Fibronectin Adsorption to Surfaces of Hydrated Crystals. An Analysis of the Importance of Bound Water in Protein-Substrate Interactions

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We have shown that fibronectin, one of the major cell adhesion promoting proteins, is adsorbed with markedly different affinities to the organized surfaces of different crystals, containing a controlled and known amount of surface-bound water molecules. Fibronectin adsorbed maximally to the purely ionic surfaces of calcite, that do not include lattice water molecules. It did not adsorb at all on the prevalent faces of brushite, that expose to solution a continuous layer of structured lattice water. In other systems including calcium (*R,R*)-tartrate tetrahydrate and calcium fumarate trihydrate, fibronectin adsorption gradually decreased as the amount of lattice water molecules on the crystal surface increased. It is thus apparent that the presence of surface bound water may be instrumental in modulating protein adsorption to surfaces. The use of crystals as substrates permits clear-cut conclusions to be reached on the molecular requirements for protein adsorption to surfaces, that were not accessible with conventional substrates.

Introduction

The structural features, the chemical properties, and the organization of biological interfaces play a fundamental role in biological interactions, often mediated by a layer of adsorbed proteins. Adsorption from solution of a layer of macromolecules on the surface of solid substrates results in modulation of the biological activity of the molecule due to changes in its conformation and orientation in the adsorbed state.[1] It has further been shown that a large number of cellular processes depend on the correct formation of contacts between cells and extracellular matrix (ECM) components. In particular, receptor-mediated binding of cells to the surface of solid substrates, requires a layer of adsorbed "adhesive proteins", which are responsible for various cellular phenomena, commonly referred to as "anchorage dependence". These include the control of cell growth and differentiation, cell locomotion, and the activation of several metabolic pathways.[2-4] Even though much information is available on the identity and structure of "adhesive proteins" such as fibronectin, vitronectin, and laminin, which may be involved in such interactions, only limited information exists on the modulation of their surface chemistry by interactions with solid substrates and the consequent effect on the activity of the protein.[5-9]

Several studies were performed in order to elucidate how proteins, in general, interact with artificial substrates and how these interactions modulate their biological activity.[10-14] A vast effort was invested in particular in the identification and characterization of the properties of synthetic substrates which promote cell adhesion and support anchorage-dependent cell behavior.[15-19] Many of these studies were based on chemical modifications of polymeric substrates, performed to achieve gradients of surface hydrophobicity/hydrophilicity,[20-23] and charge density.[24-26] While these investigations revealed some general physicochemical properties of the substrates, they provided only limited molecular information on their surface organization and structure. Most of these substrates are highly nonuniform[27] and thus terms such as hydrophobicity, hydrophilicity, wettability, rigidity, water content, and so on are macroscopic and insufficiently defined to accurately describe the structural properties of the interface.

Our long term interest is the interaction between biological macromolecules and surfaces. Recently we have focused on the fine interplay between living cells, extracellular surfaces, and proteins which mediate the adhesion between the two. Here we study the adsorption on selected substrates of plasma fibronectin (pFN), one of the best characterized adhesive glycoproteins, which plays a major

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role in cell–ECM interactions.[15,28,29] pFN is a heterodimeric secreted glycoprotein, which consists of 31 globular repeats with multiple binding sites for matrix components and surface receptors. To approach, at a molecular level, the mechanism of substrate–fibronectin binding, we selected to use crystal surfaces as substrates. The advantage of this approach lies in the high level of molecular definition of their surfaces. The three-dimensional regularity of crystal lattices allows for high resolution analysis of the parameters which regulate the adsorption of proteins onto their surfaces. The modulation of the interactions with the same molecules, exposed at different surfaces in different orientations, allows further understanding of structural and stereochemical parameters.

The parameter specifically addressed here is solvation. To approach this issue, it appears useful to examine a set of surfaces that contain bound water in specified amounts and positions, as an integral part of the crystal lattices in hydrated structures.

Calcite ($CaCO_3$), brushite ($CaHPO_4 \cdot 2H_2O$), calcium fumarate trihydrate ($CaC_4H_2O_4 \cdot 3H_2O$), and calcium (*R,R*)-tartrate tetrahydrate ($CaC_4H_4O_6 \cdot 4H_2O$) were selected as appropriate crystals. They present an interesting and well-characterized diversity in the organization of water molecules exposed on their faces. Calcite is highly ionic and does not contain lattice water molecules. Calcium tartrate and fumarate are characterized by different amounts and organization of crystalline water exposed on their surfaces. Brushite crystals expose a double layer of crystalline water on their predominant face. We determined the morphology of each crystal system and then investigated the change in morphology for crystals grown in the presence of fibronectin. Since crystal morphology is determined by the relative growth rate of the slowest-growing faces, the interaction of the additive with the growing crystal affects the overall morphology. Analysis of morphological changes is, therefore, a means of identifying the specific crystal faces that adsorb the protein (thus increasing in relative area) and thereby of studying the nature of the interactions between the two.[30–32] Immunolabeling techniques[33] have been used to further characterize the mode and selectivity of FN adsorption.

