Dihydrofolate Reductase: Low-Resolution Mass-Spectrometric Analysis of an Elastase Digest as a Sequencing Tool

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An elastase digest of a protein of unknown structure, dihydrofolate reductase, was studied by mass spectrometry. This soluble digest contained a large number of small peptides in different yields, within the ideal molecular-weight range (200–1200) for mixture-analysis mass spectrometry. Sequences of the major component peptides in the digest are reported.

Recent years have seen a continued interest in research leading to the complete sequence determination of proteins by mass spectrometry. Such an approach potentially has several advantages, not least of which is the ability to determine the sequences of peptides present in mixtures. This was first demonstrated in work on the protein silk fibroin (Geddes *et al.*, 1969; Morris, 1970) and, later, on synthetic (McLafferty *et al.*, 1970; Roepstorff *et al.*, 1971) and other protein-derived peptides (Morris *et al.*, 1971). More recently the mixture strategy has been successfully applied to several current protein-sequence problems, in work on ribitol dehydrogenase (Morris, 1972a; Hartley *et al.*, 1972; H. R. Morris, D. H. Williams, G. G. Midwinter & B. S. Hartley, unpublished work), azurin (A. Dell, H. R. Morris & D. H. Williams, unpublished work) and chloramphenicol transacetylase (A. Dell, H. R. Morris, B. D. Burleigh, B. S. Hartley & W. V. Shaw, unpublished work).

The necessity of chemical modifications of the peptides, in order to produce derivatives of sufficient volatility for examination by mass spectrometry, has in the past created severe problems, thereby limiting the potential of the method. Detailed analysis of the chemical reactions involved has led to new procedures (Morris, 1972b; Morris *et al.*, 1973) which now encompass all known amino acids, with the possible exception of cysteine.

A point of considerable significance in the evaluation of the mass-spectrometric method is that the above work on proteins has been performed without prior knowledge of the amino acid sequence or composition, and by using only low-resolution mass spectrometry (Morris *et al.*, 1971).

The size limit of the peptides that can be studied may in some circumstances be a disadvantage of using the mass spectrometer. The upper length limit is presently about ten amino acid residues, and is imposed by volatility and instrumental factors. Conventional tryptic and chymotryptic digestion of a protein will normally produce a number of peptides of length greater than ten residues. In order to obtain sequence data we have redigested mixtures of larger peptides and then refractionated to give less complex mixtures suitable for direct analysis. Clearly, an ideal proteolytic enzyme for our mass spectrometric purposes would be a hexa- to deca-peptidase. We have been examining some of the less specific enzymes (e.g. thermolysin, pepsin and subtilisin) with a view to preparing large numbers of small peptides amenable to direct analysis after simple fractionation. Our previous studies with these enzymes have not been entirely successful, either for reasons of enzyme specificity, or because of difficulties in interpretation of the spectra (H. R. Morris, D. H. Williams, G. G. Midwinter & B. S. Hartley, unpublished work). In the present communication we describe a study involving elastase digestion of dihydrofolate reductase from *Lactobacillus casei* MTX/R (Newbold & Harding, 1971).

Materials and methods

Dihydrofolase reductase was purified to homogeneity from large-scale cultures of *L. casei* MTX/R by modification of previous techniques (Newbold & Harding, 1971). The major difference was the inclusion of an anion-exchange column step before

chromatography of the reductase on the affinity column.

Elastase, prepared by the method of Shotton & Hartley (1973), was a gift from Dr. B. S. Hartley. Two equal portions of elastase were used as follows. Dihydrofolate reductase (2.8 μ mol; 40mg) was treated with the first portion of elastase at an elastase/reductase molar ratio of 1:200 at 37°C in 50mM-NH₄HCO₃, pH8.5. After 4h the second portion of elastase was added, and the digestion continued for a further 12h. The digest was freeze-dried, redissolved in water and one-fortieth of the sample examined by conventional one-dimensional paper electrophoresis, in pyridine-acetic acid buffers, pH6.5, followed by rerunning of the neutral band at pH3.5 (Ambler, 1963). This permitted the extent of the digestion to be determined on staining the electrophoretograms with cadmium acetate-ninhydrin reagent.

