Characterization of a DL-Dityrosine-containing Macromolecule from Yeast Ascospore Walls*

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We have shown previously that the outer layers of yeast ascospore walls contain dityrosine and that this amino acid is a major component of the cross-linked peptides present in the spore wall (Briza, P., Winkler, G., Kalchhauser, H., and Breitenbach, M. (1986) J. Biol. Chem. 261, 4288-4294). We now present evidence that dityrosine is located in the outermost layer and that it is in the DL-configuration. Although the proteins (peptides) of the spore wall are insoluble, the macromolecule containing dityrosine can be solubilized by partial acid hydrolysis of spore walls. Analysis of this macromolecule indicates that it contains more than 50 mol % dityrosine and a very limited number of other amino acids. Interestingly, part of the dityrosine of spore walls is present in the DL-configuration. We speculate that not only the high degree of cross-links in the outermost layer but also the D-configuration of part of the α-C-atoms of dityrosine could contribute to the spores' resistance to lytic enzymes.

We have investigated the properties of a dityrosine-containing macromolecule of the spore wall outermost layer. The chemical structure of this macromolecule seems to be different from those of other dityrosine-containing biological structures. First, the dityrosine content of the spore wall macromolecule is much higher than that of comparable biological structures (5), pointing to a highly cross-linked macromolecular network. Second, the same macromolecule contains a very limited set of different amino acids. Third, some of the dityrosine α-C-atoms exhibit the D-configuration in vivo. The implications of these findings for our understanding of the structure and biosynthesis of the dityrosine-containing macromolecule will be discussed.

EXPERIMENTAL PROCEDURES

Strains—The yeast strains AP3 (wild type with respect to sporulation) and XW285 (a glucosamine auxotroph) have been described in a previous publication (3). The mutant strain dit101 has been isolated in our laboratory. It grows normally but produces spores that lack both outer layers of the spore wall. Spores of the mutant strain dit102 are defective in synthesis of the outermost layer of the spore wall. A characterization of these mutants will be published elsewhere.

Materials and Methods—Media, growth conditions, purification of spores, spore walls and vegetative cell walls, amino acid analysis, infrared spectroscopic methods, and fluorescence spectroscopic methods have all been described in our two previous papers (2, 3).

Chemicals and Enzymes—Pronase, pepsin, trypsin, Glusulase (β-glucuronidase), zymolyase (lyticase), polytyrosine, and glycine anhydride were obtained from Sigma.

Surface Iodination of Spores and Vegetative Cells—Spores and vegetative cells were iodinated using IODO-GEN (Pierce, Oud Beijerland, The Netherlands) (Ref. 7). Cells were suspended in 0.3 ml of sodium phosphate (pH 8.2) and 100 μCi of 125I-labeled sodium iodide (Amersham Corp.) in an Eppendorf reaction vial coated with Ready-Solve® (Beckman) and counting in a liquid scintillation counter (LS 1700, Beckman) using the tritium channel.

Hydrolysis Conditions—For complete degradation of proteins, spore walls were hydrolyzed in 6 N HCl at 110 °C for 14 h. Dityrosine was identified in hydrolysates either by thin layer chromatography or by HPLC1 after removal of HCl in vacuo (2). For the liberation of dityrosine-containing macromolecules, spore walls were heated for 10 min at 110 °C in 4 N HCl in a sealed tube after flushing with nitrogen. Undissolved wall material was removed by centrifugation, and the supernatant was dried at 25 °C in a rotary evaporator.

Preparation of the Dityrosine-containing Macromolecule—The dried hydrolysate after partial acid hydrolysis was taken up in water, or by HPLC1 after removal of HCl in vacuo (2). For the liberation of dityrosine-containing macromolecules, spore walls were heated for 10 min at 110 °C in 4 N HCl in a sealed tube after flushing with nitrogen. Undissolved wall material was removed by centrifugation, and the supernatant was dried at 25 °C in a rotary evaporator.