It will be shown that FN shows a high nonspecific affinity to the ionic {104} faces of calcite. The affinity of the protein toward other crystals decreased as the crystal faces become more decorated with crystalline water molecules. FN did not adsorb on the predominant {010} faces of brushite crystals, which expose a double layer of crystalline water. It is thus apparent that the presence of surface bound water may be instrumental in modulating protein adsorption to surfaces. The use of crystals as substrates permits clear-cut conclusions to be reached that were not accessible with conventional substrates.

Materials and Methods

(a) Materials. The chemicals were all commercial analytical-grade materials and were used without further purification. Calcium chloride dihydrate, sodium hydrogen (*R,R*)-tartrate, calcium nitrate tetrahydrate, sodium dihydrogen phosphate monohydrate, and calcium hydroxide were obtained from Merck-Schuchardt (Darmstadt, F.R.G.). Fumaric acid, FITC-protein A, and bovine albumin were obtained from Sigma Chemical Company (St. Louis, MO). Thirty nanometer gold labeled goat-anti-rabbit IgG(H+L) was obtained from Amersham International (Amersham U.K.). Ammonium carbonate was obtained from BDH Chemicals, Ltd. (Poole, England). Human plasma fibronectin and rabbit antibodies against fibronectin were kindly provided by Dr. M. Werber, BioTechnology General (Rehovot, Israel). Sterile polystyrene cell culture dishes (Falcon 3 and 5 cm diameter) were obtained from Becton Dickinson, U.S.A. Spectra/por dialysis membranes with M_r cut-off 6000–8000, were obtained from Thomas Scientific, U.S.A. Double-distilled water was used in all solutions. Activated sulforhodamine B was obtained from Polysciences, Inc. (Warrington, U.S.A.).

(b) Crystallization Experiments. For each crystal system the optimal conditions of crystallization from aqueous solution were determined, so that the crystals were well formed, homogeneous, and reproducible with respect to their morphology. The morphology of typical crystals and the relative areas of the developed faces were measured on a Weissenberg camera using standard techniques.[30] Tartrate crystal morphology was measured on a Nonius CAD-4 diffractometer using Cu or Mo Kα radiation. The effect of fibronectin on the crystal morphology was examined by adding the protein in appropriate amounts (0.1–10 μg/mL) to the crystallization solutions. The morphology of crystals grown in the presence of the protein was compared to that of crystals grown in parallel without protein. All crystallization experiments were performed at room temperature.

(*i*) *Calcite.* Crystallization was induced by slow diffusion of ammonium carbonate vapor into cell culture dishes containing 2 mL of 7.5 mM $CaCl_2 \cdot 2H_2O$ (pH 6.5) in a closed desiccator for 3 days.[34]

(*ii*) *Calcium (R,R)-Tartrate Tetrahydrate.* A 10-mL portion of a solution of 3.2 mM sodium hydrogen tartrate was mixed with 10 mL of solution containing 4.4 mM $CaCl_2 \cdot 2H_2O$ at pH 6.5. Crystals form within 1 day.[34]

(*iii*) *Calcium Fumarate Trihydrate.* Fumaric acid (164 mg) and $Ca(OH)_2$ (110 mg) were suspended in 10 mL of double-distilled water, and the mixture was acidified with 1 M HCl until all the compound dissolved (pH 2.5) and then titrated back to pH 7.0 with 1 M NaOH. Crystals form within 1 to 2 days.[34]

(*iv*) *Brushite.* Crystallization was induced by slow diffusion between a solution of 2 mL of $Ca(NO_3)_2 \cdot 4H_2O$ (4 mM, pH 6.5), contained in a dialysis tube, and 15 mL of $NaH_2PO_4 \cdot H_2O$ (5 mM, pH 6.5) in 5-cm cell culture dishes, for a week.[35] All the solutions were autoclaved and contained 0.01% sodium azide, to prevent contamination. The pH was adjusted to the desired value with concentrated NaOH or HCl. The crystal fraction formed in the dialysis tube was used. Proteins were added into the dialysis tube.

