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Chimeric Human–Simian Anti-CD4 Antibodies Form Crystalline High Symmetry Particles

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A chimeric human–simian IgG, antigen specific for CD4, when exposed to 0.5 M $SO_4^=$ containing 0.4% polyethylene glycol or Jeffamine, self-assembles into discreet, roughly spherical particles 23 nm in diameter. Increasing $SO_4^=$ to 1.55 M induces the IgG particles to crystallize in either a hexagonal or a monoclinic form. From X-ray diffraction, the former crystal is of space group P622, with one IgG particle in the unit cell; thus the particle itself must have 622 point group symmetry. Both crystal forms have been imaged using atomic force microscopy. Detailed features of the duodecamer were evident, including the symmetry and a large solvent channel along the sixfold axis. The particles in some ways resemble the hexameric IgG aggregates believed to activate compliment upon antigen binding and, therefore, may have physiological relevance. Investigation of seven other IgGs of diverse origins and subclasses indicates that many, if not most, IgGs form similar particles. To our knowledge, this is the first observation of the assembly of IgG into high symmetry aggregates in the absence of antigen or their crystallization. © 2000 Academic Press

Key Words: X-ray diffraction; atomic force microscopy; light scattering; immunoglobulin; compliment.

INTRODUCTION

The monoclonal antibody clenoliximab, designated IDEC 151, is produced by IDEC Pharmaceutical Company and has been approved for clinical use in patients with rheumatoid arthritis. The antigen is CD4 on T lymphocytes, and its properties have been reviewed elsewhere, (Newman *et al.,* 1992; Newman and Ryskamp, 1995). IDEC 151 is of isotype IgG4/λ and is a primatized human immunoglobulin. The variable regions of both the heavy and light chains are derived from a monkey, while the remainders of both the light and heavy chains are of human origin (Anderson *et al.,* 1997). One particular variant of this antibody is a double mutant in which

serine 241 has been replaced by proline and leucine 248 is substituted by glutamic acid. The purpose of the mutations was to reduce the number of half molecules produced because of intrachain disulfide formation in the hinge between C239 and C242. This mutated antibody is known as IDEC 151 and is the molecule described here.

A number of intact antibodies, both myeloma proteins and monoclonal immunoglobulins, have been crystallized for X-ray diffraction analysis and this work has been reviewed elsewhere (Harris *et al.,* 1999). Both concentrated salt conditions and low ionic strength conditions in the presence of PEG have seen use. The myeloma proteins which have been crystallized are, of course, all of human origin, but both of the monoclonal antibodies whose structures have been solved were murine, one an IgG2a (Harris *et al.,* 1997) and the other an IgG1 (Harris *et al.,* 1998). No human, or simian, intact monoclonal antibody and no chimeric antibody has, to our knowledge, been reported crystallized.

In the course of identifying crystallization conditions for a number of intact monoclonal antibodies for X-ray structure determination (McPherson, 1982, 1998), some unusually productive conditions were encountered involving $LiSO_4$ plus the presence of small amounts of polyethylene glycol. As part of the optimization regimen an investigation was carried out using quasi-elastic light scattering (QELS) to monitor IgG aggregation as a function of $LiSO_4$ and polymer concentration. Results with one antibody were particularly noteworthy, and this was the chimeric human–simian IgG designated IDEC 151.

MATERIALS AND METHODS

IDEC 151 antibody against CD4 was provided at 5 mg/ml in DPBS by IDEC Pharmaceutical (La Jolla, CA). Other antibodies examined by QELS and on which crystallization was attempted were supplied in DPBS by the sources indicated in Table I. The antibody was dialyzed for 24 h at room temperature against several changes of distilled water and concentrated to 10 mg/ml

