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Direct imaging of the surfaces of poly(β) hydroxybutyrate and hydroxybutyrate oligomers by atomic force microscopy

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Attempts have been made to image the fold surface of a single crystal of polyhydroxybutyrate (PHB) using the relatively new technique of atomic force microscopy (AFM). To overcome the obscuring of the fold surface by loose loops of polymer and chain ends, two different approaches were used. We first studied the single crystals of an oligomer of 32 HB units, which is known to fold once very tightly within a crystal, using AFM. Secondly, studies were made of single crystals of PHB which have been chemically degraded with methylamine to etch away the "amorphous" layer of loosely folded material, in an attempt to expose the fold surface. The crystals of the 32-mer had a similar morphology to those of the polymer PHB. However, at high magnification, lines of ridges were observed which ran parallel to the crystallographic *b* axis with a spacing of 0.7 nm, similar to the dimensions of the unit cell (0.58, 1.32, and 0.60 nm). It was not possible to differentiate between chain ends and folds. The partially etched PHB crystals maintained enough integrity to permit imaging by AFM, although surface detail could not be resolved on a molecular scale.

1. Introduction

The degree of regularity of folding of the molecular chains in polymer single crystals has been a major issue in polymer science ever since the initial recognition that the chains must fold back into the thin lamellar crystals. Initially two extreme models were proposed: complete regular adjacent re-entry with tight folds; and the 'switchboard' model, where the chains were imagined to re-enter the crystal at random positions. Over the years the models have been refined and limitations on the degree of randomness and regularity imposed. One point of controversy has centred on the amount of tight, adjacent folding that must occur to avoid a "density defect" at the crystal surface [1]. Most authors now agree that there is some minimum fraction of chains which do indeed fold back sharply into the crystal. However, there is still an ongoing discussion as to how regular such folding is, and to the number of adjacent folds which run along a particular crystal face.

The tantalizing possibility of directly imaging the folds has been the focus of renewed interest due to the advent of a new class of microscopy, known as scanning probe microscopy (SPM). SPM is the generic name for a family of ultra-high resolution surface probes yielding three-dimensional images (see, for examples, [2]). One major advantage of SPM is the ability to image at extremely high resolution while operating in gaseous or liquid environments [3]. The atomic force microscope, AFM, is the member of the SPM family which is currently proving most valuable in the study of polymer structures on length scales from tens of micrometres to molecular scale [4]. The AFM can image polymer specimens without the need for staining or etching. The main types of synthetic–polymer specimens that have so far been studied by AFM are solid-state drawn sheets [5–7], melt-drawn [8–10] and solution-spun [11] fibres of semicrystalline polymers, and solution-grown single crystals [12, 13]. The study of fold surfaces of crystals of cyclic alkanes [14] and polyethylene [8] have recently been reported. In the work reported here, we describe our initial studies using AFM directed towards the eventual imaging of the folding in polyhydroxybutyrate (PHB) single crystals.

However, as with the application of any new technique to such a problem, there are many difficulties to be overcome before a definitive image of a fold surface can be observed, and correctly interpreted. To achieve molecular resolution, a flat, ordered crystallographic surface is required. In polymer crystals there will be some parts of the molecules from the loose folds, loops, ends, etc. which will lie on top of the fold surface and obscure the underlying fold structure. Even if the

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folding is predominantly regular and adjacent, a small number of loose loops would be expected. This "amorphous" material would be expected to make imaging of the folds more difficult. Crystallinity data on polymer single crystals suggest that the amorphous layers are typically about 10% of the total crystal thickness, i.e. in the case of PHB single crystals, about 0.6 nm [15,16]. Clearly, if we are to use SPM to examine the underlying folds we will need to penetrate through this "amorphous" layer. One solution would be to etch away this surface layer progressively, removing the looser material and exposing the tighter folds to the microscope. Previous degradation studies have shown that it is possible to degrade single crystals of polyethylene [17] and PHB [18] so that the fold surface and "amorphous" layers are preferentially removed. As an alternative strategy, oligomers which form crystals with thicknesses equal to integral multiples of their length (implying tight folding) are available as model materials [19].