(c) Calcium (*R,R*)-Tartrate Tetrahydrate Structure Refinement. A tartrate crystal (0.4 × 0.4 × 0.3 mm) was mounted on a Rigaku AFC5 diffractometer equipped with a rotating anode, operated at 40 kV and 250 mA. A silver rotating target was used to eliminate adsorption of the crystal (μ = 0.379 cm^{-1}). Data collection was performed at 120 K, until 2θ = 70° (resolution of 0.488 Å). The tartrate structure was refined using SHELXL-92 to R = 0.023 (supplementary material). The initial coordinates were taken from Ambady.[36]

(d) Immunofluorescence Labeling. Routinely, all the adsorption experiments were performed at room temperature, in solutions saturated with respect to the specific crystal system. Unless otherwise specified, crystals were incubated for 2 h with rabbit antiserum anti-fibronectin, at 1:10 dilution. The crystals were washed (×3, 200 μL) in the appropriate saturated solution and incubated either for 2 h with fluorescein (FITC)-labeled protein-A (50 μg/mL) or for 2 h with gold-labeled goat anti-rabbit IgG at 1:10 dilution. The fluorescently labeled crystals were air-dried on a microscope slide and examined using a Zeiss

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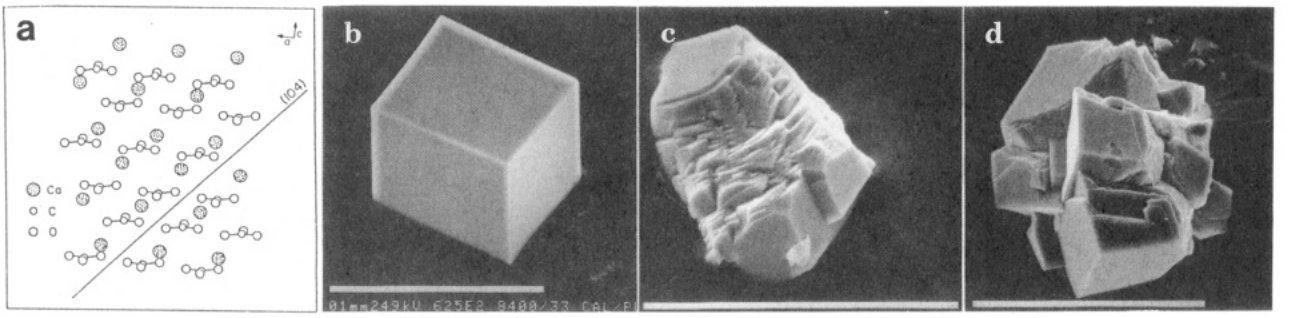

Figure 1. (a) Packing arrangement of calcite, viewed on the (010) plane and rotated by 5° around the *c* and *a* axes to facilitate the understanding of the structure. The (104) plane, that delineates the naturally developed crystal faces, is viewed edge-on to the drrawing to show the profile of the emerging ions. Calcite crystals grown in the absence of pFN (b) or in the presence of 0.5 μg/mL (c) or 1.0 μg/mL (d) fibronectin. Bars = 100 μm.

