Direct Observation of Phosphorylase Kinase and Phosphorylase *b* by Scanning Tunneling Microscopy†

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ABSTRACT: The molecular structures of phosphorylase *b* and phosphorylase kinase have been visualized by scanning tunneling microscopy (STM). STM is a near-field technique that can resolve structures at the nanometer level and thus can image individual molecules. Phosphorylase *b* can be seen in dimeric and tetrameric forms as well as linear and globular aggregates. The linear arrays consist of side by side dimers with the long axis of the dimer perpendicular to the aggregated chain. Individual molecules of phosphorylase kinase appear to be planar, bilobate structures with a 2-fold axis of symmetry and a central depression.

Scanning tunneling microscopy (STM) allows direct visualization of individual molecules of phosphorylase kinase and its substrate glycogen phosphorylase. Phosphorylase kinase is a large multisubunit enzyme that catalyzes the phosphorylation of glycogen phosphorylase *b* to form the active species phosphorylase *a*. The enzyme used in these studies was isolated from rabbit muscle and has an M_r of 1.3×10^6. There are four types of subunits, each occurring in four copies—$(\alpha\beta\gamma\delta)_4$ (Pickett-Gies & Walsh, 1986). It has been established that the α (M_r 145 000) and β (M_r 128 000) subunits have a regulatory role, activating the enzyme when they have been phosphorylated by cAMP-dependent protein kinase (Pickett-Gies & Walsh, 1985). The γ subunits (M_r 45 000) contain the catalytic sites (Paudel & Carlson, 1987), and the δ subunits are calmodulin (M_r 17 000), each capable of binding 4 Ca^{2+}. Attempts to crystallize phosphorylase kinase have thus far been unsuccessful. Electron microscopy and chemical cross-linking studies of the molecule indicate that it is a dimer of octamers $(\alpha_2\beta_2\gamma_2\delta_2)_2$ (Trempe et al., 1986; Fitzgerald & Carlson, 1984). Electron micrographs of freeze-dried, shadowed phosphorylase kinase have displayed a variety of bilobate structures that have been described as chalices or butterflies (Cohen, 1978; Schramm & Jennissen, 1985; Trempe et al., 1986).

Glycogen phosphorylase is the muscle enzyme responsible for converting the glucose residues stored in glycogen into the metabolically active form, glucose 1-phosphate (Madsen, 1986). The enzyme can occur as dimers or tetramers of identical subunits, each having a molecular weight of 97 400. More highly aggregated forms also are known to exist (Chignell et al., 1968). Phosphorylase *b* is easily crystallized and was an early candidate for X-ray crystallography. Its molecular structure has been resolved to 1.9 Å with unit cell dimensions of 128.5 Å by 116.3 Å (Sprang et al., 1988). The dimer has been reported to have the molecular dimensions 11.0 × 6.5 × 5.5 nm by electron microscopy (Chignell et al., 1968) and X-ray scattering (Puchwein et al., 1970).

In scanning tunneling microscopy, a sample placed on an atomically flat, conductive surface (pyrolytic graphite) is scanned by a Pt/Ir wire probe attached to a computer-driven ceramic piezoelectric cylinder (Hansma & Tersoff, 1987; Zasadzinski, 1989). The computer rasters the wire probe across the surface in the x–y plane. The distance between the probe tip and the substrate (0.2–2.0 nm) is set by a bias potential (<200 mV), which results in a tunneling current (<1 nA). As an object on the surface is encountered, an image is generated on the basis of the distance the probe must be moved in the z direction to maintain a constant tunneling current. Although the theoretical basis for imaging biological materials by STM is not well understood, resolution of structures as small as 2 nm is possible (Beebe et al., 1989).

Experimental Procedures

Phosphorylase kinase (Cohen, 1973) and phosphorylase *b* (Fischer & Krebs, 1959) were prepared from rabbit muscle and dialyzed versus buffer containing 10 mM imidazole, pH 7, 100 μM $CaCl_2$, and 100 μM $MgCl_2$ or 50 mM HEPES, pH 7, 20 mM NaCl, and 0.1 mM EDTA. The final concentration of each protein solution was adjusted to 1 mg/mL, two applications of 0.5 μL each were applied to the graphite substrates, and the solutions were dried under a stream of nitrogen for a few minutes.