TABLE I

Antibodies Used and Their Sources

Antibody	Source	Organism	Subclass	Antigen/target
Clenoliximab	IDEC Pharmaceutical Co. (La Jolla, CA)	Human–simian	IgG4/λ	CD4 on human lymphocytes
Rituxan	IDEC Pharmaceutical Co. (La Jolla, CA)	Mouse–human	IgG1/κ	CD20 on human lymphocytes
CE9.1	IDEC Pharmaceutical Co. (La Jolla, CA)	Human–simian	IgG1/λ	CD4 on human lymphocytes
2B8	IDEC Pharmaceutical Co. (La Jolla, CA)	Mouse	IgG1/κ	CD20 on human lymphocytes
T84.66	City of Hope Hospital (Duarte, CA)	Human–mouse	IgG1/κ	Carcinoembryonic antigen (CEA) on human carcinoma cells
Mab 231	Synbiotics Pharmaceutical Co. (San Diego, CA)	Mouse	IgG2a/κ	Canine lymphoma cells
Mab 61.1.3	QED Pharmaceutical Co. (San Diego, CA)	Mouse	IgG1/κ	Phenobarbital
(Unnamed)	I. Sandlie, T. E. Michaelson (Univ. of Oslo and Natl. Inst. of Public Health Oslo, Norway)	Human	IgG4/κ	3-Iodo-4-hydroxy-5-nitrophenylacetate (NIP)

in Centricon concentrators (Millipore Co., Bedford MA). Jeffamine ED2000 was from Hampton Research (Laguna Niguel, CA) and polyethylene glycol 4000 from Fluka.

QELS. QELS measurements were carried out using a Malvern 4700c submicroparticle analyzer (Malvern Instruments, Inc., Southborough, MA) as described for earlier QELS investigations of the crystallization of viruses and proteins (Malkin and McPherson, 1994; Malkin *et al.,* 1993). Before any experiments were conducted all solutions were centrifuged for 10 min at 12 000g and passed through 0.22-μm syringe filters (Millipore Co.) to remove foreign particles or aggregates. Samples analyzed by QELS had total volumes of 75 μl in square microcuvettes (Starna Cells, Inc., Atascadero, CA). The sample cell was maintained at the center of a glass vat containing double distilled water that was continuously recirculated through a 0.22-μm filter. The cell temperature was maintained at 22° ± 0.1°C. The concentration of antibody in samples was 5 mg/ml.

The photomultiplier was positioned at 90° to the incident laser beam throughout the studies (Innova 70-3, 1-W argon laser, Coherent Laser Products Division, Palo Alto, CA). The digital signal from the photomultiplier was processed through a 256-channel correlator and passed to the computer for analysis. Light scattering measurements were made in photon correlation mode. The theory, technique, and methods of determination of particle size distribution from the analysis of the autocorrelation function have been described elsewhere (Berne and Pecora, 1976). In practice, this technique permits calculation of the translational diffusion coefficient of particles undergoing Brownian motion. If particles are assumed to have spherical shapes, essentially true in this study, the hydrodynamic radius, r_h, can be calculated using the Stokes-Einstein equation.

AFM. Samples verified by QELS to contain antibody particles 23 nm in diameter were adsorbed onto freshly cleaved mica and examined both in air and in their mother liquor in sealed fluid cells. Crystals, grown *ex situ,* were transferred to sealed fluid cells of about 60 μl in volume, and the crystals were fixed to the glass substrate by pinning them beneath fine glass fibers and examined under solutions identical to their mother liquors, including antibody. All operations were carried out at 22°C on more than a dozen antibody crystals. The AFM instrument was a Digital Nanoscope E (Digital Instruments, Santa Barbara, CA) and images were collected in tapping mode using silicon nitride oxide sharpened tips. Most procedures were those described in detail in earlier work on the crystallization of macromolecules as studied by AFM (Kuznetsov *et al.,* 1997, 1999; Malkin *et al.,* 1999).

Crystallization. Antibody particles were crystallized using the vapor diffusion technique in Cryschem plastic trays (Hampton Research), with 12-μl protein mother liquor droplets and 0.6-ml reservoirs. Mother liquor and crystallization conditions are specified in the legend of Fig. 2. The particular crystal form obtained, otherwise from the same initial conditions, was determined by the temperature of the crystallization experiment and the reservoir LiSO$_4$ concentration. Crystals of the monoclinic form have also been grown by substituting 2.2 M ammonium sulfate in place of the 1.75 M LiSO$_4$. Crystals initially appear after about 10 days and grow over several weeks.

X-ray diffraction. Both hexagonal and monoclinic crystal forms were examined preliminarily using an *R*-axis detector with a Rigaku RU-200 rotating anode source (Molecular Structures Corp., Houston, TX). Images were recorded at both 19°C using conventional capillary mounts (McPherson, 1982) and −173°C, the latter after replacement of the crystal mother liquor by peritone oil (Hampton Research). Freezing was not observed to have any substantial effect on the extent of the diffraction pattern, nor the mosaicity, but did very significantly prolong the crystals' lifetime in the X-ray beam.