1 The abbreviations used are: HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.

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RESULTS

Dityrosine Is Located in the Outermost Layer of Yeast Ascospore Walls—To test for the presence of dityrosine on the surface of yeast spores, spores were purified on a Percoll gradient as described earlier (2, 11) and treated with [125I]potassium iodide. This procedure labels phenolic residues present on the surface of cells. Mid-log phase vegetative cells were collected by centrifugation, washed, and similarly surface labeled with [125I]. In both experiments, the same number of cells or spores (3.3 x 10^7) was used and treated with exactly the same amount of [125I] (50 μCi). The total amount of radioactivity incorporated was measured by liquid scintillation counting. The spores were found to bind 60 times more 125I than vegetative cells. Both labeled preparations were then treated with Pronase, and the kinetics of the liberation of 125I-labeled material was monitored. 80% of the iodine incorporated was measured by liquid scintillation counting after evaporation of the solvent (modified from Ref. 9). In control experiments employing a racemization catalyst, 100% of the iodine activity incorporated was measured by liquid scintillation counting as described above.

Circular Dichroism—Circular dichroism spectroscopy of spore wall dityrosine (and controls) was performed on a Dichrograph Mark III (Jobin-Yvon, France) instrument.

Properties of the Surface Layer—Both intact spores and purified spore walls were used for the experiments described here. Treatment with a number of commercially available enzyme preparations (Pronase, pepsin, trypsin, Glusulase, zymolyase) did not lyse or kill yeast spores. The same treatment failed to liberate soluble compounds containing dityrosine (as monitored by its fluorescence) from spores or purified spore walls. Treatment of intact spores with the enzymes mentioned above also resulted in no detectable morphological changes (monitored by electron microscopy; data not shown). However, the inner layers of purified spore walls could be digested with Glusulase or zymolyase, leading to very thin "walls" that seemed to consist only of layers 1 and 2 but still retained the shape of the spore walls as well as their dityrosine component.

Analysis of a Dityrosine-containing Macromolecule from Spore Walls—Although treatment of intact spores or purified spore walls with proteases, Glusulase, or zymolyase did not release any soluble compounds containing dityrosine, we found that partial acid hydrolysis of spore walls (4 N HCl, 110 °C, 10 min) liberated a water-soluble macromolecular fraction. The fluorescence spectrum of this fraction was nearly identical with that of dityrosine. A fluorescent macromolecule with very similar properties was liberated by treatment of spore walls with trifluoromethanesulfonic acid, a reagent believed to cleave specifically the glycosidic bonds of sugar residues (12).

The fluorescent macromolecule present in the acid hydrolysate of spore walls was partially purified by ethanol precipitation and reversed phase HPLC (Fig. 2). Two fluorescent peaks were resolved. Peak 1 was sharp and free of polysaccharides. Peak 2 (Fig. 2) was very broad and contained most of the fluorescent macromolecular material along with soluble chitosan (3). The molecular mass of both fluorescent substances from peak 1 and peak 2 was nonhomogeneous, producing a broad smear on SDS-polyacrylamide gel electrophoresis ranging from about 10 to about 100 kDa (Fig. 3A). Upon analytical isoelectric focusing, the fluorescent material of the two peaks behaved similarly, forming a reproducible pattern of about seven bands (Fig. 3B). The most likely interpretation of a regular pattern such as this one is that the macromolecule contains regular repeats of an ionizable substructure. The substance constituting peak 2 contained a Coomassie-positive contaminant (soluble chitosan and minor amounts of protein). The substance constituting peak 1 was free of any nonfluorescent contaminating chitosan or protein. Therefore, the amino acid composition of peak 1 is characteristic of the dityrosine-containing macromolecule.
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The amino acids were determined in acid hydrolysates of HPLC fractions (peak 1 and peak 2) and of purified spore walls.

| Amino acid | Amount |
|------------|--------|
|            |  mol % |
| Ser        | 2.5    | 6.3  | 16.5 |
| Asx        | 2.8    | 11.9 |    7.9 |
| Glx        | 12.6   | 9.2  |    9.3 |
| Arg        | 2.2    | 2.0  |    2.1 |
| Thr        | 2.9    | 6.3  |    17.9 |
| Gly        | 31.8   | 8.2  |    4.8 |
| Ala        | 4.3    | 5.1  |     8.5 |
| Met        | —      | 0.9  |     — |
| Pro        |      | 5.6  |     8.7 |
| Val        | 2.5    | 4.2  |     4.6 |
| Phe        | 4.4    | 2.8  |     2.0 |
| Leu        | 4.4    | 3.9  |     2.8 |
| Ile        | 4.4    | 2.2  |     4.6 |
| Lys        | 2.2    | 4.2  |     4.6 |
| His        | —      | Traces | 2.8 |
| Tyr        | Traces | Traces | 4.2 | 4.4 |
| Di-Tyr*    | 53.7   | 32.9 | 19.9 | — |

* — , not detectable.