The hydroxybutyrate (HB) oligomer studied here was a sample of a 32-mer, which has been shown to have the same crystallographic structure as PHB and to fold once very tightly within the crystal [19]. Therefore, this 32-mer should provide a good model material for the polymer PHB. In this paper we report our preliminary results which demonstrate that we can image the surfaces of the 32-mer crystal. We show that we can visualize structures due to the chain ends and folds, although we have not been able to distinguish between them. We also present AFM images of the surfaces of PHB single crystals before and after an etching treatment. We have not been able to find any structure relating to molecular folding in either of these preparations.

2. Experimental procedure

2.1. Sample preparation

The oligomers were synthesized as described previously [19]. The 32-mer contained 10% 16-mer, 10% 31-mer, and 80% 32-mer. Owing to the synthesis procedure, it has two protecting end-groups which are different from the usual end groups of PHB (Fig. 1). Crystals were grown in solution in propylene carbonate, by dissolving the oligomer at a concentration of 0.1% (wt/vol), at 110 °C. The solution was then quenched to 0 °C, and left to crystallize for several days. A drop of the crystal suspension was put on to a sheet of freshly cleaved mica, and put under vacuum to evaporate the solvent.

The PHB (M_w 38 000 and polydispersity 2.4) was also crystallized from a 0.1% (wt/vol) propylene carbonate solution. The solution was self-seeded [20] at 105 °C, and crystallized at 60 °C for several days, before hot filtering with fresh solvent. After exchanging the solvent for acetone, a drop of the crystal suspension was put on to freshly cleaved mica. The acetone was evaporated under vacuum.

PHB crystals, grown as above, were degraded by methylamine in aqueous solution (40% wt/wt). 2.5 mg of PHB crystals suspended in 1 ml acetone were added to 2 ml of the methylamine solution and left to degrade for 24 h. Previous studies carried out in Bristol [21] show that degrading single crystals of PHB with methylamine in these concentrations for 24 h removes the amorphous layer and fold surface, leaving a lattice of short chains of PHB behind. Degraded crystals were prepared for AFM in the same way as the undegraded polymer.

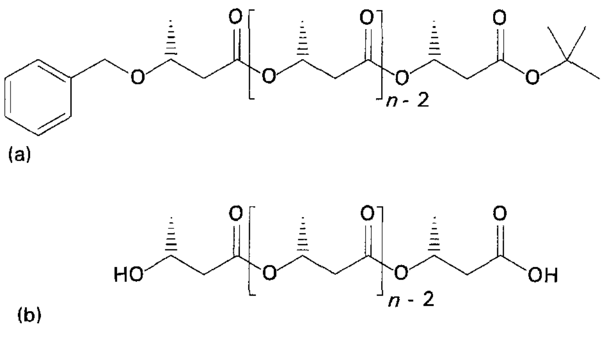

Figure 1 Schematic diagram showing the structure of (a) a protected HB oligomer of *n* monomer units, and (b) an unprotected HB oligomer, also of *n* monomer units.

2.2. Atomic force microscopy

AFM studies were performed using a Nanoscope III with a multimode AFM head (Digital Instruments, Santa Barbara, USA). The instrument was operated in both the contact mode and the "tapping" mode. Contact-mode imaging was performed in both air and in *n*-propanol, which reduces the capillary force between the tip and the sample, allowing a smaller and more stable imaging force of about 1 nN to be used. As its name implies, the tip in the "tapping" mode is in intermittent contact with the surface. Therefore, the lateral forces and potential sample damage that are possible with a cantilever in permanent contact are minimized. The force–distance plot was frequently checked to ensure that the applied force was kept to as low a value as possible. Cantilevers of nominal spring constant 0.06 $\mathrm{N\,m^{-1}}$ and integral pyramidal tips were used. For "tapping" mode imaging, cantilevers had a spring constant of 40 $\mathrm{N\,m^{-1}}$ and images were recorded in air. The D- and J-scan tubes were used, with maximum scan ranges of 12 and 125 μm respectively. Most of the images were recorded at constant force by using feedback control to maintain the tip-sample force at a constant value. For the degraded material, data were obtained by monitoring the variable deflection (and hence variable force) of the cantilever as the sample was scanned at a constant *z*-height. All images were recorded at room temperature.

