Prefracture and Cold-fracture Images of Yeast Plasma Membranes

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ABSTRACT Fracture-temperature related differences in the ultrastructure of plasmalemma P faces of freeze-fractured baker's yeast (Saccharomyces cerevisiae) have been observed in high-resolution replicas prepared in freeze-etch systems pumped to 2 x 10⁻⁷ torr in which the specimens were protected from contamination by use of liquid nitrogen-cooled shrouds. Two major P-face images were observed regardless of the source of the yeast, the age of the culture, the growth temperature, the physiological condition, or the suspending medium used: (a) a "cold-fracture image" with many strands closely associated with tubelike particles (essentially the same image as those previously published for yeast freeze-fractured at 77°K), and (b) a "prefracture image" characterized by the presence of more distinct tubelike particles with few or no associated strands (for aging cultures, the image recently referred to as "paracrystalline arrays" of "craterlike particles"). Both types of P-face image can be found in separate areas of single replicas and occasionally even within a single plasma membrane. Whereas portions of replicas known to be fractured at any temperature colder than 218°K reveal only the cold-fracture image, prefracture images are found in cells intentionally fractured at 243°K and in cracks or fissures which develop during the freezing of other specimens. These findings demonstrate that the prefracture image results from the fracturing of specimens at some temperature above 230°K, not from fracturing specimens at some temperature between 173° and 77°K, and not from the use of "starved" yeast cells.

In 1971 McNutt and Weinstein (6) recommended use of the circular depressions which they observed on the "B" faces (herein referred to as E faces [1]) of freeze-fractured baker's yeast, as a "resolution standard" for "freeze-cleave and etch replication techniques." They prepared their specimens in a modified Bullivant type II freeze-cleave device (Ladd Research Industries, Inc., Burlington, VT) (2, 18) and reported that these depressions have been seen only in high-quality replicas. Two years later, complementary stereo electron micrographs of a portion of the plasmalemma of one of many baker's yeast cells freeze-fractured and replicated at 77°K in a modified Denton freeze-etch module (Denton Vacuum Inc., Cherry Hill, N. J.) were published (14). These micrographs revealed the same ringlike depressions on the E face and irregular tubular structures ("donuts") along with numerous strands on the true complementary P face.

More recently, Gross et al. (5) criticized "conventional freeze-etching," and reported that the topographic resolution and complementarity of morphological details of yeast plasmalemma fracture faces can be improved significantly by fracturing the specimens at 77°K and replicating them under ultrahigh vacuum (UHV) conditions (i.e., with the entire vacuum chamber at 2 x 10⁻⁹ torr). They presented micrographs of E faces which compare favorably with those of the earlier reports (6, 14) and of P faces that reveal what they called "paracrystalline arrays" of "craterlike substructure" which are quite different from any previously published. Because they did not note significant improvement in images of specimens prepared at 173°K under UHV conditions, they concluded that "the improvement is achieved primarily by fracturing at lower temperature" and that "UHV conditions are needed only to keep the fracture surfaces clean."

Sleytr and Messner (8) criticized this claim and have demonstrated their ability to obtain electron micrographs of the paracrystalline arrays of craterlike particles on P faces and the ringlike depressions on E faces in their system, in which a
pressure of $2 \times 10^{-7}$ torr was obtained and the fracture faces were protected by liquid nitrogen-cooled shrouds. They reported that, regardless of the fracture temperature in the range of 173° to 77°K, craterlike particles appear on the P faces of "starved" yeast cells whereas "dome-shaped particles" appear on the P faces of "resting" yeast cells, and conclude that the "local vacuum conditions around the specimen," rather than the "complicated UHV technology," are important for the production of high-resolution replicas.

Studies in this laboratory with baker's yeast cultured under controlled conditions led us to believe that the hexagonal arrays of tubelike particles without strands (the paracrystalline arrays of craterlike particles of Gross et al. [5]) appear only on P faces of "prefractured" cells (those fractured when natural cracks or fissures developed during the solidification of water into ice). Consequently, the present study was undertaken to examine the effect of freezing conditions, fracturing temperature, and physiological state of the yeast on the fracture face images obtained. A preliminary report of this work has appeared elsewhere (17).