† Di-Tyr, dityrosine.

As shown in Table I, the dityrosine-containing macromolecule (peak 1) consists of very few different amino acids. The most abundant amino acid is dityrosine, followed by glycine, glutamic acid, alanine, and lysine. We do not know at present if there are other, non-amino acid components present in that macromolecule. Despite its proteinaceous nature, the macromolecule, like the surface layer of intact spores, was not degraded by the proteases and lytic enzymes mentioned above. This fact together with the very unusual amino acid composition of the macromolecule raised the question of whether this molecule is a polypeptide at all. We investigated the infrared spectrum of the substance constituting peak 1 (Fig. 4). The spectrum confirmed the absence of polysaccharides and the presence of dityrosine. It did not show the diagnostic peaks of standard trans-amide bonds at 1640 and 1520–1530 cm⁻¹ (13). The substance constituting peak 1 shows prominent bands at 1685 and 1435 cm⁻¹. These bands do occur in the infrared spectrum of free dityrosine. They are also characteristic of cis-amide bonds (13) as observed, for instance, in diketopiperazines, and at present we cannot exclude the presence of such cis-amide bonds in the dityrosine-containing macromolecule of peak 1.

The chromophore of dityrosine seemed to be relatively undisturbed in the macromolecule, as both the UV and the fluorescence excitation and emission spectra of the macromolecule were very similar to those of free dityrosine (Fig. 5). They also showed exactly the same pH dependence (data not shown). This showed that the bonding of dityrosine in the macromolecule cannot occur via the phenolic hydroxyl groups. Free phenolic hydroxyl groups are essential for the observed fluorescence, as only the phenolate form of the molecule...
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FIG. 5. Spectroscopic properties of the dityrosine-containing macromolecule. A, material of peak I of Fig. 2; 2, dityrosine. A, UV/visible absorption spectra; B, fluorescence emission spectra; C, fluorescence excitation spectra.

Dityrosine is probably bound to the macromolecule via its amino and/or carboxylate groups.

Optical Configuration of Spore Wall Dityrosine—Amino acid analysis of a hydrolysate of yeast spore walls yielded two closely related peaks of dityrosine in about equal amounts, which have been assigned to the meso- and racemic forms of dityrosine, respectively (2). Here we demonstrate that the meso-dityrosine is present in vivo and is not an artifact of acid hydrolysis.

First, LL-dityrosine was not measurably converted to the meso-form under the conditions used for acid hydrolysis of yeast spore walls (6 N HCl, 110 °C, overnight, i.e. “standard conditions”). Second, trypsin was oxidatively cross-linked by performic acid treatment and hydrolyzed under standard conditions. The dityrosine recovered was entirely in the LL-form.

Third, we isolated spore wall dityrosine under the mildest possible conditions (6 N HCl, 40 °C, overnight). The relative amount of meso-dityrosine recovered was the same as under standard conditions. Fourth, the residual dityrosine present in our dityrosine-less mutant ditl01 was analyzed after standard hydrolysis and purification. The mutant contained nearly exclusively the LL-dityrosine peak. Similarly, spores of a mutant auxotrophic for glucosamine (15) contained nearly exclusively the LL-dityrosine peak (Fig. 6). These experimental results support the notion that DL-dityrosine does occur in vivo in wild-type spores.

As a more direct test for possible racemization during hydrolysis of spore walls we measured the incorporation of 3H into spore wall dityrosine using tritiated HCl (7) (Table II). The samples used for the tritium measurements were also checked for the presence of the two stereoisomeric forms of dityrosine by HPLC analysis. Phenolic compounds do show a small amount of 3H incorporation even in the absence of racemization (7). Table II shows the amount of background 3H incorporation into LL-dityrosine under standard hydrolysis conditions without racemization. Hydrolyzing spore walls under standard conditions showed no significant increase in 3H incorporation over the background level. Using LL-dityrosine and a catalyst (benzaldehyde) in tritiated 80% acetic acid (10), a 15-fold increase in 3H incorporation and a DL-dityrosine peak (HPLC) were found, indicating partial racemization.