STM was performed with a Nanoscope II (Digital Instruments Inc., Santa Barbara, CA). All the data manipulations and image processing were carried out with the Digital Instruments software. The instrument settings are listed with the figures.

Results

A typical image of phosphorylase kinase is shown in Figure 1A. Many scans were performed with both samples in both buffers, and in each case, the butterfly pattern shown here was observed as the predominant form of the unaggregated enzyme. The largest dimension across the wing tips is 23.7 ± 3.2 nm (n = 15), and at the narrowest point the wings are 12.1 ± 1.4 nm (n = 15). The length of a wing (parallel to the edge) is 21.3 ± 2.0 nm (n = 20), and the bridge between the wings is 15.0 ± 1.0 nm wide (n = 15). The thickness of the molecule was measured to be 1.43 ± 0.03 nm (n = 9) on the basis of the calculated average displacement of the probe tip from the graphite while the molecule was scanned. In addition to in-

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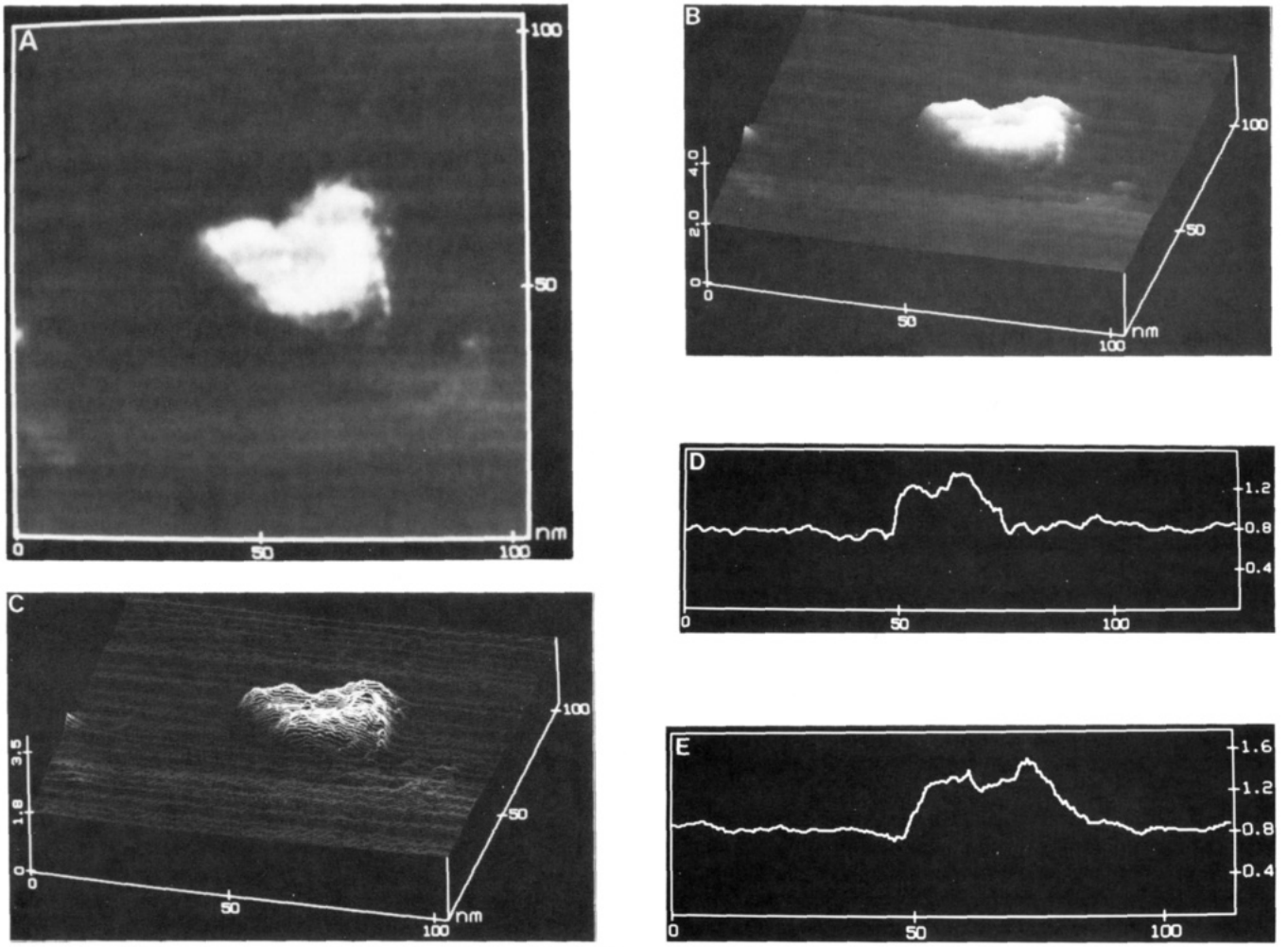