The crystals, under cryogenic conditions (Garman and Schneider, 1997), were subsequently examined using the highly intense, focused beam at the Advanced Light Source at the Lawrence Berkeley Laboratories (Berkeley, CA). Following determination of the unit cell parameters and symmetry with the aid of the program DENZO (Otwinowski and Minor, 1997), a complete set of diffraction data over 180° of reciprocal space was collected and processed on the monoclinic crystal form. The data set contains 5460 independent reflections.

RESULTS

As shown in Fig. 1, when the antibody IDEC 151 is titrated with LiSO$_4$ (or (NH$_4$)$_2$SO$_4$) in the presence of 0.4% Jeffamine ED2000, at 0.4 to 0.6 M salt a transition in size distribution occurs. The monomeric IgG, initially yielding a single discreet intensity peak corresponding to a hydrodynamic diameter of about 9 nm, begins aggregating to yield a second peak corresponding to a diameter of 23 nm. A peak at about 10 to 16 nm is also observed during the experiment but is transient and ultimately disappears. Presumably it represents some assembly intermediate. Eventually all of the IgG is consumed into the specie at 23 nm diameter, which remains stable over time, forming no significant amount of higher aggregates. Combination of either the polymer or the salt alone with the antibody produces no
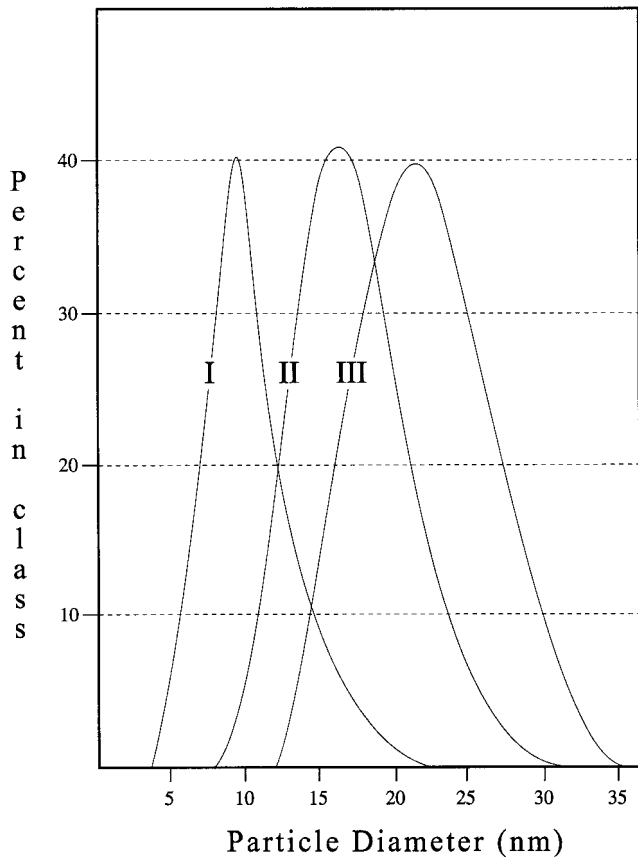
FIG. 1. Quasi-elastic light scattering spectra were recorded using a Malvern 4700c submicroparticle analyzer by procedures described under Materials and Methods. Ten independent measurements of 1 min each were made at 22°C, under each of eight conditions, on 75-μl samples in a quartz cuvette. The conditions were (1) 5 mg/ml IDEC 151 Ab in DPBS, (2) Ab plus 0.20% Jeffamine ED 2000, (3) Ab plus 0.40% Jeffamine, (4) Ab plus 0.40% Jeffamine, 0.2 M LiSO$_4$, (5) Ab plus 0.40% Jeffamine, 0.4 M LiSO$_4$, (6) Ab plus 0.40% Jeffamine, 0.6 M LiSO$_4$, (7) Ab plus 0.40% Jeffamine, 0.8 M LiSO$_4$, (8) the previous condition after an additional 4 h. Distribution I was produced by the antibody alone, condition (1), and did not change for conditions (2) through (4). The polymer alone, therefore, did not induce aggregation, even in the presence of 0.2 M LiSO$_4$. At 0.4 M LiSO$_4$, condition (5), the peak broadened and began shifting slowly to the right, implying that aggregation was taking place. Distribution II was recorded under condition (6), where the LiSO$_4$ concentration had been raised to 0.6 M, but ultimately Distribution III was produced. Increase of LiSO$_4$ to 0.8 M concentration immediately produced Distribution III. After 4 additional h, condition (8), Distribution III was unchanged, indicating that the 23-nm-diameter particle produced by the Jeffamine plus LiSO$_4$ was stable. These experiments were reproduced, with essentially the same results, for the series of other antibodies presented in Table I. Among these were four murine Abs of subclasses IgG1 and IgG2a, a second human–simian chimer, a human–murine chimer, and a human Ab of subclass IgG4.

