Application of high-performance liquid chromatography to the purification and characterization of ribosomal protein L3 from trichodermin-resistant yeast mutants

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A new h.p.l.c. cation-exchange method has been used to separate proteins from 60S ribosomal subunits prepared from strains of *Saccharomyces cerevisiae* sensitive or resistant to trichodermin. Ribosomal protein L3 was identified in column eluates by one-dimensional and two-dimensional gel electrophoresis and purified further by reverse-phase h.p.l.c. The protein was cleaved with CNBr and the products were analysed, again by reverse-phase h.p.l.c. A marked difference was observed in the peptide profiles between preparations from trichodermin-sensitive and trichodermin-resistant yeast strains. These results provide the first direct demonstration that, in yeast, mutationally induced resistance to trichodermin can alter the covalent structure of ribosomal protein L3. They convincingly demonstrate the potential of the experimental technique for the rapid and preparative separation of a selected yeast ribosomal protein and its subsequent characterization.

INTRODUCTION

A number of *Saccharomyces cerevisiae* mutants have been isolated as resistant to selected antibiotics that inhibit protein synthesis, and in many cases (Cooper *et al.*, 1967; Skogerson *et al.*, 1973; Grant *et al.*, 1974; Vázquez, 1979; González *et al.*, 1981) resistance is associated with an alteration in a r-protein. Cycloheximide resistance, for example, involves a change at position 37 of r-protein L29 (for nomenclature see Fried & Warner, 1981; Michel *et al.*, 1983), where glutamine is replaced by glutamic acid (Stocklein & Piepersberg, 1980; Stocklein *et al.*, 1981; Kaufer *et al.*, 1983). Certain mutants are resistant, at the ribosome level, to the trichothecene antibiotic trichodermin (Schindler *et al.*, 1974; Jiménez *et al.*, 1975), which is a specific inhibitor of the peptidyl-transferase centre on 80S ribosomes. Indirect evidence from Fried & Warner (1981) indicates that the protein involved in controlling trichodermin resistance in a *S. cerevisiae* strain designated CLP-1 is r-protein L3. The gene encoding this protein is the *tcm-1* gene, which has been sequenced (Schultz & Friesen, 1983). Nevertheless, no actual amino acid alteration has been inferred for the mutated form of r-protein L3, since the allele for the unchanged protein has not been isolated.

Extensive investigations have been carried out on two additional strains of *S. cerevisiae* that are resistant to trichodermin. These strains have easily distinguishable phenotypes. Strain CLP-8 has the same parent (strain A224A) as strain CLP-1, but is independently derived (Schindler *et al.*, 1974). In strain CLP-8, pre-rRNP particles containing 20S pre-rRNA accumulate in the nucleus and are only slowly transported to the cytoplasm, where formation of 18S rRNA is also slowed (Carter & Cannon, 1980). In sharp contrast, this processing pathway is normal in strain TR-1, which is derived from a parent strain Y166. To explain this difference, the processing

defect in strain CLP-8 has been postulated to be due not solely to the presence of the trichodermin resistance allele. Rather this allele may allow the expression of a 'silent' lesion in strain A224A (Carter *et al.*, 1980; Mitlin & Cannon, 1984). Indeed, strain A224A expresses a processing defect when grown for several generations at 36 °C, which is indistinguishable from that seen in strain CLP-8 (Mitlin & Cannon, 1984). The control of ribosome biogenesis in strain A224A is thus critically balanced and apparently can be perturbed in different ways to yield similar phenotypes.

To facilitate the analysis of mutants with altered ribosomes, we have developed a rapid preparative method for the isolation of r-proteins. This method involves the use of an ion-exchange h.p.l.c. column and represents a new approach for yeast. To illustrate its potential, we have now applied the method to a study of trichodermin resistance in *S. cerevisiae*. The mutation causing trichodermin resistance in each of the strains CLP-8 and TR-1 maps in the same gene (Grant *et al.*, 1976). Nevertheless, the alteration need not be identical in the two cases, since a different amino acid change in the same protein could result in the distinguishable phenotypes observed. In the present work we have used a combination of ion-exchange and reverse-phase h.p.l.c. to purify r-protein L3 from each of the four *S. cerevisiae* strains Y166, TR-1, A224A and CLP-8. The latter technique was subsequently used to separate CNBr digests of the purified proteins. Our results clearly demonstrate that ion-exchange h.p.l.c. is a powerful technique for the rapid separation of large amounts of r-proteins, which are thus made readily available for final purification and partial or complete characterization. Our results also show directly that trichodermin resistance in yeast is associated with a structural alteration in r-protein L3 and indicate that the same or