3. Results and discussion

Fig. 2 shows AFM images of crystals of PHB and the 32-mer oligomer of HB, illustrating morphologies which are elongated "lath-like" crystals, rather similar to crystals seen in transmission electron microscopy (TEM) studies of these samples (Fig. 3). Electron diffraction studies show that the long axis of a PHB crystal is its crystallographic *a* axis [22]. Even at the resolution of these pictures, AFM has the advantage

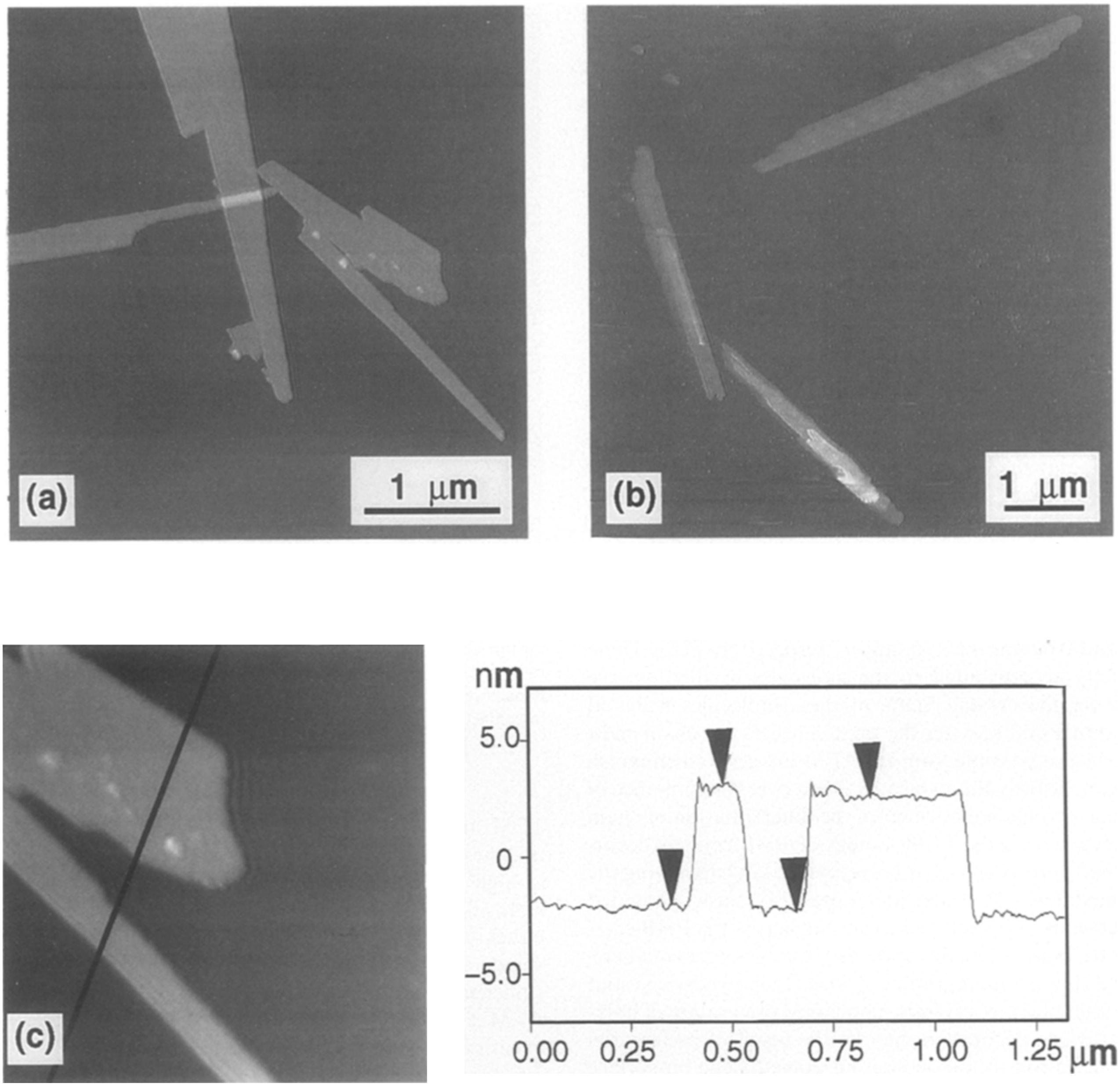

Figure 2 Low-magnification AFM images of PHB and 32-mer crystals. (a) Tapping mode, constant force image of PHB. A 3.9µm × 3.9 µm scan area with a black to white height range of 30 nm. (b) Contact mode constant force image of 32-mer crystals. Height range is 60 nm. Evidence of multilayering of the crystals is seen in this image. (c) Line profile data of part of (a). Note the sharp edges and flat tops of the crystals.