FIGURE 1: (A) Top view scan of a phosphorylase kinase molecule. Increasing brightness corresponds to elevation of the protein surface above the graphite. STM was carried out at an 80–120-mV bias and set points of 0.4–0.6 nA. The Nanoscope II (Digital Instruments, Inc., Santa Barbara, CA) was used with the D head and a Pt/Ir (80/20) wire as the probe. All dimensions in figures are in nanometers. (B) Computer-constructed three-dimensional surface of the molecule shown in (A). (C) Line scan of the same image that allows easier visualization of the central depression. (D) Cross section through the phosphorylase kinase molecule perpendicular to the apparent plane of symmetry. (E) Cross section along the plane of symmetry between the two lobes.

dividual molecules, aggregates could also be observed as well as fragments that appeared to be the size of half-molecules (octamers). It is possible that the fragmentation was spontaneous or it may be an artifact of mechanical or electrical forces in the scanning process. There was no evidence of the chalice form of phosphorylase kinase described in an electron microscopy study of the molecule (Schramm & Jennissen, 1985).

A computer-processed view of the same molecule is shown in Figure 1B. A smoothed three-dimensional image is presented. The central portion of the molecule between the lobes appears to be depressed with respect to the outer areas. This depression is especially evident in the line scan of the image seen in Figure 1C. A cross section of the molecule perpendicular to the midline is shown in Figure 1D. The section along the midline is shown in Figure 1E. As discussed below, the absolute thickness of the molecule is not accurately described by these measurements. Nevertheless, the relative heights shown here should be proportional to absolute thickness.

Glycogen phosphorylase molecules in two aggregation states are shown in Figure 2. The tetramer in Figure 2A is made up of two dimers, each of them 11 nm long and 5.3 nm wide. In addition to the tetramers, long chains of phosphorylase *b* molecules were observed. A portion of one 710 nm long chain is shown in Figure 2B (top view), and in Figure 2C a three-dimensional surface view is shown. The width of the chain is about 11 nm (between the arrows in Figure 2F), and it has a periodic pattern along the chain shown in the cross-section view of Figure 2D. Figure 2E is a Fourier transform of the pattern in Figure 2D and shows a repeat distance of 5.67 ± 0.33 nm. In addition to that periodicity, examination of Figure 2A or Figure 2B indicates an alternating pattern where every other dimer appears to be oriented differently than its adjacent partners.

Discussion

The images presented here demonstrate that scanning tunneling microscopy is capable of imaging protein molecules at a level of resolution better than previously obtained by electron microscopy. Details of protein shape and subunit organization are clearly discernible.

The photographs of phosphorylase kinase show that it has the bilobar structure reminiscent of the butterfly images from electron microscopy (Trempe et al., 1986). The protein is clearly thicker at the edges of the lobes than in the central region. The central depression may correspond to the space between the "bridges" seen in electron micrographs (Trempe et al., 1986). In the STM images, there is clearly material in that space. The size of the molecules in this study (24 × 21 nm) is comparable to that found in electron microscope studies. The STM images confirm the plane of symmetry between the lobes, indicating that the holoenzyme is indeed