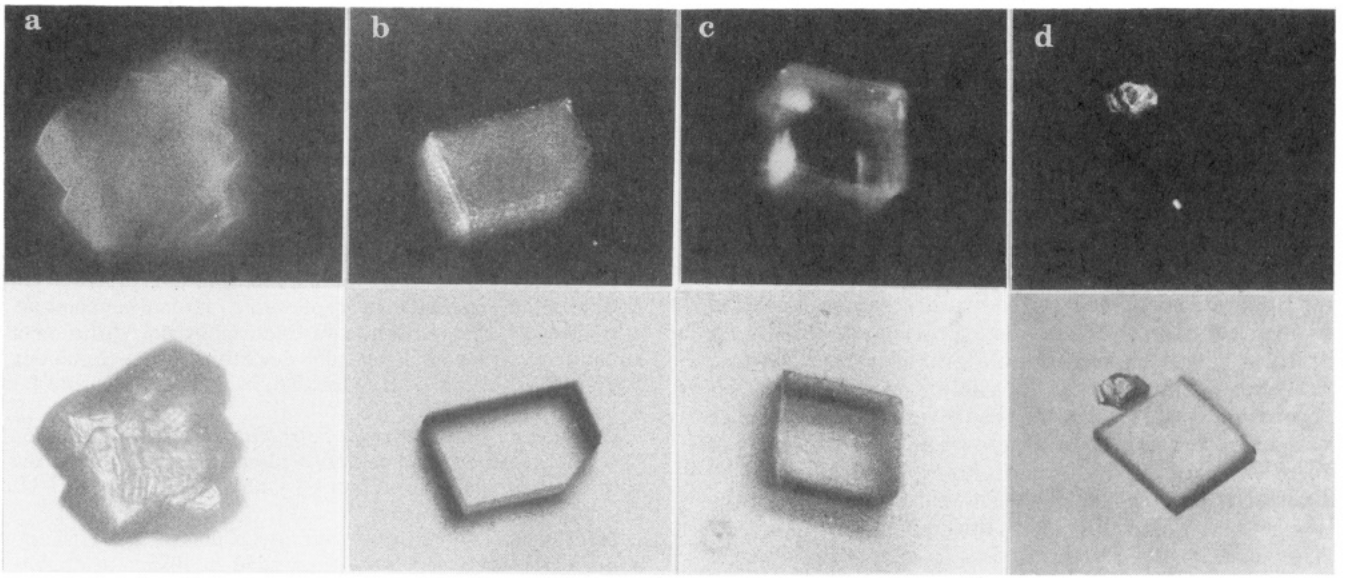

Figure 2. Bright field (bottom panel) and fluorescent (top panel) micrographs of calcite crystals. Magnification ×160. (a) Crystals grown in the presence of 1 μg/mL rhodamine-labeled fibronectin. (b) Crystals incubated with 10 μg/mL pFn and immunolabeled with anti-fibronectin antibodies and FITC–protein A. (c) Crystals incubated with 10 μg/mL fibronectin and 30 μg/mL albumin, immunolabeled with anti-fibronectin antibodies and FITC–protein A. (d) Control crystal immunolabeled as in part b, without incubation with fibronectin.

16 microscope equipped with filter sets for rhodamine and fluorescein fluorescence. The gold labeled crystals were dehydrated with alcohol, using Micropours Spec Cap (Spi Supplies, U.S.A.) and critical point dried (CPD) with CO_2. Following CPD, the crystals were placed on carbon-coated stubs (Spi) and sputter coated with gold during 12 min at 6 mA (S150 Edwards, USA). The stubs were examined at accelerating voltage of 10 kV using a JEOL 6400 scanning electron microscope.

Results

A series of experiments were performed to characterize the adsorption of plasma fibronectin (pFN) on the various crystals. For each system, the crystal structure will be briefly described first. The morphological and immunolabeling experiments will follow. Routinely, all the adsorption and immunolabeling experiments were performed in solutions saturated with respect to the specific crystal system, under known and controlled conditions.

Adsorption of Plasma Fibronectin to Calcite Crystals ($CaCO_3$). Calcite is a calcium carbonate polymorph with hexagonal structure $R\bar{3}c$ ($a = 4.99$, $b = 4.99$, $c = 17.06$ Å, $\gamma = 120°$, $Z = 6$). The structure is characterized by having layers of calcium and carbonate ions alternating along the *c* axis. The planar carbonate ions lie on the (001) plane.[37] Pure calcite develops the "cleavage rhombohedron" habit, delimited by six equivalent faces, the {104}[38] faces according to the hexagonal nomenclature. The face is highly ionic and is characterized by its dense distribution of cationic and anionic groups (Figure 1a,b).

1. Morphological Effects. The growth of calcite is strongly inhibited at pFN concentrations varying from 0.1 to 10 μg/mL (Figure 1c,d). At the macroscopic level, a general loss of the well-defined crystal edges and shapes was observed, whereas preservation of the {104} faces was observed at the microscopic level. This behavior is typical of nonspecific adsorption of protein on the faces initially exposed during growth.

2. Fluorescence Tagging and Immunofluorescence Experiments. Tagging pFN with rhodamine allows direct observation of the adsorbed protein on the growth crystals. The direct binding experiments enable visualization of adsorbed pFN, irrespective of its conformational state. The results are in full agreement with the crystal grown experiments. Fibronectin is strongly adsorbed on calcite, resulting in uniformly fluorescent crystals (Figure 2a). The levels of fluorescence were not significantly affected by the duration of the incubation period (2–24 h). We further observed that when pFN was added to the crystallization

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(38) The notation {*hkl*}, e.g. {101}, {011}, or {104}, indicates a set of identical, symmetry related faces.