MATERIALS AND METHODS

Freeze-Etch Systems

Three freeze-etch systems were used in this study: (a) The prototype Denton Vacuum DFE-2 freeze-etch module (12), extensively modified in this laboratory (13, 15, 16) and operated on a Denton DV 503 vacuum system; (b) a current production model Denton DFE-3 freeze-etch module mounted on a Denton DV 502 vacuum system; and (c) a modified Balzers BAF 301 freeze-etch unit (Balzers Corp., Hudson, N. H.) with a Pfeiffer TPH-450 turbomolecular pump (A. Pfeiffer GMBH, Wetzlar, W. Germany) backed by a Balzers type D forepump. Modifications to the Balzers system consisted of: (a) the attachment of a crude copper plate shroud (Fig. 1) fitted to the knife support arm; (b) the use of gold complementary specimen holders (14) with hinges removed, and (c) installation of our modified evaporators (16) with source-to-specimen distance of 7 cm as in the Denton units.

A resistance sensor and monitor, and the evaporating procedure described elsewhere (16) were employed with each unit for controlled deposition of the thickness of Pt shadow films. The darkening of a strip of white paper was used to assess carbon deposition.

Specimens and Preparation Procedures

A culture of baker's yeast (Saccharomyces cerevisiae, ATCC 13264 = NRRL 2439), obtained from Dr. Lekh R. Batra (Mycology Laboratory, Plant Protection Institute, Agricultural Research, Science and Education Administration, U. S. Department of Agriculture, Beltsville, Md.), was maintained in the yeast extract, malt extract (YEME) medium (for yeast) (19) with 0.05 ml/200 ml medium of Dow Corning Antifoam A spray silicone defoamer (Dow Corning Corp., Midland, Mich.) added to prevent foaming. Cultures were grown with aeration at 319°, 299°, and 294°K.

Aliquots of these cultures collected at different stages of growth were concentrated into pellets by low-speed centrifugation and used for each type of experiment. Most of the experiments were repeated using: (a) a thick suspension of Fleischmann's active dry yeast reconstituted by the addition of water; (b) a thick suspension prepared by adding water to Fleischmann's active yeast cake; or (c) the pellet from low-speed centrifugation of a suspension of Fleischmann's active yeast cake (diluted 1/100 [wt/vol] in distilled water) and stirred for 15 min. Small portions of these pellets or thick suspensions were transferred by use of a sharpened applicator stick into the 0.3- by 0.5- by 1.0-mm specimen chamber of regular complementary specimen holders (a of Fig. 18.1c of reference 14), or onto the side of specially constructed holders (Fig. 2). To avoid air drying, each sample for the primary studies was frozen in liquid Freon 22 at 118°K immediately after being placed in the holder. Several specimens were prepared for each freeze-etch operation.

Additional specimens were prepared with the following variations: (a) Some were frozen as small samples, less than one-tenth the usual volume, on the side of the specially constructed holders (Fig. 3); (b) some were frozen by immersion in liquid nitrogen; (c) some were frozen by conduction through the stage of a Denton unit to each of the following temperatures (243°, 233°, 223°, 218°, and 193°K), fractured at these temperatures, then cooled rapidly to below 173°K; (d) some were frozen and fractured at 230°, 233°, 218°, and 213°K in Freon 22, then immediately transferred to Freon 22 at 118°K. Where necessary for the removal of contaminants (variations c and d), the specimens were etched for 2 min at 175°K.

Specimens were fractured at various temperatures, then shadowed and replicated immediately at fracture temperature, or cooled to 77°K for shadowing and replicating. Pressure in the three units was between $2 \times 10^{-7}$ and $2 \times 10^{-5}$ torr during Pt and C evaporations. The liquid nitrogen-cooled shroud was used as a cold trap in all experiments to prevent specimen contamination. Freeze-fracture specimens were shadowed and replicated within 10 s after being fractured, and...
replicas were cleaned in chrome sulfuric acid cleaning solution in the usual manner (14, 16).

Electron micrographs were obtained with a JEOL JEM 100-B transmission electron microscope equipped with a 60° top entry goniometer stage. During operation of the microscope, all cold traps were cooled with liquid nitrogen. Specimen tilt between electron micrographs of stereo pairs was 10°. To provide electron micrographs with black shadows for publication, contact negatives of the original Kodak glass electron image plates were made on medium contrast Kodak projector slide plates. These were then used like any negative to make either contact prints, slides, or enlargements. This transfer can be made easily without excessive increase in contrast or loss of resolution.