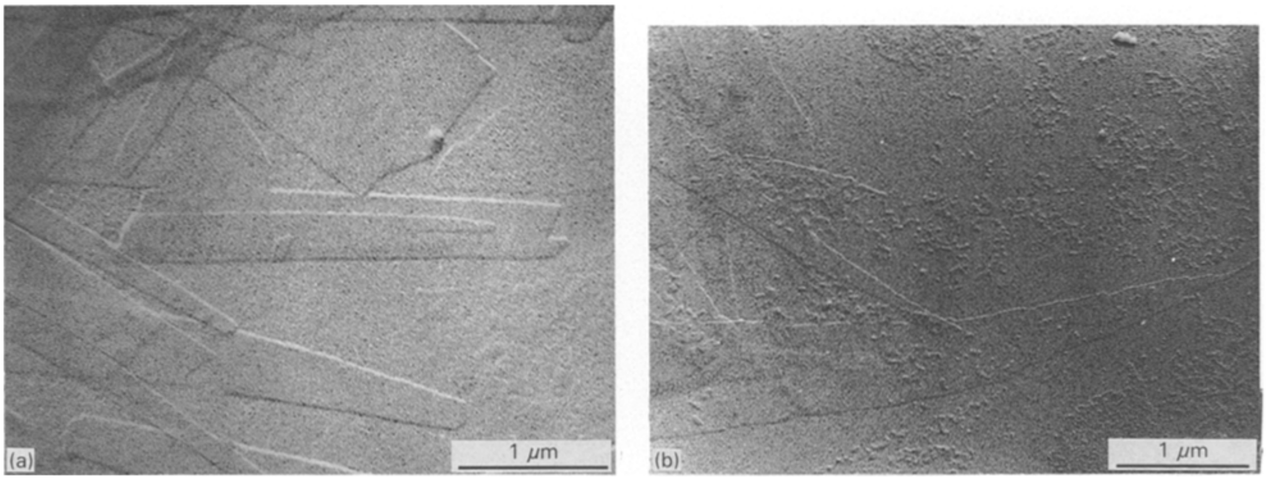

Figure 3 Transmission electron micrographs showing images of crystals of (a) PHB, and (b) 32-HB.

over TEM of giving direct information about the height of the crystals. Typical measurements of the thicknesses of crystals yielded values of 5.0 nm for the PHB (see Fig. 2c) and 5.5 nm for the 32-mer. These data compare well with the long periods obtained from small angle X-ray scattering (SAXS), 4.9 and 5.2 nm respectively. The thickness of the oligomer crystals is equivalent to the length of 16 monomers (assuming the same helical conformation as a PHB chain). This indicates that the chains fold back on themselves adopting a hairpin conformation [19]. 16 monomer HB units correspond to an approximate length of 4.8 nm, and the end groups are approximately 0.2 nm long.