Abbreviations used: r-protein, ribosomal protein; pre-rRNP, pre-ribonucleoprotein; pre-rRNA, ribosomal precursor RNA.

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an equal change is found in the *S. cerevisiae* strains CLP-8 and TR-1.

EXPERIMENTAL

Yeast strains and their maintenance and growth

The following strains of *S. cerevisiae* were used in the present work: strain A224A (*a*, *leu-2*⁻, *can-1*⁻); strain CLP-8 (*a*, *leu-2*⁻, *can-1*⁻, *tcm-1*⁻); strain Y166 (*α*, *trp-5*⁻, *his-4*⁻); strain TR-1 (*α*, *trp-5*⁻, *his-4*⁻, *tcm-1*⁻). All strains were maintained at 30 °C on YEPD plates (Gritz *et al.*, 1982) and grown in liquid YEPD medium at this temperature.

Large-scale growth of yeast strains

Colonies were picked from plates to inoculate several 10 ml cultures. After 10–18 h of growth, four of these cultures were used to inoculate four separate 1-litre cultures, which were incubated at 30 °C with stirring overnight. These cultures were examined microscopically for possible bacterial contamination and two were selected and used to inoculate a 150 litre fermenter. Cells were grown to an A_{600} of 2.0 and then cooled slowly to 8 °C to induce ribosome run-off (Carter *et al.*, 1980). Cells were harvested with a Sharples centrifuge, and washed twice in distilled water with centrifugation at 10000 *g* for 5 min at 2 °C.

Breakage of yeast cells

Each yeast preparation (approx. 100 g wet wt. of cells) was resuspended in 300 ml of buffer (100 mM-Tris/HCl, pH 7.4, 12.5 mM-magnesium acetate, 80 mM-KCl, 5 mM-dithiothreitol). Cells were then disrupted by four passages through a French Pressure Cell (Aminco) at 7000 lbf/in² (48.3 MPa), and the lysates were centrifuged at 20000 *g* for 15 min at 2 °C. The supernatant fractions were then centrifuged at 20000 *g* for 30 min at 4 °C to clear any remaining debris and kept at 4 °C before ultracentrifugation.

Preparation of ribosomal subunits

Supernatant fractions, prepared as described above, were centrifuged for 2 h at 45000 rev./min at 4 °C in a Spinco Ti50.2 rotor. The pellets obtained were resuspended in buffer (100 mM-Tris/HCl, pH 7.4, 12.5 mM-magnesium acetate, 80 mM-KCl, 5 mM-dithiothreitol) and the centrifugation was repeated. The resultant pellets were again resuspended in the same buffer and then sedimented through a cushion of 20% (w/v) sucrose, prepared in the same buffer, for 2 h at 45000 rev./min. Ribosomes were then resuspended in approx. 50 ml of buffer (50 mM-Tris/HCl, pH 7.4, 5 mM-magnesium acetate, 500 mM-KCl, 5 mM-dithiothreitol). This buffer dissociates 80S ribosomes into their constituent 60S and 40S subunits. The ribosome resuspension was then subjected to zonal ultracentrifugation in a Spinco Ti-15 zonal rotor. Approx. 10000 A_{260} units were loaded on to a 1-litre 10–35% linear sucrose gradient with a 400 ml 45%-sucrose cushion prepared in the same buffer. Centrifugation was at 23000 rev./min for 17 h at 12 °C. The 40S and 60S peaks from the zonal separation were collected, and the ribosomal subunits were precipitated with 2 vol. of 95% (v/v) ethanol at -20 °C overnight. The precipitates were spun down and resuspended in buffer (10 mM-Tris/HCl, pH 7.4, 10 mM-magnesium

acetate). Protein was extracted from the purified ribosomal subunits by the acetic acid method of Hardy *et al.* (1969).