RESULTS

Cracks or fissures (Fig. 4), made visible by use of a dissecting microscope, were observed to form as small samples of yeast suspensions froze. Many of the fissures penetrate only part way into the frozen sample, but it is not unusual for them to pass clear through the frozen block. Similar cracks were observed to form in samples frozen within glass capillary tubes (Fig. 5).

Examination of freeze-fracture replicas of specimens that have been frozen in Freon 22 reveals the presence of many cubic crystals on surfaces which have been exposed to the Freon. These crystals appear on unfractured outer surfaces of the frozen sample, on fracture faces of samples which have cross-fractured while in the Freon, and the faces exposed within the above-mentioned cracks or fissures (Fig. 6). These cubic crystals do not appear in replicas of samples frozen in liquid nitrogen and not exposed to Freon 22, nor on samples frozen in Freon 22, then etched for 1 min or more at 175°K.

In unetched freeze-fracture specimens, these cubic crystals serve as convenient markers for the identification of areas which have fractured prematurely (before the time of intentional fracturing). Although fracturing can and occasionally does occur during transfer of the specimens into the freeze-etch unit or during other manipulations within the unit, it can generally be assumed that, when our complementary holders are used and specimens are frozen in Freon 22, areas of freeze-fracture specimens that do not have cubic crystals or other clearly observable contaminants were fractured at the intended specimen fracture temperature.

In more than 200 freeze-fracture preparations which were frozen in Freon 22, hexagonal arrays of tubelike particles with few or no strands (the paracrystalline arrays of craterlike particles of Grosset al. [5]) were found only on P faces of yeast plasma membranes that had cubic crystals (Figs. 6 and 7, bottom of Fig. 8, and Fig. 9). We call these “prefracture images.” Furthermore, areas of the same replicas that do not have cubic crystals invariably reveal many elongated particles (“strands”) closely associated with the tubelike particles (top of Fig. 8, and Fig. 10a) referred to hereafter as “cold-fracture images.” We also find areas with cubic crystals which reveal cold-fracture or intermediate type images. The two major types of P-face image are observed regardless of the yeast culture examined or the intended fracture temperature between 218° and 77°K. The only physiologically related difference which we could detect was that the tubelike particles of P faces and the complementary ringlike depressions of the E faces were well dispersed over the fracture faces of continuous culture cells (Fig. 11) instead of being present in hexagonal arrays as
FIGURE 6 Stereo electron micrographs of prefractured P-face image of cultured yeast plasma membrane, intentionally freeze-fractured at 77°K in the Denton DFE-3 freeze-etch module, revealing the cubic crystals and the hexagonal arrays of tubelike particles found on prefractured surfaces of specimens frozen in Freon 22. X 100,000.

FIGURE 7 Stereo electron micrographs of prefracture P-face image of aging cultured baker's yeast freeze-fractured at 77°K in the modified Denton DFE-2 freeze-etch module. This portion of the prefractured area was selected to avoid the cubic crystals that were clearly visible on surrounding parts. Figs. 7A and 7B are parts of identical stereo images with A printed to show white shadows which make the particles appear craterlike and B printed to show black shadows that reveal the true tubelike nature of the particles. These figures demonstrate the extreme variability in appearance of the individual tubelike particles.

Regardless of the freeze-etch unit used, or the intended fracture temperature, if below 218°K, we find the same two major P-face images. Furthermore, although no telltale cubic crystals are present to mark the prefractured areas these same two P-face images are seen in freeze-fracture specimens frozen in liquid nitrogen.

By using very small samples in specially designed complementary specimen holders (Figs. 2 and 3), we have been able to prepare freeze-fracture replicas of yeast suspensions frozen in Freon 22 without any fractured areas containing cubic crystals. These samples were small enough that a crack or fissure in the fracture zone between the two halves of the holder would most likely continue clear across the specimen and cause the holder to open prematurely in which case the specimen would be discarded. Specimens prepared in this manner reveal only the cold-fracture P-face image if the specimen temperature at the time of fracturing is between 175° and 77°K.