change in the monomeric IgG peak, and no assembly of molecules into higher aggregates is observed. We did note that more rapid and efficient formation of

the 23-nm particles was achieved when the IgG was exposed first to the polymer for several minutes or more, before addition of the salt.

The assembly process, which can be reversed upon reduction of the salt and polymer concentration, produces a unique particle 23 nm in diameter. Identical experiments conducted on the series of other monoclonal antibodies, presented in Table I, of diverse specificities, subclasses, and architectures demonstrated that the phenomenon is not confined to IDEC 151, but is shared by many, if not all, IgG class antibodies. No IgG was tested which failed to form particles. We observed from QELS, however, that the diameters of particles formed by various antibodies did, in some cases, differ from that of the IDEC 151 antibody particles by 10 to 15%.

Increase, by vapor diffusion (7,8), of the LiSO$_4$ concentration to between 1.55 and 1.85 M leads to the crystallization of the 23-nm particles in either a hexagonal or monoclinic form, as shown in Fig. 2. Often the two forms coexist in the same sample, though the hexagonal form appears to be favored by lower temperature and lower final salt concentration. X-ray diffraction analysis revealed the space group of the hexagonal crystals to be P622 ($a = b = 23.0$ nm and $c = 24.0$ nm). There can be but one IgG particle in the crystallographic unit cell; thus the particle itself must have 622 point group symmetry and be composed of 12 identical units so arranged. Analysis of the monoclinic crystals showed them to have an unexpectedly large unit cell having space group C2 ($a = 25.9$ nm, $b = 31.3$ nm, $c = 75.7$ nm, and $\beta = 91.7°$), which contains eight particles. Neither crystal diffracts to beyond about 10 Å resolution. A complete set of diffraction data has been recorded to the resolution limit of the monoclinic crystals at the Advanced Light Source at Lawrence Berkeley Laboratories along with preliminary data on the hexagonal form.