Separation of r-proteins

The extracted proteins were dialysed into 5% (v/v) acetic acid and freeze-dried. The freeze-dried samples were resuspended in buffer A (8 M-urea, 20 mM-KH₂PO₄, 0.5 mM-dithiothreitol). The pH was adjusted to 5.6 with methylamine. The protein extract (20–120 mg) was applied to a preparative Bio-Rad SP-5-PW cation-exchange column (150 mm × 21.5 mm) and eluted with a 0–0.8 M-KCl gradient in buffer A. The flow rate was 1.5 ml/min and fractions were collected for 2 min each.

Identification of r-proteins by gel electrophoresis

Two types of acid/urea gels were run, in the buffer system of Reisfeld *et al.* (1962). A 10%-monomer/0.75%-bisacrylamide 'hard gel' was used for the initial identification of peaks from the h.p.l.c. column (system 1). A 10%-monomer/0.15%-bisacrylamide 'soft gel' was used as the first dimension in the two-dimensional system (system 3). The urea/SDS gel system of Martini & Gould (1971) was used as the second dimension, and as a one-dimensional system (system 2) to perform secondary identification.

Reverse-phase h.p.l.c.

A preparative SynChropak RP-P C₁₈-silica column was used with a two-component water/acetonitrile system in 0.1% trifluoroacetic acid as described by Kerlavage *et al.* (1983) for further purification of r-protein L3 and to analyse its CNBr cleavage products. The acetonitrile gradient was changed for these different uses. Samples from the column eluates were freeze-dried directly.

CNBr cleavage of purified r-protein L3

Freeze-dried r-protein L3 was dissolved in water, and then formic acid was added to give a final concentration of 70%. This was followed by the addition of 50–100 mg of solid CNBr. The mixture was incubated at 37 °C for 6 h, after which it was diluted with 5 vol. of water and frozen for freeze-drying. The freeze-dried sample was resuspended in 1 ml of 8 M-urea to run on the reverse-phase h.p.l.c. column.

RESULTS AND DISCUSSION

Reverse-phase h.p.l.c., on large-pore C₁₈-silica columns, has been used successfully to separate mixtures of peptides, polypeptides and proteins, and in particular has provided a rapid, sensitive and highly reproducible method for resolution of r-proteins from *Escherichia coli* (Kerlavage *et al.*, 1983), rabbit reticulocytes (Ferris *et al.*, 1984) and yeast (Kerlavage *et al.*, 1985). Kerlavage *et al.* (1983) considered the possibility of using ion-exchange h.p.l.c. columns to separate r-proteins, but concluded that the highly basic nature of these components would prevent their satisfactory resolution. Indeed, although Dalrymple *et al.* (1983) showed clearly that ion-exchange h.p.l.c. could resolve simple test proteins, such as lysozyme and ricin, the separation achieved with a rat liver r-protein preparation was, at the best, only adequate. Despite these considerations, however, Kamp *et al.* (1984) have indicated that ion-exchange h.p.l.c. can be useful for isolating *E. coli* r-proteins under selected

Fig. 1. Separation of TP-60 by ion-exchange h.p.l.c.

Approximately 60 mg of protein, dissolved in 3 ml of buffer A after extraction from the large ribosomal subunit of *S. cerevisiae* strain TR-1, was applied to a preparative SP-5-PW column as described in the Experimental section. Both the A_{280} of the eluate (—) and the KCl concentration of the eluting buffer (----) are indicated.

conditions, and a successful separation of these components has recently been achieved by M. Capel & G. R. Craven (unpublished work). We have now developed ion-exchange h.p.l.c. as a rapid preparative method for the isolation of yeast r-proteins. We describe here the application of the method to the purification and analysis of r-protein L3 from yeast strains sensitive or resistant to trichodermin.