The remainder of the digest was fractionated on a column (54cm $\times$ 0.9cm) containing Locarte cation-exchange resin (code L1/5; Locarte Co., Northend Road, London W.14, U.K.). The peptides were eluted by using a pyridine-acetic acid gradient from pH3.1 to 5.0, formed from 180ml (0.2M in pyridine) and 350ml (2.0M in pyridine). The pumping speed was 20ml/h and fractions were collected at 10min intervals. A portion of each fraction was examined by electrophoresis as described above to determine the resolution of the column and allow a rational choice of fractions for pooling.

One-half of each freeze-dried pool was acetylated and permethylated (Morris, 1972b; Morris *et al.*, 1973), and examined directly by low-resolution mass spectrometry. Instrument parameters and the techniques used were as described previously (Morris *et al.*, 1971). Assignment of the peptide sequences from the mixture spectra was aided in some cases by a knowledge of fragmentation pathways gained from our other protein studies (see the introduction).

Results and discussion

Although dihydrofolate reductase is a small (mol.wt. approx. 14000) pyridine-containing protein, we have encountered several difficulties during sequencing procedures. Tryptic and cyanogen bromide digests of homogeneous *L. casei* dihydrofolate reductase have yielded products partially insoluble in simple buffers. Extremes of pH or high concentrations of, for example, urea will render the digests completely soluble, but these measures introduce new problems. In such cases losses due to precipitation on columns or difficulties in salt-free isolation of small peptides have been found to be unavoidable.

In contrast, the elastase digest of dihydrofolate reductase is completely soluble in water, enabling simple chromatography to be performed in relatively

high yield. Separation on the ion-exchanger, in association with electrophoretic 'mapping' and subsequent pooling of column fractions, provided samples varying in complexity from single peptides to mixtures of four major components. The degree of heterogeneity of the pools obtained in this way was acceptable for direct mixture analysis by mass spectrometry, thus obviating the necessity of further fractionation of the pooled samples.

Table 1. Sequences of the major components of the elastase digest

For experimental details see the text. Sequences incompatible with overlaps generated from studies of other digests can be confirmed, or otherwise, by stable-isotope labelling (for references see the text).

Asn-Val-Val-Leu
 Gly-Gly-Ala-Gln-Leu
 Ala-Gly-Ser-Phe-Glu-Gly
 Asp-Thr-Lys-Met-Leu
 Met-Val-Val
 Val-Val-Val
 Phe-Ala-Tyr-Ala
 Trp-Ala-Gln
 Lys-Gln-His-Leu
 Tyr-Ala-Lys-Gln-His-Leu
 Phe-Leu-Trp
 Asp-Val
 Met-Leu-Pro
 *Thr-Ala-Phe-Leu-Trp-Ala-Gln
 Leu-Met-Val-Val
 Phe-Thr
 Ala-Ala
 Trp-Gln
 Phe-Lys
 Gly-Lys-Leu
 Arg-Leu-Ala
 Glu-Lys-Lys
 Leu-Pro-Leu-Asn-Trp
 Thr-Tyr
 His-Thr-Thr
 Glu-Asp-Thr
 Asp-Thr-Asn
 Val-Val-Val-His-Asp-Val-Ala
 Glu-Val-Trp

*Other data (H. R. Morris & J. Bridgen, unpublished work) establish this peptide as the N-terminus of the protein.

The number of ninhydrin-positive spots on the analytical electrophoretogram of the column effluent was indicative of a small average peptide length. This has been borne out by the sequences obtained. Table 1 shows sequences of the major components of the digest obtained by our low-resolution mixture-analysis approach. Attention is drawn to the sites (underlined) of potential overlap with peptides from conventional digests, and to the small average size of the peptides. An absence of large peptide fragments, whose presence can be detected by low or zero electrophoretic mobilities and also by thermal decomposition in the mass spectrometer, is noted. A number of other sequences of minor component peptides have also been established, and these await confirmation by comparison with data from other digests.

The strategy described here allows for the retention of a portion of each of the pooled fractions which may be re-examined should the alignment of a particular peptide in the total sequence prove difficult. Veracity of the proposed sequence will then be sought by mass-spectrometric analysis of stable-isotope derivatives (Morris, 1970; Roepstorff & Brunfeldt, 1972).

In the present work we have found elastase to be an ideal proteolytic enzyme for combination with the mass-spectrometric study of dihydrofolate reductase, in generating a large number of small peptides suitable for rapid screening by our mixture-analysis technique. It is clear that other groups, applying this mixture approach to their own studies, should find elastase a valuable enzyme for this purpose. We

emphasize the need for only one column fractionation in the present work on this size of protein.

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