Immobilization of the IgG particles in a periodic lattice offered, in addition to X-ray crystallography, an opportunity for *in situ* imaging of the assemblies, in the mother liquor, using atomic force microscopy (Kuznetsov *et al.,* 1997, 1999; Malkin *et al.,* 1999). Indeed, as illustrated in Figs. 3 and 4, this proved of substantial value in revealing some overall structural details of the particles. When IgG in 0.6 M LiSO$_4$ plus polymer, prior to crystallization, is adsorbed onto cleaved mica, it is visualized in Fig. 3a as a field of discrete, 23-nm-diameter, roughly spherical particles, consistent with QELS results. AFM images of the crystals reveal additional features. In Figs. 3b through 3f particles are seen within the surface layers of crystals, but consistently along their unique axes. The hexagonal symmetry is apparent, as are large channels of about 5
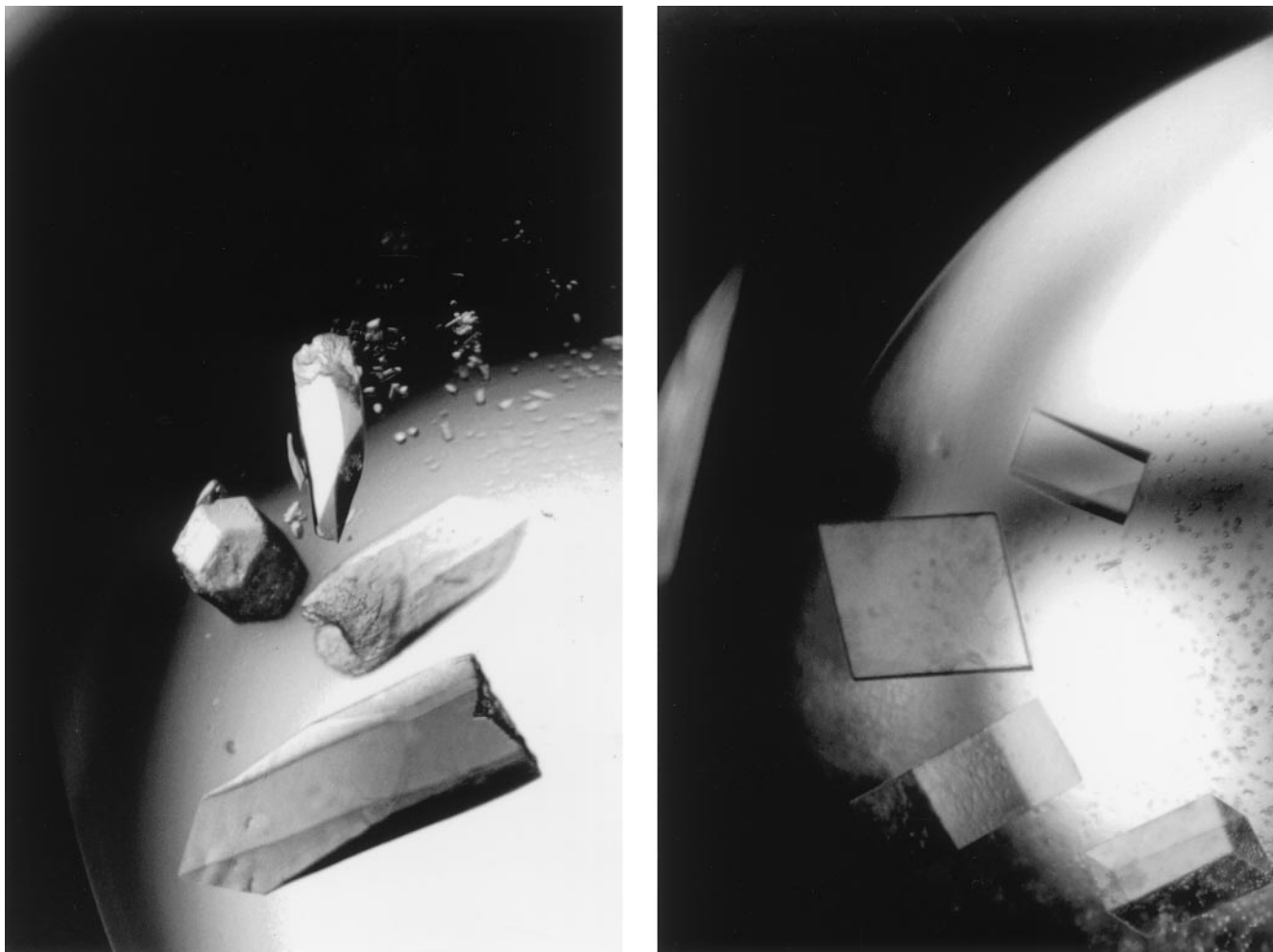
FIG. 2. On the left hexagonal and the right monoclinic crystals of the 23-nm-diameter particles of the IDEC 151 antibodies against CD4. The crystals were grown from 12-μl droplets by vapor diffusion against 1.55 M LiSO$_4$ at 2 C° and 1.75 M LiSO$_4$ at 19 C°, respectively. The droplets in both cases contained, initially, 4.2 mg/ml protein, 0.4% Jeffamine ED 2000, and 0.67 M LiSO$_4$. The pH appeared irrelevant between 4.5 and 8.0. The growth time was 2 to 6 weeks. The longest dimension of the hexagonal crystals was about 0.5 mm; the longest dimensions of the monoclinic crystals were 0.75 mm. The hexagonal crystals are frequently striated and many imperfections are visible. Under the microscope, most of the monoclinic crystals appear visually perfect.

nm in diameter running through the centers of the particles along the sixfold axes. When the particles are imaged perpendicular to their sixfold axes, as in Fig. 4, their appearance is distinctly different. In this orientation particles are seen to be demarcated by a longitudinal depression, which separates the complex into sections, thereby producing a "double dome" arrangement. The dimensions, symmetry, and packing of particles observed in these AFM images are consistent with those from the X-ray analyses and, in fact, provide a packing explanation for the unusually long c axis of the monoclinic crystals, as well as the relationship between the hexagonal and monoclinic lattices.