Specimens frozen by conduction to 243° and 233°K then fractured (see Materials and Methods, variation c) revealed areas with the prefracture type image (Figs. 12 and 13, respectively) and samples frozen and fractured at 218°K or colder revealed areas with what appeared to be the cold-fracture type image (Fig. 14). Specimens frozen and fractured in Freon 22 (variation d) at 230°K reveal the same type of prefracture image (Fig. 15a) as well as an intermediate type image (Fig. 15b). Specimens intentionally fractured between 243°K and 175°K had to be etched to remove contaminants. Therefore, the cubic crystals were not present.

Although we did not prepare samples in an unmodified conventional Balzers system for comparison, we were able to...
FIGURE 8 P-face images of cold-fracture and prefracture areas within a single freeze-fractured yeast plasmalemma. Strands on cold-fracture area (A) are restricted to regions where arrays of tubes are located. Hexagonal arrays of tubes without strands are seen in the prefractured area (B) which, in this instance, appears to be at a slightly lower level within the membrane than is the cold-fracture plane. Variability in appearance of particles because of differences in fracture plane, fracture temperature, shadow angle, or other reasons is clearly demonstrated by the appearance of hexagonally arrayed particles in this stereo pair. Tubular particles in cold-fractured areas are usually somewhat obscured by the strands, whereas most of the particles in the upper left corner of the prefractured area are clearly tubular and most of those in the lower central portion of the prefractured zone are domelike. X 38,000.

FIGURE 9 Stereo electron micrographs of prefracture P-face image prepared in a modified Balzers BAF-301 freeze-etch unit. Intended fracture temperature was 103°K. Area was selected to avoid crystals that were present in the surrounding zone.

prepare replicas at 173°, 123°, and 93°K that revealed both prefracture (Fig. 9) and cold-fracture P-face images, and E-face images revealing the ringlike depressions.

DISCUSSION
In contradiction to the reports of Gross et al. (5) and Sleytr and Messner (8), we show that high-resolution replicas of P faces of freeze-fractured yeast plasma membranes of specimen areas known to have been fractured at any temperature between 173° and 77°K reveal the cold-fracture image characterized by the appearance of many strands along with tubular structures (Fig. 10a), similar to that shown in 1973 (14). Furthermore, we present evidence that the more impressive hexagonal arrays of tubular particles with few or no strands
FIGURE 10  Complementary stereo electron micrographs of freeze-fracture images of cultured yeast plasma membrane faces actually fractured at 77°K. (a) P-face image revealing tubular particles in hexagonal arrays with many strands in areas where tubular particles are located. (b) E-face image complementary to a with hexagonal arrays of ringlike depressions. Complementary prints are mounted as though sample were fractured and opened like a book from top to bottom so that positions A, B, and C of the two stereo images are complementary points. The six "substructure" depressions surrounding ringlike depressions are clearly revealed in some areas (lower center) but rough fracturing in other areas has obscured it. × 200,000.

The paracrystalline arrays of craterlike substructure of Gross et al. [5]) appear in specimens that were fractured at a temperature no colder than 243°K (Fig. 12). Because this image appears in areas of specimens that were known to have fractured prematurely during freezing (4), we refer to it as the prefracture image.

In our studies we observed through a dissecting microscope the development of cracks or fissures in practically every frozen specimen examined, including samples frozen in 35% glycerol. Many of these cracks appeared as the suspension solidified into ice. The presence of cubic crystals on a fracture face of freeze-fracture specimens frozen in Freon 22 (Fig. 6) was used as a means of identifying those prefracture areas. Freeze-etch specimens, fractured at known temperatures between 243° and 77°K provided additional information useful to understanding the conditions necessary for production of the two P-face images. Although we have no way of knowing the actual specimen temperature at the time and place where each of these natural fissures occurs, a careful examination of the results of our experiments leads us to believe that they form over a range of temperatures, that the prefracture image is formed only when the fracture actually occurs at some specimen temperature above 230°K, that there is a transition temperature (probably in the range of 233°-218°K) at which intermediate images appear, and that, most likely, any part of a specimen that actually fractures at a temperature of 218°K (Fig. 14) or colder will reveal only cold-fracture images.