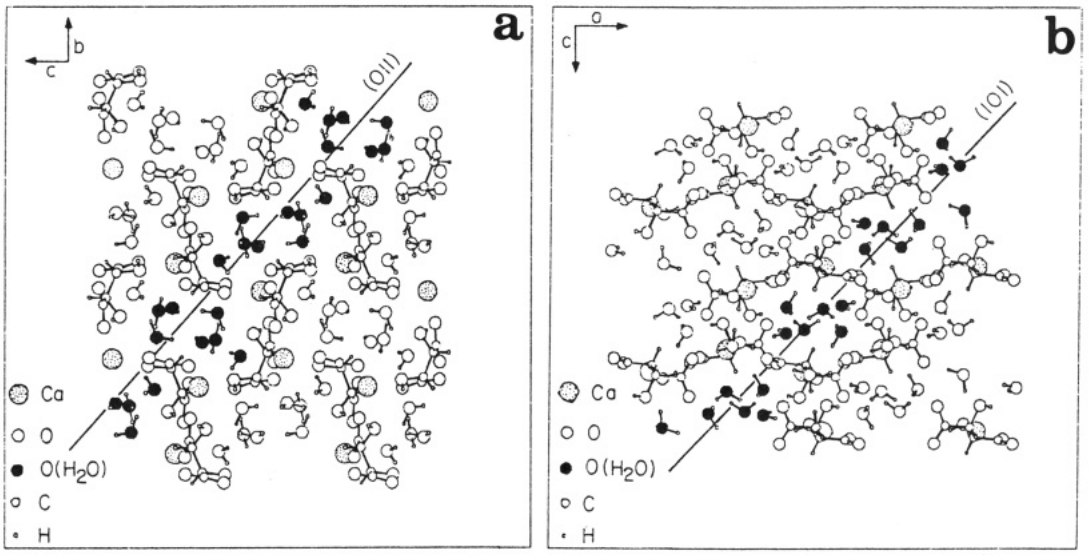

Figure 3. Packing arrangement of calcium (*R,R*)-tartrate tetrahydrate viewed on the (100) (a) and (010) (b) planes. The (011) and (101) planes delineating the developed crystal faces are viewed edge-on to the drawings to show the profile of the emerging molecules. The water molecules at the crystal surface are blackened. In part a the channels of water molecules lying along the *a* axis on the (011)[38] face are seen in section (the *a* axis is perpendicular to the plane of the drawing).

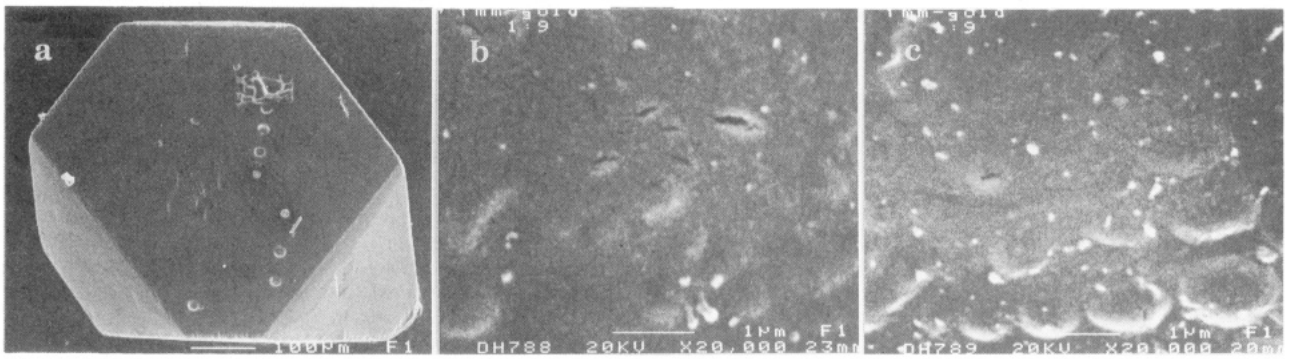

Figure 4. (a) Scanning electron micrograph of a tartrate crystal used for an immunogold-labeling experiment. The spots on the crystal face correspond to the sites where the immunogold particles were sampled, caused by electron beam damage. Bar = 100 μm. (b) Colloidal gold particles on the (011) face, after incubation of the crystal with fibronectin, followed by antifibronectin antibodies and colloidal gold-linked protein A. Bar = 1 μm. (c) Colloidal gold particles on the (101) face of the same crystal shown in part b. Bar = 1 μm.

solution during crystal growth, the fluorescent protein accumulated also within the crystals. This was established by partial dissolution of such crystals followed by examination by fluorescence microscopy.

Localization of pFN (unmodified) at the crystal surface was carried out with indirect immunofluorescence, performed by incubation of the protein-coated crystals with polyclonal antifibronectin antibodies followed by incubation with FITC-labeled protein-A. As shown, fibronectin was homogeneously distributed on the {104} crystal faces (Figure 2b), while control crystals incubated without pFN were negative (Figure 2d). The intensity of fluorescence was related to the initial concentrations of pFN added to the solutions.

The presence of two or more proteins in solution may result in competitive adsorption in which proteins which can most optimally adhere to the surface tend to displace the less firmly bound proteins (Vroman effect). Thus, addition of other proteins to the incubation solution can elucidate the complex hierarchy of adsorbed protein types developed with time. Addition of bovine serum albumin (weight ratio 3:1), only slightly suppressed the levels of the emitted fluorescence, reflecting the preferential binding of pFN to the crystal faces (Figure 2c). The high affinity of fibronectin toward the {104} faces was further verified with the use of 10% fetal calf serum (FCS), as the incubation solution. Fetal calf serum contains about 70 mg/mL total proteins fraction—including albumin, globulins, and not more than 0.3 mg/mL FN. The levels of the FN fluorescence observed did not significantly differ from those observed in the albumin experiments.

