antagonists used in cancer chemotherapy (1). Structural studies at the molecular level therefore offer the prospect of understanding both ligand binding properties and the phenomenon of drug resistance. Elucidation of the catalytic mechanism(s) of the dihydrofolate reductases will also be of particular interest from a structure-function viewpoint since with molecular weights of 15,000 to 30,000 daltons they are the smallest known pyridine nucleotide-linked dehydrogenases. As a model for the human enzyme, we have begun by studying the primary structures of dihydrofolate reductase from wild type and methotrexate-resistant strains of Lactobacillus casei. The enzyme from the methotrexate-resistant organism (molecular weight 18,000 daltons) has been purified for nuclear magnetic resonance and kinetic studies (2), and for essential work on the primary structure, reported in part here. Crystal growth and X-ray diffraction studies (University of Leeds) are

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also progressing with the recent availability of sufficient quantities of pure enzyme. Substrate analogue binding studies on the L. casei enzyme have shown that several histidine residues lie at or near the binding site (3), and chemical labelling in the presence and absence of folate analogues, together with sequence studies on dihydrofolate reductase from Streptococcus faecium suggest that the N-terminal one-third of the molecule contains the important substrate (and inhibitor) binding site (4). Here we report the corresponding portion of the structure of the L. casei dihydrofolate reductase and discuss points arising from the new data.

MATERIALS and METHODS

Enzyme was purified from a methotrexate-resistant strain of L. casei as described by Dann et al. (2) and was donated for sequence work by these authors. Trypsin and chymotrypsin were purchased from Worthington. Elastase was prepared by the method of Shotton and Hartley (5). Cyanogen bromide was purchased from Koch Light. Dihydrofolate reductase samples (1 to 4μM) were digested using trypsin, chymotrypsin or elastase (enzyme:substrate ratio 1:50) in 0.1M ammonium bicarbonate pH 8.5, for 5 hr at 37°C, followed by freeze drying. The digest was fractionated using a cation exchange column and buffer gradient described previously (6). Details of the chromatographic and mass spectrometric properties of all peptides studied will be given elsewhere together with the total primary structure which is now nearing completion. Fractionation of the fragments produced by cyanogen bromide digestion (7) was achieved by (a) extraction of the freeze dried powder into pH 6.5 pyridine acetate buffer, leaving an insoluble peptide and (b) chromatography of the soluble peptides on Whatman DE52 resin using a 10mM to 300mM ammonium acetate gradient at pH 8.0. The N-terminus of the protein (and of the CNBr fragments) was determined mass spectrometrically by a modification of the method of Gray & Valle. (8). The protein (or peptide) 4mg was dissolved in 200μl of 98% formic acid to which 100μl of acetic anhydride was then added. Formylation was terminated after 2 hr. at room temperature by evaporation under vacuum. Other aspects of the method were as described (8). Overlapping and ordering of CNBr fragments was achieved by using sequences deduced for peptides generated from the tryptic, chymotryptic and elastase digests of the intact protein. Peptide sequences were determined by mass spectrometry using acetyl permethyl derivatives (9, 10) and instrument techniques previously described (11, 12). As a check on two overlap assignments, some residues in the sequence were confirmed by partial runs on a Beckmann automated sequencer by courtesy of Dr. J. Walker and colleagues. Mass spectra were recorded on AE1 MS902, MS50 and MS30 instruments.

RESULTS and DISCUSSION

Figure 1 shows the sequence, determined mass spectrometrically (see Materials and Methods) for the N-terminal region of dihydrofolate

L. casei:	Thr-	Ala-	Phe-	Leu-	Trp-	Ala-	Gln-	Asp-	Arg-	Asp-	Gly-	Leu-
E. coli:	1	1	1	1	2	0	1	1	2	0	1	1
S. faecium:	2	2	1	1	0	0	0	0	1	1	0	0

L. casei:	Ile-	Gly-	Lys-	Asp-	Gly-	His-	Leu-	Pro-	Trp-	His-	Leu-	Pro-
E. coli:	0	0	1	1	2	2	1	0	0	1	0	0
S. faecium:	0	0	0	0	0	1	0	0	0	1	0	0

L. casei:	Asp-	Asp-	Leu-	His-	Tyr-	Phe-	Arg-	Ala-	Gln-	Thr-	Val-	Gly-
E. coli:	1	0	0	2	2	0	1	2	2	0	1	2
S. faecium:	0	0	1	1	1	0	0	2	1	0	1	1

L. casei:	Lys-	Ile-	Met-	Val-	Val-	Gly-	Arg-	Arg-	Thr-	Tyr-	Glu-	Ser-
E. coli:	2	2	1	1	1	0	0	1	0	2	0	0
S. faecium:	0	0	1	0	1	0	0	1	0	0	0	1

L. casei:	Phe-	Pro-	Lys
E. coli:	1	2	1
S. faecium:	2	2	0

Figure 1

N-terminal sequence of dihydrofolate reductase from a methotrexate-resistant strain of Lactobacillus casei. Numerals indicate the minimum number of base changes necessary to convert any amino acid into its corresponding residue in the E. coli (13) or S. faecium (4) reductase sequences. Underlined residues show positions of identity in all three dihydrofolate reductases.

reductase from the methotrexate-resistant strain of L. casei. Below the sequence we show the minimum number of base changes which would be required to interconvert the L. casei sequence with those of the Escherichia coli (13) and Streptococcus faecium (4) enzymes respectively.

By comparing the sequences of dihydrofolate reductases from E. coli and S. faecium Gleisner et al. (4) have concluded that the enzymes have two areas of homology. The largest of these, the N-terminal one-third of the molecule, they suggest contains the pteridine binding site. The data in Figure 1 emphasises the importance of the N-terminal region of dihydrofolate reductase, showing that the L. casei enzyme has 17 identities with that of E. coli and 30 identities with that of S. faecium in the first 51 residues. These are far higher proportions of sequence identity than exist in the whole of the remaining two-thirds of the molecule (the mass spectrometric study on the total sequence is now nearing completion). Further, we see that all three enzymes share 14 identities in the N-terminal region, and these are presumably conserved because of their importance to substrate binding. However, two identities previously noted between the S. facium and E. coli enzymes (Met-42 and Gly-51 (4)) are absent in our structure, and therefore not essential for enzyme function. Looking at the sequence identities in Fig. 1 from a chemical viewpoint, Asp-26, Glu-47 and Arg-43 are likely candidates for direct involvement in binding the pteridine and glutamate moieties of the enzyme substrate or inhibitors, whilst other residues could be essential for folding or hydrophobic interaction. Similarly, the conservation of Trp-21 and Phe-30 may indicate the intimate involvement of their aromatic side chains in stabilisation of the binary enzyme-substrate complex.

Roberts et al. (3), in nuclear magnetic resonance studies on this L. casei enzyme, have observed resonance shifts of the imidazole C-2 protons of three histidine residues upon binding of substrate analogues. Three histidine residues (His-18, -22 and -28) are present in the postulated substrate binding domain (Fig. 1), and whilst they may be the ones observed in the resonance experiments

(3), the fact that they are not conserved residues (Fig. 1) indicates that any resonance shift would be caused by substrate proximity rather than direct involvement in binding.

It is expected that the total enzyme sequence (now nearing completion) together with the nuclear magnetic resonance and X-ray diffraction data (in progress) will elucidate the substrate and coenzyme binding and catalytic mechanism of this important enzyme.

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