DISCUSSION

The observation that the self-assembly of IgG into duodecameric particles of high symmetry occurs for a variety of monoclonal antibodies, not only in the presence of LiSO$_4$ but in other salts as well, suggests that the phenomenon reported here is not highly specific, but may be general. Certainly IgG may exist which does not aggregate to form the particles we report here, but none was among those available to us. Although we have no evidence, we would be surprised if particle formation was without physiological relevance. This is particularly true given the similarity between the properties of the particles visualized here and those of the hexameric assembly
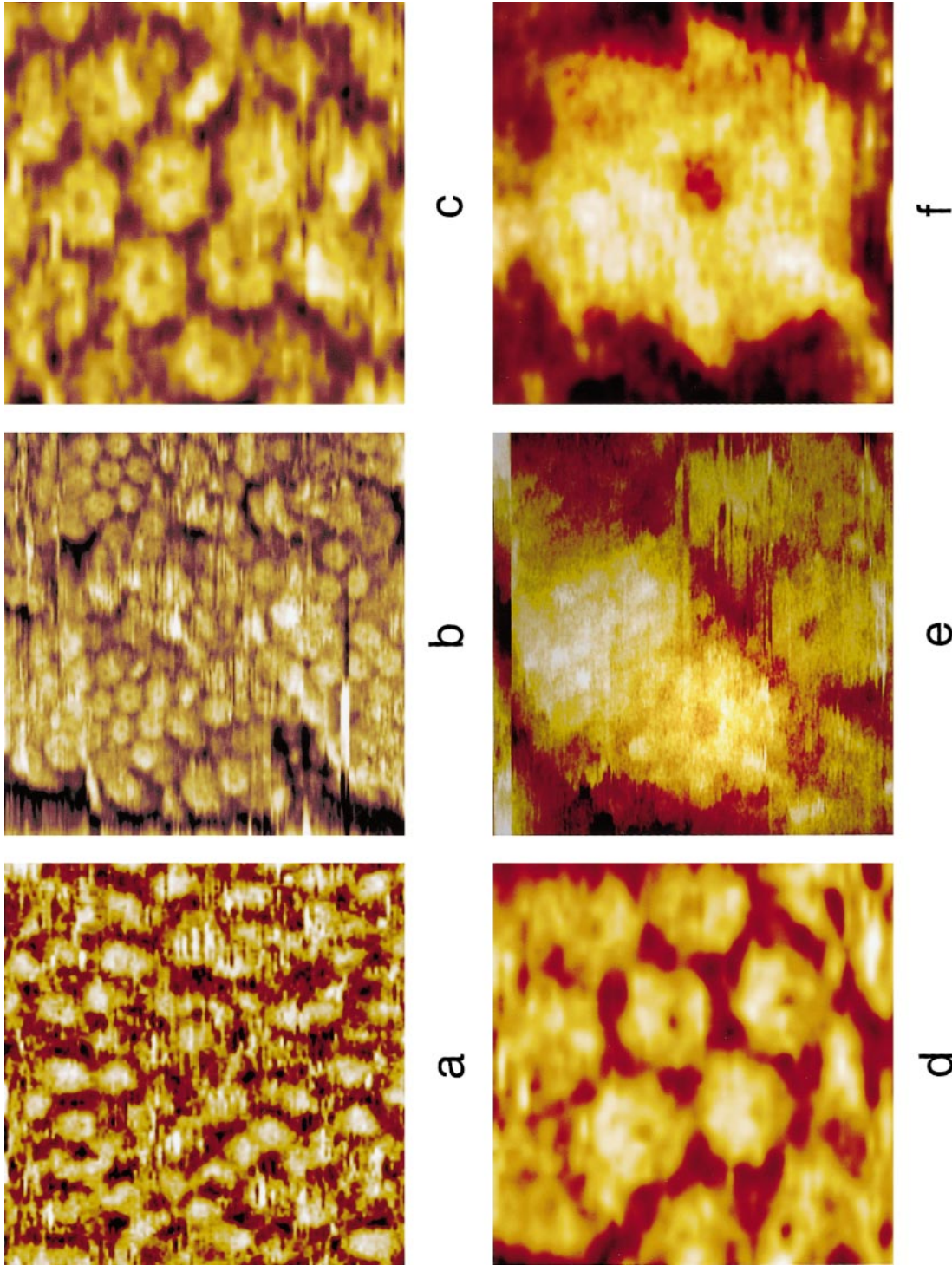
FIG. 3. Atomic force micrographs of the anti-CD4 antibody particles. In (a) individual IgG assemblies are seen as roughly spherical particles of 23 nm average diameter, consistent with QELS results, when, in 0.6 M LiSO$_4$ plus 0.4% Jeffamine ED 2000, they are spread on the surface of freshly cleaved mica. In (b) the rough <001> face of a hexagonal crystal provides small areas where groups of particles making up the hexagonal lattice can be seen along their sixfold axes. In (c) and (d), groups of the IgG particles on