On inspecting the surfaces of the crystals at higher magnification, Fig. 4, repeating lines of spacing 0.7 ± 0.1 nm can be seen, across the width of the oligomer crystals parallel to the crystallographic *b*-axis. This feature has not been observed as yet on the PHB crystals. Perhaps it is not surprising that fine detail on the polymer crystals is not observed because a relatively small amount of mobile amorphous material and loose folds on the surface of the crystals could prevent the imaging of any ordered material. The ridges which are seen in the oligomer have a spacing which is similar to the unit cell dimensions: 0.58, 1.32 and 0.60 nm for *a*, *b*, and *c*, respectively [23]. These may be attributed to the molecules as they emerge from the crystal. Some of these molecules will fold tightly and re-enter the crystal, others are chain ends; it is not possible from the AFM images to distinguish conclusively the two cases. Moreover, the presence of end groups might obscure the differentiation of chain ends and folds. TEM studies of PHB crystals decorated with evaporated polyethylene [24], following the method of Wittman and Lotz [25], show elongated crystals of polyethylene running across the PHB crystals, in the same direction as the ridges observed here, i.e. the crystallographic *b* axis. In polyethylene and other polymer crystals, a preferred orientation of polyethylene decoration may be the result of preferential nucleation of the decorating polyethylene on surface features on the crystal caused by tight folds, e.g. epitaxy in troughs between parallel rows of folds. For these samples we appear to have a direct image by AFM of features which can only be inferred indirectly by TEM studies.

Fig. 5 shows PHB crystals which have been etched with methylamine. Generally the crystals have less well defined boundaries than unetched crystals. The edges and top surfaces of degraded crystals are much less clearly delineated than undegraded crystals. This can be seen in comparison of line profile data for degraded and undegraded crystals, Figs 5b and 2c, respectively. The crystal shown in Fig. 5a has shallow pits where some of the fold surface has been removed. It would appear that degradation occurs preferentially in the centre of the crystals, although the mechanism is as yet unclear. The holes vary in outline shape, lateral dimensions and depth; the hole in Fig. 5b is 1.2 nm deep.

4. Conclusions

We have successfully imaged crystals of both PHB and an oligomer of 32 HB units. These images show the morphology of the crystals, confirming results of TEM and SAXS on the lateral growth habits and thicknesses. On the surface of crystals of the oligomer at high magnification, the tight folding of the oligomers allows us to image a repeating structure due to either chain folds or chain ends. Finally, we have shown that partially etched PHB crystals can retain sufficient mechanical integrity to permit imaging by AFM. Future work will attempt to conduct a series of experiments examining the AFM images as the etching progressively removes more of the "amorphous" layers, to reveal the degree of regularity in the underlying tight folds.

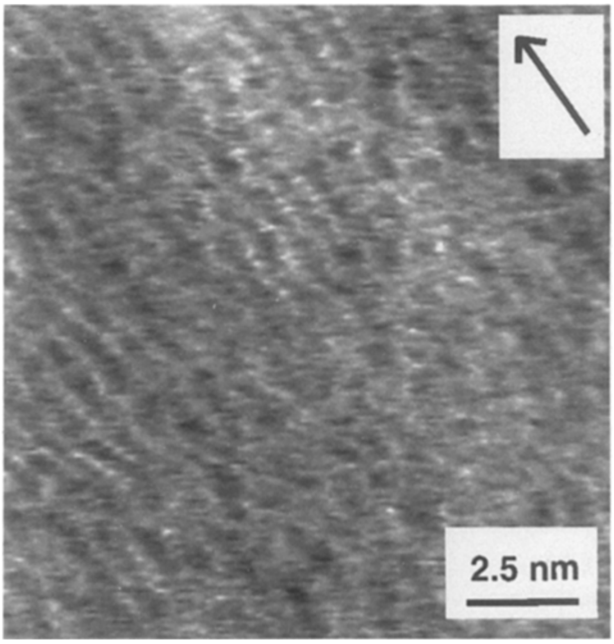

Figure 4 High-magnification image of the surface of a 32-mer crystal. The height of the ridges, which are 0.7 nm apart and run from the top left to the bottom right of the micrograph, is in the range 0.3 to 0.6 nm.The arrow represents the crystallographic *b* axis (determined from a lower magnification image). The image was obtained in contact mode at a force of 1 nN.