Adsorption of Plasma Fibronectin to Calcium (*R,R*)-Tartrate Tetrahydrate Crystals ($Ca^{-}OOC$–CH(OH)–CH(OH)–COO^{-}). Calcium tartrate tetrahydrate crystallizes in an orthorhombic structure ($P2_12_12_1$) characterized by a net of combined ionic interactions and hydrogen bonds.[36] The unit cell dimensions were redetermined (supplementary material) as $a = 9.152$, $b = 10.504$, $c = 9.554$ Å, $Z = 4$. The large prismatic crystals are delimited by {101} and {011} faces (Figure 3a,b and 4a). The differences between the faces are structural and stereochemical. Both surface types display a mixed character. The carboxylate and the hydroxyl orientation to the surface are however different, and so is, interestingly, the water molecules organization. The {011} faces display a corrugated surface, with chains of water molecules hydrogen bonded along *a*, alternating to tartrate molecules lying along the *a* axis. The hydroxyl groups of every second tartrate molecule emerge at the surface adding to the hydrogen bond character of the face (Figure 3a). On the {101} faces, in contrast, the carboxylate group of one tartrate molecule emerges perpendicular to the face,

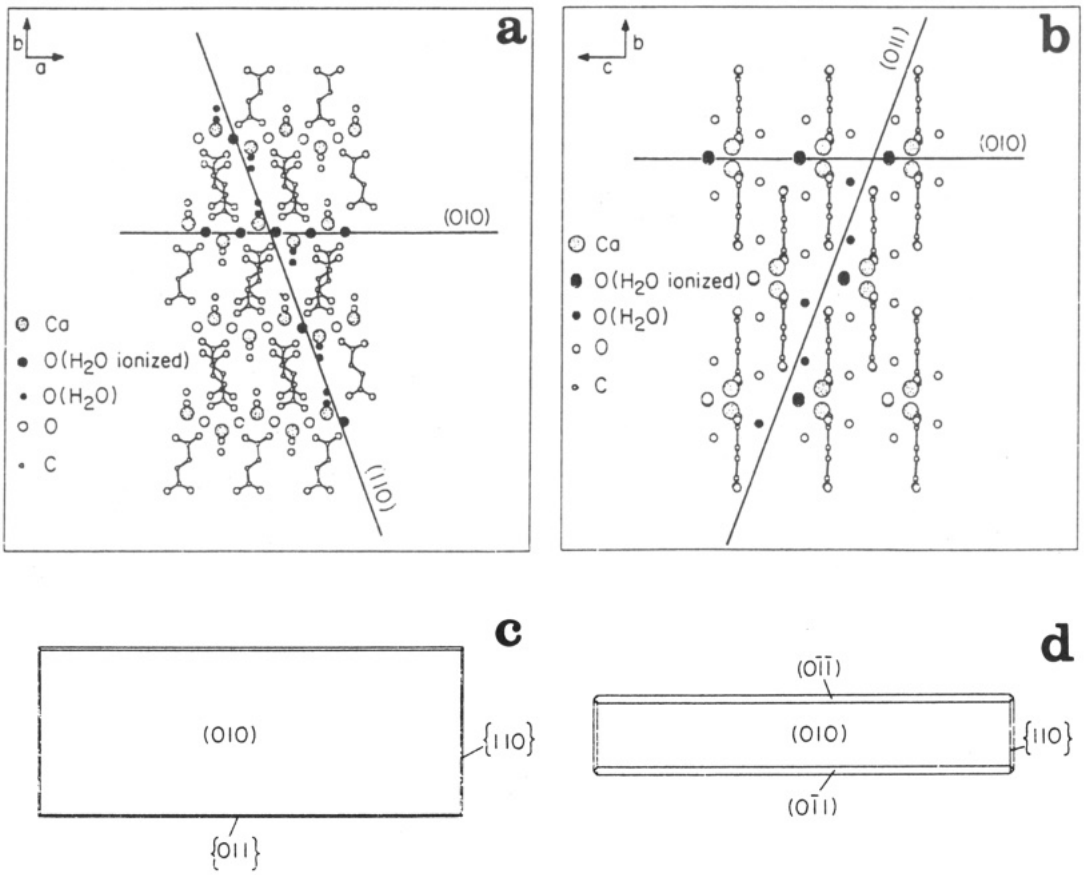

Figure 5. Packing arrangement of calcium fumarate trihydrate viewed on the (001) (a) and (100) (b) planes. The (110), (010), and (01$\bar{1}$) planes, delineating the developed crystal faces, are viewed edge-on to the drawing to show the profile of the emerging molecules. The water molecules at the crystal surfaces are blackened. The partially ionized water molecules are represented in a different size, to distinguish them from regular water molecules. Computer-drawn morphologies of a fumarate pure crystal (c) and of a crystal grown in the presence of 10 μg/mL fibronectin (d).

surrounded by a patch arrangement of water in deep "pools" (Figure 3b). A second tartrate molecule emerges with the carboxylate oxygens edge-on to the face. These faces have thus a more ionic character than the {011} faces.

1. Morphological Effects. The calcium tartrate growth morphology was not affected by the presence of fibronectin in the solution, in a concentration range of 5–10 μg/mL.