the surfaces of hexagonal crystals are imaged which clearly show the central channels along the sixfold axes. In (e) and (f), individual antibody particles on the <001> faces of monoclinic crystals are recorded at higher magnification. These images show the central channels through the particles as well as the approximately hexagonal star arrangement of the IgG within the particles. Scan sizes are (a) 252 nm^2, (b) 317 nm^2, (c) 104 nm^2, (d) 89 nm^2, (e) 62 nm^2, and (f) 29 nm^2.
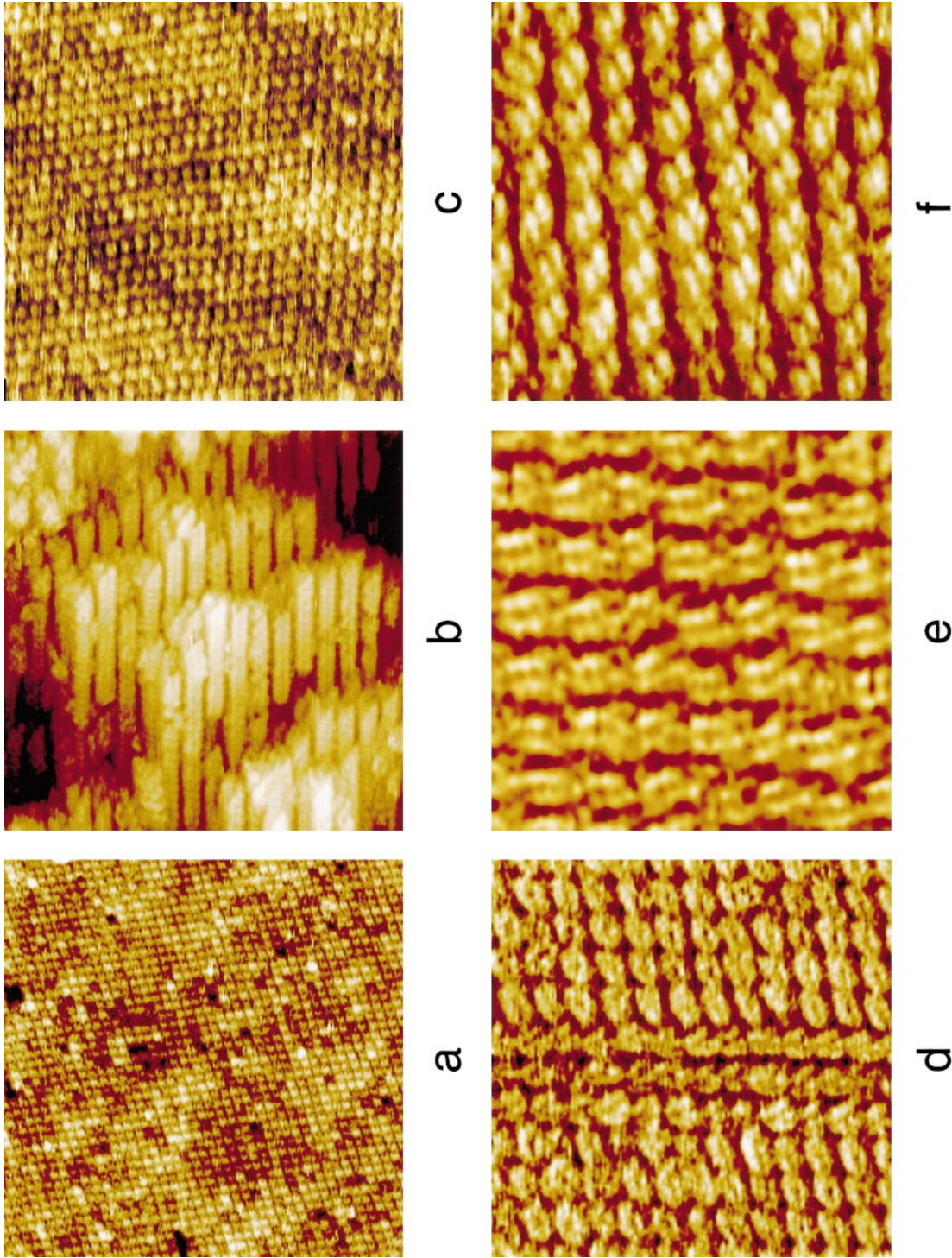
FIG. 4. In (a) through (c) are images of the 23-nm-diameter IgG particles organized in the surface layers of different faces of the monoclinic crystals, including the <010> face in (b). Lattice spacings measured from the AFM images are strictly consistent with those measured from the crystals by X-ray diffraction. In (d) through (f) higher magnification, higher resolution images of the antibody particles on the <100> crystal faces are presented. In this view, it is evident that the particles are delineated by longitudinal depressions consistent with a very open arrangement of the antibodies within the assemblies. The scan sizes are (a) 1 μm^2, (b) 1.5 μm^2, (c) 600 nm², (d) 260 nm², (e) 165 nm² and (f) 238 nm².