Because the specimens of Gross et al. (5) were contained within brass capillary tubes and were held at 173°K for 15 min, there would be little chance that cubic crystals would appear on their replicas even though they were frozen in Freon 22. Furthermore, they would have no way of knowing whether the fracture actually occurred at the intended fracture temperature or as a premature fissure within the capillary, such as those we observed in samples frozen in glass capillaries. Examination of their micrographs leads us to believe that the specimen they intended to fracture at 173°K in their UHV unit (10a of reference 5) was actually fractured at 173°K or prefractured at a temperature below 218°K (it appears to be a cold-fracture image) whereas the specimen which they intended to fracture at 77°K (Fig. 11 a of reference 5) actually fractured within the capillary tube at some temperature above 230°K. (It represents a characteristic prefracture image.)

Because Sleytr and Messner (8) froze their specimens in nitrogen slush, even prematurely fractured areas would not have the tell-tale cubic crystals. Therefore, they would have no way of knowing that their specimens prefractured. Our complementary specimen holders are designed with a loose hinge
which permits the movement of one-half of the holder with respect to the other, to provide a means for expansion and release of tensions as the sample freezes. We have observed that, when the hinges are too tight, many of our specimens upon freezing, prefracture across the space between the two parts of the holder. Because the specimens of Sleytr and Messner (8) were frozen within complementary specimen holders consisting of hollow rivets held together with a modified pair of forceps (9), it is easy to believe that most of their specimens partially or totally fractured during freezing. This might well explain the apparent ease with which they obtained prefracture images. What we cannot explain is how they obtained their hexagonal arrays of “domelike particles” in resting cells. Because we obtained both cold-fracture and prefracture images with all samples examined, we can say with certainty that the prefracture image is not dependent, as they believed, upon the use of starved cells (see also Bullivant et al. [3]). The only difference in the tubelike particles and their complementary ringlike depressions that we can attribute to the physiological state of the yeast is that in continuous culture cells, they are well dispersed (Fig. 11), as reported by Sosinsky (10), whereas in aging cells they are found in the hexagonal arrays irrespective of the specimen temperature when fractured (Figs. 6–10, 12–15).

At this time one cannot be certain why the prefracture and cold-fracture images differ. Nevertheless, we do have a plausible suggestion. Assume that the strands that appear in the cold-fracture P-face images are caused by plastic deformation as reviewed by Sleytr and Robards (7). Assume also that the same plastic deformation occurs at any temperature below the freezing point of the specimen. If these assumptions are true, a simple explanation for the appearance of the two fracture images would be that, at specimen fracture temperatures above 223°K, the plastically deformed molecules are able to “snap back” or return to a more stable condition which may or may not be their original shape, whereas at specimen fracture temperatures...
temperatures below 223°K, the deformed molecules refreeze in the extended position before they can snap back into a more ordered array. Our results are in agreement with those of Sleytr and Messner (8) that UHV conditions as defined by Gross et al. (5) are not necessary for the production of high-resolution replicas and that the important thing is to have a reasonably good vacuum ($10^{-6}$ or $10^{-7}$ Torr) and a zone near the specimen free of

Figure 12. Stereo electron micrographs of P face of yeast plasma membrane intentionally fractured at 243°K. Hexagonal arrays of short tubes and dome-shaped particles without strands are observed. × 200,000.

Figure 13. Stereo electron micrographs of P face of yeast plasma membrane fracture intentionally at 233°K. Even though membrane surface is distorted, the hexagonal arrays of tubular and dome-shaped particles without strands are clearly observed. × 200,000.

Figure 14. Stereo electron micrographs of P-face yeast plasma membrane fractured intentionally at 218°K. The usual cold-fracture image is clearly observed even when the specimen is fractured at this temperature. × 200,000.
condensables. A properly designed liquid nitrogen cold trap between the vacuum pump and the chamber and a liquid nitrogen-cooled shroud, as provided by Steere (11) and used by Sleytr and Messner (8) and for the experiments reported here will serve the purpose. Whereas these conditions are useful in the production of high-resolution replicas, it must be stressed that a relatively high-fracture temperature rather than the high-resolution replicas, is the critical factor in the production of the prefracture P-face images.

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