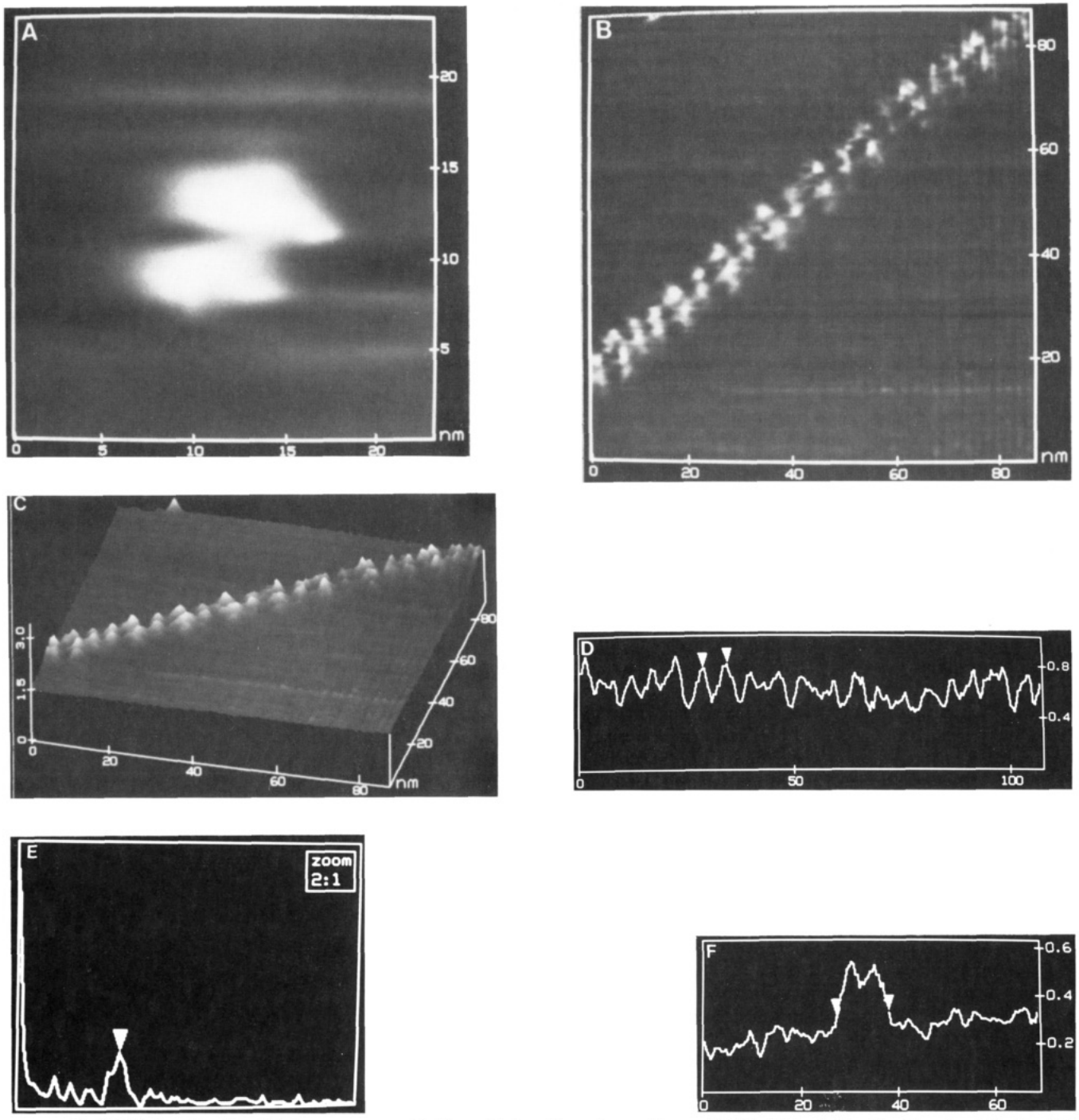

FIGURE 2: (A) A phosphorylase *b* tetramer composed of two side by side coplanar dimers. (B) A chain of phosphorylase *b* molecules. (C) Three-dimensional surface view of the chain. (D) Cross section through the chain, parallel to the long axis of the polymerized phosphorylase *b*. (E) Fourier transform of the curve in (D) showing the major periodicity at 5.67 nm. (F) Cross section perpendicular to the chain through the fifth dimer from the left in (B) and (C).

a dimer of octamers. However, the images invariably show an asymmetry in which there is always a narrow end and a wide end to the butterfly image. This asymmetry of the hexadecamer requires that the octamer must have at least one axis of asymmetry.