Fig. 1 illustrates a typical separation of r-proteins isolated from 60S ribosomal subunits (TP-60) prepared from the trichodermin-resistant *S. cerevisiae* strain TR-1. The proteins were separated on the cation-exchange column SP-5-PW (sulphopropyl) as described in the Experimental section. Approx. 30 protein peaks can be detected in this experiment, with excellent resolution of 20 major components. Similar separations were obtained with r-proteins prepared from 60S ribosomal subunits of

each of the *S. cerevisiae* strains A224A, Y166 and CLP-8 (results not shown).

In order to make a preliminary identification of r-protein L3, peak fractions (labelled 1–16 on Fig. 1) were analysed by one-dimensional gel electrophoresis (system 1). The results are illustrated in Fig. 2. The gel system used contains urea and separates r-proteins on the basis of both size and charge. Since L3 is the largest of the yeast r-proteins (Fried & Warner, 1981), this protein is likely

Fig. 2. One-dimensional polyacrylamide-gel electrophoresis of peak fractions from the TP-60 separation illustrated in Fig. 1

Electrophoresis was carried out as described in the Experimental section, system 1. Tracks 1 and 19 contain TP-60 (0.2 mg); tracks 2 and 20 contain TP-60 (0.4 mg); tracks 3–18 contain 4–15 μ g (30 μ l) of the peak fractions 1–16 identified in Fig. 1.

Fig. 3. One-dimensional SDS/polyacrylamide-gel electrophoresis of selected fractions from the TP-60 separation illustrated in Fig. 1

Electrophoresis was carried out as described in the Experimental section, system 2. Tracks 1 and 6 contain TP-60 (60 μ g); track 2 contains peak fraction 9 (6 μ g); track 3 contains peak fraction 9 (6 μ g) plus TP-60 (60 μ g); track 4 contains peak fraction 12 (6 μ g); track 5 contains peak fraction 12 (6 μ g) plus TP-60 (60 μ g).

Fig. 4. Two-dimensional polyacrylamide-gel electrophoresis of peak fractions 9 and 12 from Fig. 1

Electrophoresis was carried out as described in the Experimental section, in system 3. The left side of each gel shows the pattern for 6 µg of the sample alone; the right side of each gel shows the pattern for 6 µg of the sample plus 60 µg of TP-60. Gel (a) contains peak fraction 9; gel (b) contains peak fraction 12. Migration in the first dimension was from right to left and in the second from top to bottom.

Fig. 5. Final purification of r-protein L3 by reverse-phase h.p.l.c.

The r-protein L3 fractions purified by h.p.l.c. on the SP-5-PW column were further purified by reverse-phase h.p.l.c. with a preparative C_{18} -silica column as described in the Experimental section. The profile illustrated is of r-protein L3 isolated from strain A224A; corresponding profiles for the other three preparations were essentially identical. The peaks were pooled as indicated and freeze-dried. The samples were then run on urea/SDS gels (system 2) to assess the purity of the r-protein L3 peak; the gels are shown as an inset in the Figure.

to be the one present in either peak fraction 9 or peak fraction 12 of the gel depicted in Fig. 2. This interpretation is supported by the data illustrated in Fig. 3. In this experiment the peak fractions 9 and 12 from Fig. 1 were again analysed by one-dimensional gel electrophoresis. In this case, however, the gels used (system 2) contained SDS, which separates r-proteins primarily on the basis of size. As Fig. 3 shows, peak fraction 12 contains the largest r-protein in the preparation analysed, and we infer that peak 12 contains r-protein L3.

Further evidence that our identification of r-protein L3 is correct was obtained by two-dimensional gel electrophoresis (system 3) of the peak fractions 9 and 12 (Fig. 1). These analyses used a first dimension similar to system 1 and a SDS gel (system 2) in the second dimension. For this analysis, the conditions closely resemble those in the

two-dimensional system I (acid SDS) used by Michel *et al* (1983) to correlate r-protein nomenclature (see also Fried & Warner, 1981). Accordingly, an unambiguous identification of r-proteins can be achieved. In this experiment samples of the r-proteins suspected to be r-protein L3 were mixed with a low concentration of a TP-60 r-protein preparation from *S. cerevisiae* strain CLP-8. The single r-protein added is in relative excess over its counterpart in the TP-60 preparation and stains heavily on the gels. The results are illustrated in Fig. 4. These experiments identify the peak fraction 12 (Fig. 1) as r-protein L3.