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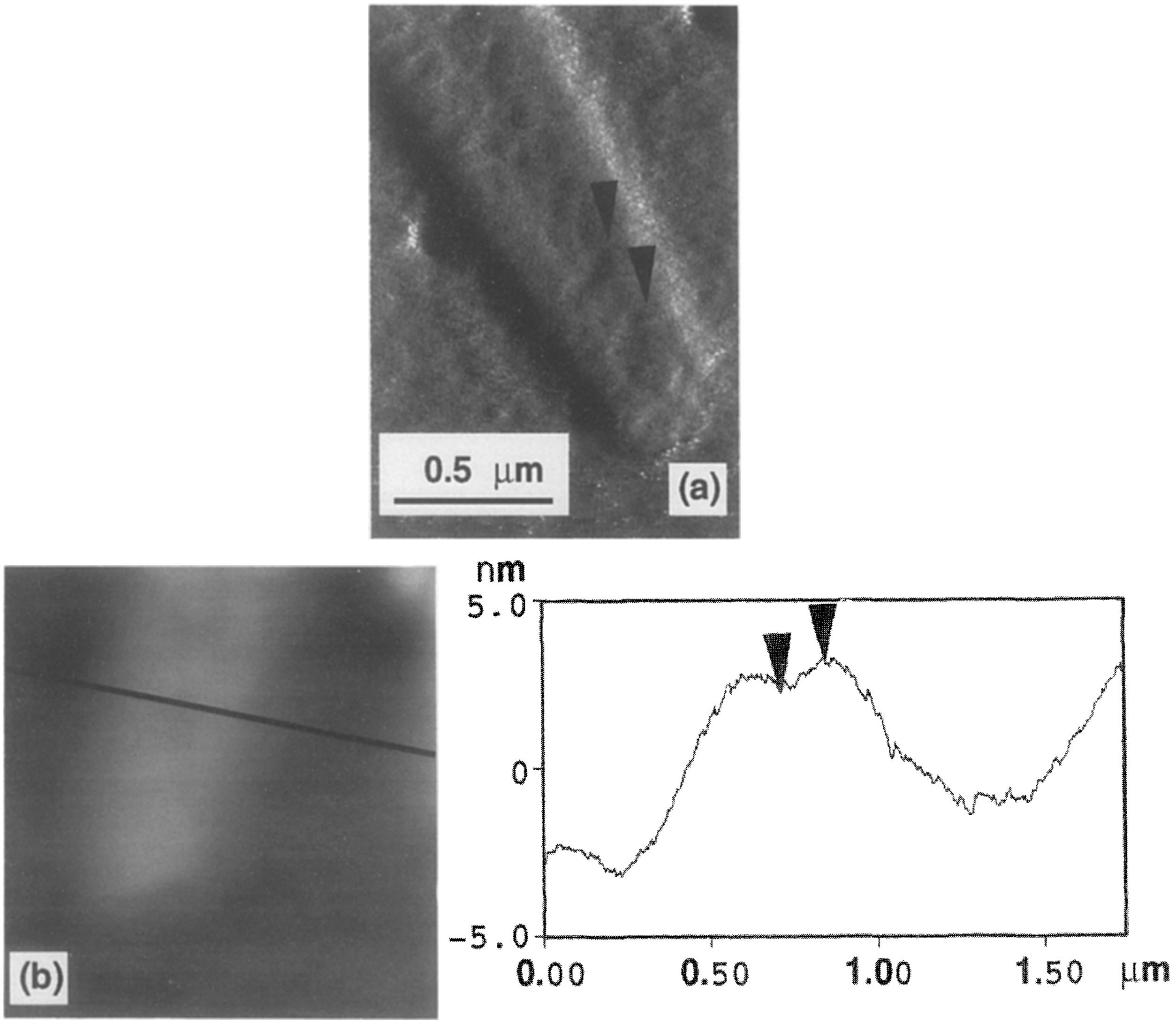

Figure 5 AFM images of degraded crystals of PHB. (a) Tapping mode, deflection image of a methylamine-degraded PHB crystal. The arrows indicate two of the more prominent holes on the crystal surface. (The dark 'trough' on the left side of the crystal is a result of the scanning process, and not a real depression.) (b) Line profile across another degraded PHB crystal. The height difference between the arrows is 1.2 nm. Note the rounded edges of the crystal profile in contrast to the undegraded crystal (Fig. 2c).

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