2. Immunofluorescence and Gold Labeling Experiments. Immunofluorescence studies, direct and indirect, indicated that the adsorbed fibronectin is accumulated at a higher amount on the {101} faces. Those observations could not however be conclusive, due to artifacts induced by high internal reflection of the fluorescent beam, in this crystal system. Furthermore, the control crystal, incubated with FITC–protein A without fibronectin, also showed moderate levels of fluorescence. Addition of albumin or surfactant, and dilution in the concentration of the antibodies or FITC–protein A did not eliminate the fluorescence observed in the control crystals.

The use of colloidal gold-conjugated protein A, instead of FITC–protein A, can at least eliminate artifacts due to internal reflection of fluorescence. The 30-nm colloidal gold particles, deposited on the crystal faces are then directly detected and quantitated by scanning electron microscopy. In the control crystals, the amount of gold detected on the {011} faces was 98 ± 37 and 216 ± 48 particles/100 μm^2 on the {101} faces. This indicates a high nonspecific adsorption of protein A and/or the polyclonal antibodies on both surfaces, with a more than 2-fold preference for the {101} faces. Following pretreatment with pFN, the number of particles was (104 ± 41)/100 μm^2, for the {011} faces (Figure 4b), and (276 ± 55)/100 μm^2 on the {101} faces (Figure 4c). We deduce that the {101} faces have, in general, a greater tendency to adsorb proteins. The slightly higher labeling following incubation with pFN and its absence from the {011} faces may indicate some binding specificity of the protein for the former faces.

Adsorption of Plasma Fibronectin to Calcium Fumarate Trihydrate Crystals. Calcium fumarate trihydrate crystallizes in an orthorhombic structure $Pna2_1$ (a = 6.62, b = 17.63, c = 6.97 Å, Z = 4).[39] The crystals are thin {010} platelets, elongated along the a axis. The predominant faces are {010}, with thin {011} and {110} side faces (Figure 5a,b,c). The flat fumarate molecules lie all elongated along the b axis, with the carboxyl groups emerging perpendicular to the {010} faces (Figure 5a). In one row of molecules along the a axis, the carboxyls are ionized and bind calcium in a double chain motif. In the alternating row the carboxyls are only partially ionized and bind calcium through partially ionized water molecules. Two other nonionized water molecules complete the coordination polyhedron of the calcium ion. As a result of this peculiar arrangement, all the faces have a mixed charged and hydrogen bond character, with water and carboxyls contributing to both.

1. Morphological Effects. The crystals grown in the presence of pFN showed morphological changes which indicated preferential interactions with some crystal faces. The crystals preserve their platelet character, but the adsorbed protein in a concentration range of 5–10 μg/mL

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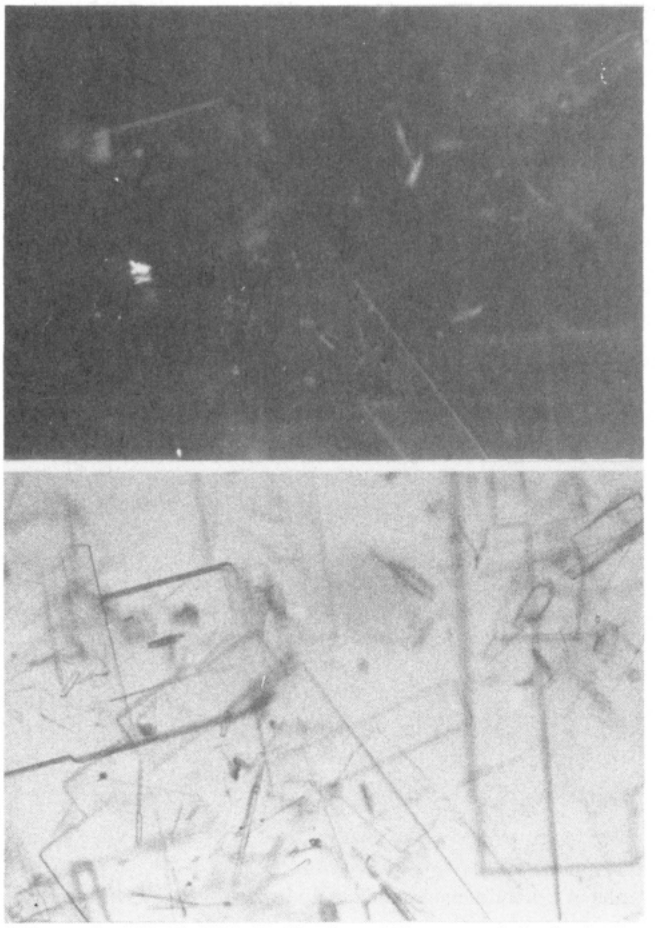

Figure 6. Bright field (bottom) and fluorescent (top) photomicrographs of calcium fumarate trihydrate crystals incubated with 10 μg/mL fibronectin followed by immunolabeling with anti-fibronectin antibodies and FITC–protein A, ×160.

causes a selective increase in the relative area of the {011} faces (Figure 5d) relative to the {110} faces.