To maximize yields, the fractions comprising r-protein L3 and illustrated in Fig. 1 were pooled. A similar exercise was carried out for r-protein L3 isolated by ion-exchange h.p.l.c. from each of the other three yeast strains, with the

protein again being identified by the procedures outlined above. An initial estimate of purity was obtained by analysing samples from the pooled fractions by one-dimensional gel electrophoresis (systems 1 and 2) (results not shown). In all four cases the pooled r-protein L3 peaks were found to be contaminated with lower- M_r proteins. In order to purify r-protein L3 further, the pooled fractions were subjected to reverse-phase h.p.l.c. as described in the Experimental section. The results of such a purification for r-protein L3 from strain A224A are shown in Fig. 5. The major component seen in the profile is essentially uncontaminated r-protein L3, as shown by polyacrylamide-gel electrophoresis (see insert in Fig. 5).

As detailed in the Introduction, the *trm-1* gene, which apparently encodes a mutated form of r-protein L3 in the *S. cerevisiae* strain CLP-1, has been cloned and the complete DNA sequence determined (Schultz & Friesen, 1983). The DNA sequence is consistent with an encoded protein of 387 amino acids, of which there are 7 methionine residues widely dispersed throughout the polypeptide. To compare, at the peptide level, the mutant L3 proteins from strains CLP-8 and TR-1 with the corresponding proteins from their parent strains, we cleaved the four proteins with CNBr and analysed the digestion products by reverse-phase h.p.l.c. The resultant peptide profiles are reproduced in Fig. 6. The profiles of the preparations derived from the trichodermin-sensitive parent strains Y166 and A224A are essentially identical.

In contrast, although digests from the trichodermin-resistant strains TR-1 and CLP-8 give very similar elution profiles, there are marked differences between parent and mutant preparations. We believe that the peptides marked with arrows in Fig. 6 demonstrate that a covalent modification in r-protein L3 has taken place in the two mutant strains as compared with the parent strains and that this results in a reproducible alteration in chromatographic characteristics. Although the molecular basis underlying this difference remains elusive, our data provide the first direct demonstration that an alteration in the covalent structure of r-protein L3 is responsible for causing trichodermin resistance in yeast, and thus confirm the conclusion inferred from the cloning work of Fried & Warner (1981). An amino acid change is almost certainly involved. Our data also show clearly that the relative retention times of the altered peptides of the two mutant L3 proteins are virtually identical, which suggests that the same or equal change exists within the two proteins. If this is the case, the difference in the phenotypes of strains CLP-8 and TR-1 with respect to ribosome biogenesis is not the result of a different mutation in the gene encoding r-protein L3, but relates to a difference in the genotypic backgrounds of the strains. Accordingly, our results support the concept of a 'silent' lesion in strain A224A (Carter *et al.*, 1980; Mitlin & Cannon, 1984), with the implication that r-protein L3 has an important role in controlling some aspect(s) of ribosome biogenesis in yeast.

Fig. 6. Separation, by reverse-phase h.p.l.c., of fragments of r-protein L3 generated after cleavage with CNBr

Preparations of r-protein L3 purified from each of the *S. cerevisiae* strains Y166, TR-1, A224A and CLP-8 were digested with CNBr and separated by reverse-phase h.p.l.c. as described in the Experimental section. The amount of protein loaded was as follows: Y166, 290 μ g; TR-1, 330 μ g; A224A, 770 μ g; CLP-8, 68 μ g. The A_{230} scale is linear for all four parts.

The results presented demonstrate conclusively that ion-exchange h.p.l.c. can be applied to the rapid and preparative separation of a selected yeast r-protein. Used in conjunction with reverse-phase h.p.l.c. it offers an excellent experimental system for the preliminary detection of amino acid changes in r-proteins isolated from yeast mutants. Furthermore, protein fragments obtained from enzymic or chemical digestion of purified components can be isolated in good yields, thereby providing the material for subsequent sequencing work. The preparative method has the potential to allow the complete characterization of yeast r-proteins. Identification and purification of these components will aid the development of reconstitution techniques for eukaryotic ribosomes, with important implications for studying structure-function relationships in this organelle.

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