The thickness measurements of phosphorylase kinase gave values of only one-fourth to one-half of what we would expect for a protein of its molecular weight, but they are consistent with height measurements obtained for DNA (Lee et al., 1989). While distance measurements in the x–y plane are based on displacement of the probe, height measurements involve a complex combination of physical distances and surface work functions, i.e., the energy required to remove an electron from the surface. Since the substrate, graphite, and the adsorbed protein have different electronic work functions, the measured vertical distance will not in general coincide with the physical distances.

The images of phosphorylase *b* are entirely consistent with the measurements based on X-ray crystallography, X-ray scattering, and electron microscopy. It is clear that the short chains of phosphorylase seen in electron micrographs (Chignell et al., 1968) can extend to a length of several hundred dimeric units. Whether this is a natural state for phosphorylase or is a result of condensation on the graphite surface is not known.

This study provides the most detailed STM images of proteins yet obtained. The ability to image protein molecules

such as phosphorylase kinase and phosphorylase *b* and its aggregates suggests that near-field techniques such as STM can make a major contribution to protein chemistry. STM techniques have resolved individual atoms in crystalline structures under optimal conditions. It is possible that they could be used for imaging multiprotein complexes and enzyme–substrate complexes.

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Registry No. Phosphorylase *b*, 9012-69-5; phosphorylase kinase, 9001-88-1.

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Modeling with in Vitro Kinetic Parameters for the Elaboration of Transfer RNA Identity in Vivo†

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ABSTRACT: A tRNA with "double identity" was created, and this tRNA was demonstrated in vitro to aminoacylate quantitatively with either of two amino acids. In contrast, acceptance of only one of these amino acids was observed in vivo, and a simple manipulation determined which one was accepted. Kinetic parameters were obtained for aminoacylation with each amino acid of the tRNA with double identity and of related tRNAs. Modeling with these parameters largely explains which amino acid specificity is observed in vivo. The results delineate some of the kinetic boundaries for the design and accommodation of tRNA sequence variations in the elaboration of identity in vivo.

The identity of a tRNA is established by its interaction with the cognate aminoacyl-tRNA synthetase (Schimmel & Soll, 1979; Schimmel, 1987). Investigations of the nucleotide determinants that are responsible for identity have been carried out either in vivo or in vitro (Schulman & Abelson, 1988; Yarus, 1988; Schimmel, 1989). A fundamental difference between the two approaches is the environment in which the specificity of a tRNA is established. The specificity of aminoacylation in vitro is evaluated on the basis of kinetic parameters of a tRNA substrate with a specific enzyme (Schulman & Pelka, 1985, 1988; Park et al., 1989; Sampson et al., 1989). Misacylations have been observed in vitro, although the overall catalytic efficiency of aminacylation, expressed as the ratio (k_{cat}/K_m) of the catalytic rate constant k_{cat} to the Michaelis constant K_m for tRNA, is greater for the cognate tRNA than for the noncognate tRNA by a factor of 10^4 or more (Schimmel & Soll, 1979; Schimmel, 1987).

For studies in vivo, variants of an amber-reading tRNA suppressor are created so as to alter the amino acid that is inserted at an amber codon (Normanly et al., 1986a; Hou & Schimmel, 1988; McClain & Foss, 1988a,b; McClain et al., 1988; Roger & Soll, 1988). This system examines the identity of a tRNA in the context of 20 aminoacyl-tRNA synthetases, each of which can compete for the same tRNA substrate. The relative amounts of synthetases and tRNAs have been shown to be important for the specificity of aminoacylation in some cases (Yarus, 1972; Hoben et al., 1984; Yarus et al., 1986; Swanson et al., 1988). In addition, the aminoacylated tRNAs are rapidly sequestered by the abundant elongation factor EF-Tu so that the degree to which editing can be achieved by a specific enzyme may be limited. Recent work suggested that editing may have a role in the overall recognition process in vivo (Hou & Schimmel, 1988).

Both approaches have demonstrated that a set of individual nucleotides can be transferred from one tRNA to another and thereby confer upon it the identity of the "donor" tRNA.

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