TABLE II
Incorporation of 3H into dityrosine of spore walls and controls in acid hydrolysis experiments

| Experiment          | cpm  | Relative peak area of meso-dityrosine |
|---------------------|------|--------------------------------------|
| LL-Di-Tyr/3HCl      | 5,596| 0.0                                  |
| Spore wall/3HCl     | 6,516| 0.8                                  |
| LL-Di-Tyr/3HAc⁺     | 1,460| 0.0                                  |
| LL-Di-Tyr/3HAc/Benz.⁺ | 22,776| 0.4                                |
| Spore wall/3HAc    | 3,243| 0.8                                  |

* Di-Tyr, dityrosine.
⁺ HAc, acetic acid.
⁻ Benzaldehyde.

Sensitive enough to detect even moderate amounts of racemization during acid hydrolysis. LL-Dityrosine in 80% acetic acid, with no added catalyst, was not racemized and incorporated little tritium. Spore walls were hydrolyzed in 80% acetic acid, the peak ratio of the two forms of dityrosine was normal, and little 3H was incorporated (Table II). As there was no increase in 3H incorporation into dityrosine (i.e. no racemization) when spore walls were hydrolyzed, we conclude that meso-dityrosine fluoresces (14). Therefore, dityrosine is probably bound to the macromolecule via its amino and/or carboxylate groups.

WAVELENGTH (nm)

P. Briza, A. Ellinger, G. Winkler, and M. Breitenbach, unpublished observations.
The dityrosine of the spore wall is not an equilibrium mixture of the two stereoisomeric forms. As shown by the circular dichroism measurements (Fig. 7), spore wall dityrosine contains a preponderance of L-configurated α-C-atoms. (The equilibrium mixture should show no circular dichroism signals.) In other words, spore wall dityrosine was mostly in the DL- and LL-form with very small amounts of the DD-form.\(^1\) It would be interesting to know the molecules to which dityrosine is bound in the macromolecule, but only indirect evidence is available. The chromophore of dityrosine is unknown at present. The neuropeptide of frog skin, dermorphin, contains D-alanine (20), is very probably biosynthesized from L-alanine, glutamic acid, and lysine whereas typical bacterial peptidoglycans contain the amino acids glycine, alanine, glutamic acid, and lysine (or diaminopimelic acid, depending on the species).\(^2\)

It has been shown here that about 50% of the spore wall dityrosine is DL-configurated \textit{in vivo}, and we have recently obtained evidence (to be published elsewhere\(^3\)) that D-alanine and D-glutamic acid, which are well known components of bacterial peptidoglycans, do occur in the yeast spore wall. On the other hand, aspartic acid and valine are moderately abundant in the spore wall, are not part of the dityrosine-containing macromolecule (Table I), and exist exclusively in the L-configuration.

(iii) The dityrosine-containing macromolecule is closely associated with a structural polymer consisting of \(\beta(1,4)\)-linked glucosamine residues. This is the chitosan of the second outer layer of the spore wall (3), which is hard to separate from the dityrosine-containing macromolecule and may be covalently bound to it \textit{in vivo}. The corresponding structure in the prokaryotic cell wall is the \(\beta(1,4)\)-linked polymer of N-acetylglycosamine and N-acetylmuramic acid.

However, the structure of the dityrosine-rich macromolecule also exhibits one feature different from a peptidoglycan structure. Dityrosine cross-links have not been found in bacterial cell walls. They have been found in extremely small quantity in a bacterial spore wall, possibly due to an oxidative artifact during workup (17).

It would be interesting to know the molecules to which dityrosine is bound in the macromolecule, but only indirect evidence is available. The chromatophore of dityrosine is unknown at present. The neuropeptide of frog skin, dermorphin, contains D-alanine (20), is very probably biosynthesized from L-alanine, glutamic acid, and lysine whereas typical bacterial peptidoglycans contain the amino acids glycine, alanine, glutamic acid, and lysine.

\(\text{DISCUSSION}\)

The data presented here support a model for the yeast ascospore wall in which the outermost layer is of importance for the resistance of the spore against enzymatic attack. Ultrastructural analysis of wild-type spores shows that this layer is very thin. The iodination experiments indicate that dityrosine is exposed on the surface of the spore. Further evidence that the dityrosine-containing macromolecule is located exclusively in the surface layer of yeast spores was provided by the electron microscopy of spores of the yeast mutant dit102 recently isolated in our laboratory.\(^4\) This mutant produced spores that were almost completely devoid of dityrosine and sensitive to Glusulase yet viable and lacking only the outermost layer of the spore wall. This suggests that only the outermost layer contains dityrosine and confers resistance to Glusulase. The glucosamine auxotroph gcn1 (15) produced spores that lacked both outer layers and were sensitive to Glusulase but were viable. The results of the iodination experiment and of electron microscopy of mutant spores lacking dityrosine show that the dityrosine of yeast spore walls is located in the surface layer.