of IgG postulated to activate serum compliment upon antigen binding (Burton, 1990; Burton and Woof, 1992). The images of the particles are also similar in some ways to the hexameric arrangement formed by IgG binding to antigen immobilized on a membrane (Uzgiris and Kornberg, 1983), as visualized by Reidler *et al.* (1986) using electron microscopy.

The IgG particles formed in the presence of polymer and sulfate are consistent with a roughly spherical model, both the top and the bottom of which consist of six antibody molecules arranged with C_6 symmetry about a pole of the unique axis, presumably through Fc–Fc interactions between neighbors (Burton, 1990; Burton and Woof, 1992). The two rings of six Fc segments form the channel about 5 nm in diameter through the center. The top and bottom rings of particles are identical and related by D_2 symmetry, that is, by twofold axes perpendicular to the sixfold axis. Fab segments extend downward and outward toward the periphery, where they interact at maximum particle radius with Fabs from antibodies in the D_2 related ring. The Fc need not be in the same plane as the Fab segments, nor need any molecular symmetry be maintained, consistent with the X-ray structures of intact monoclonal antibodies (Harris *et al.,* 1997, 1998, 1999).

We suggest that, by chance, we may have come upon conditions that induce IgG to assume a similar arrangement to that formed by antigen-complexed antibody assembled to interact with its effector molecules. It is hard to imagine that the capability of the IgG to self-assemble into discrete, highly symmetrical, stable particles is entirely coincidental and not reflective at least of some natural function.

We are not certain what effect Jeffamine (or PEG) has on IgG in the presence of salt, only that it is essential for particle formation and for subsequent crystallization. We note, however, that this is not the first instance of proteins exhibiting unusual behavior or crystallizing in mixed polymer–salt solutions. Indeed, W. J. Ray discussed these systems extensively for the case of rabbit phosphoglucomutase (Ray, 1992; Ray and Puvathingal, 1986).

While the currently available X-ray diffraction data are only of limited resolution they nevertheless contains more than 5000 independent reflections. Given that the constituents of the particles, the IgG molecules, are known to atomic resolution (Harris *et al.,* 1997, 1998, 1999), the high symmetry of the particles, and their gross features apparent from AFM images, the X-ray data in hand should be adequate to test potential models of the assembly. That effort is in its initial stages, as are attempts to extend the diffraction resolution of the crystals and to obtain crystals of particles of other antibodies.

Because the known symmetry of the IDEC 151 particles is not expressed in the monoclinic crystallographic symmetry, the structure of the particles is also amenable to direct determination using the same approaches now employed for virus crystallography.

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