Experiments with the fluorescent stains fluorescein isothiocyanate (FITC) and primulin (3) show some inferences to be made about the permeability of the surface layer. Both fluorescein isothiocyanate-concanavalin A and primulin showed little reactivity toward intact spores but readily stained spore wall preparations, indicating that the surface layer was impermeable to those molecules in intact spores.

Both the intact surface layer and the soluble, dityrosine-rich macromolecule described here are resistant to the attack of all proteases tested and to the enzyme mixtures Glusulase and zymolyase. Mutant spores that lack only the outermost layer of the spore wall and are devoid of dityrosine are readily lysed by Glusulase or zymolyase. The experiments described here indicate that the molecular structure of the surface layer protects the spores against lytic enzymes. The model suggested by these experimental results is that of a dityrosine-rich, two-dimensionally cross-linked (and therefore dense and insoluble) macromolecule covering the surface of the spore.

What are the chemical composition and structure of this macromolecule? We speculate that the dityrosine-rich macromolecule may be similar in structure to a prokaryotic peptidoglycan. This is based on the following observations.

(i) The amino acid composition, aside from dityrosine, of the purified macromolecule (Table I) is similar to that of a peptidoglycan (16). The macromolecule contains dityrosine, glycine, alanine, glutamic acid, and lysine whereas typical bacterial peptidoglycans contain the amino acids glycine, alanine, glutamic acid, and lysine.

(ii) D-Amino acids do occur in the macromolecule. We have shown here that about 50% of the spore wall dityrosine is DL-configurated \textit{in vivo}, and we have recently obtained evidence (to be published elsewhere\(^3\)) that D-alanine and D-glutamic acid, which are well known components of bacterial peptidoglycans, do occur in the yeast spore wall. On the other hand, aspartic acid and valine are moderately abundant in the spore wall, are not part of the dityrosine-containing macromolecule (Table I), and exist exclusively in the L-configuration.

The dityrosine of the spore wall is not an equilibrium mixture of the two stereoisomeric forms. As shown by the circular dichroism measurements (Fig. 7), spore wall dityrosine contains a preponderance of L-configurated α-C-atoms. (The equilibrium mixture should show no circular dichroism signals.) In other words, spore wall dityrosine was mostly in the DL- and LL-form with very small amounts of the DD-form.
sized as a conventional peptide encoded in the frog genome, and is post-synthetically epimerized to its diastereomeric form containing D-alanine (21). Only this form is biologically active (20). Cyclosporin A of the fungus Tolympodium inflatum and other cyclic depsipeptide antibiotics produced by fungi contain D-amino acids (22). They are biosynthesized nonribosomally like the bacterial D-amino acid-containing antibiotics (23) and the pentapeptides of bacterial peptidoglycan (16). The origin of the D-amino acids in peptidoglycan is due to the action of racemases or transaminases acting on the free L-amino acids (16). The origin of the D-amino acids is unknown in the case of cyclosporin A.

It is an open question as to which of the above biosynthetic possibilities applies to the yeast spore wall structure discussed here. The situation is more complex here than in the other examples of eukaryotic peptides containing D-amino acids because epimerization or racemization of dityrosine (or tyrosine) could occur before, during, or after the oxidative cross-linking of tyrosines to form dityrosine. Natural spore wall dityrosine is not an equilibrium mixture of the two stereoisomeric forms as shown by circular dichroism measurements. Both epimerization of specific positions in the macromolecule and the direct incorporation of D-tyrosine into the corresponding positions of a precursor peptide seem probable. L-Tyrosine is incorporated in vivo into dityrosine, as we have shown earlier (2). If our hypothesis elaborated above is correct, and the yeast spore wall dityrosine-containing macromolecule has a structure similar to a prokaryotic peptidoglycan, then its biosynthesis should also be similar to that of a peptidoglycan. Then we would expect a nonribosomal incorporation of amino acids into a peptidic structure catalyzed by specific enzymes for every amino acid to be added. Both D- and L-amino acids could be incorporated in this way.

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