2. Immunofluorescence Experiments. Indirect immunofluorescence studies were performed on pure crystals incubated with pFN at 5–10 μg/mL and on crystals grown in the presence of the protein. In the former crystals, all the faces exhibited moderate fluorescence levels (Figure 6). The crystals grown in the presence of the protein showed a slightly higher level of fluorescence, specifically from the {011} faces.

Adsorption of Plasma Fibronectin to Brushite Crystals ($CaHPO_4 \cdot 2H_2O$). Calcium hydrogen phosphate dihydrate (brushite) crystallizes in the monoclinic structure *Ia* (a = 5.812, b = 15.180, c = 6.239 Å, β = 116°, Z = 4).[40] Under the conditions used, the crystals form as irregular platelets delimited by prominent plate faces {010} and side faces {$h0l$} (Figure 7). In the unit cell, the calcium and the hydrogen phosphate ions form bilayers parallel to the (010) plane, intercalated by bilayers of water molecules which are also parallel to the (010) plane (Figure 7a). Thus in principle, the crystal may expose at the interface of the {010} faces a bilayer either of structured water or of calcium and phosphate ions. When the crystals are immersed in water, the interface layer is presumably a structured water layer, because the concentration of water is much higher (55 M against millimolar concentration of the salts). The side faces have a mixed ionic character intercalated by ribbons of water.

1. Morphological Effects. Brushite morphology was not affected by the presence of fibronectin in the crystallization solution up to concentrations of 10 μg/mL.

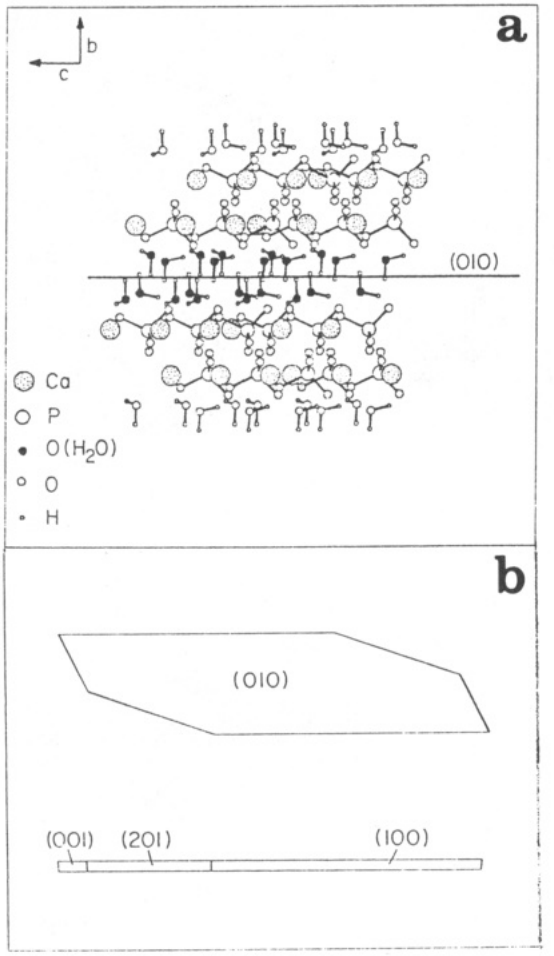

Figure 7. (a) Packing arrangement of brushite viewed on the (100) plane. The (010) plane, delineating the large plate face, is viewed edge-on. The water molecules of the bilayer emerging at the crystal surface are blackened. (b) Computer-drawn morphology of a measured crystal of brushite, showing the developed faces.

2. Fluorescence Tagging and Immunofluorescence Experiments. In the immunofluorescence studies, the crystals fluoresce from all the side faces, predominantly the {100} faces. Fluorescence was not detected from the {010} faces either when rhodamine labeled pFN was added to the crystallization solution (Figure 8a) or following indirect immunolabeling. The control specimens did not show any fluorescence (Figure 8b). Long term incubation of the crystals (24 h) with fibronectin, in the presence of bovine albumin as competitor protein (weight ratio 3:1), did not change the level of fluorescence observed from the side faces. The {010} plate faces were still not fluorescent. The same phenomenon was observed in the presence of 10% FCS, as the incubation solution.

Discussion

We have shown that fibronectin, one of the major cell-adhesion promoting proteins, is adsorbed with drastically different affinities to the organized surfaces of crystals, containing a controlled and known amount of surface-bound water molecules.

Fibronectin adsorbed maximally to the purely ionic surfaces of calcite, that do not include lattice water molecules. It did not adsorb at all on the prevalent faces of brushite, that expose to solution a continuous layer of structured lattice water. In the intermediate systems of

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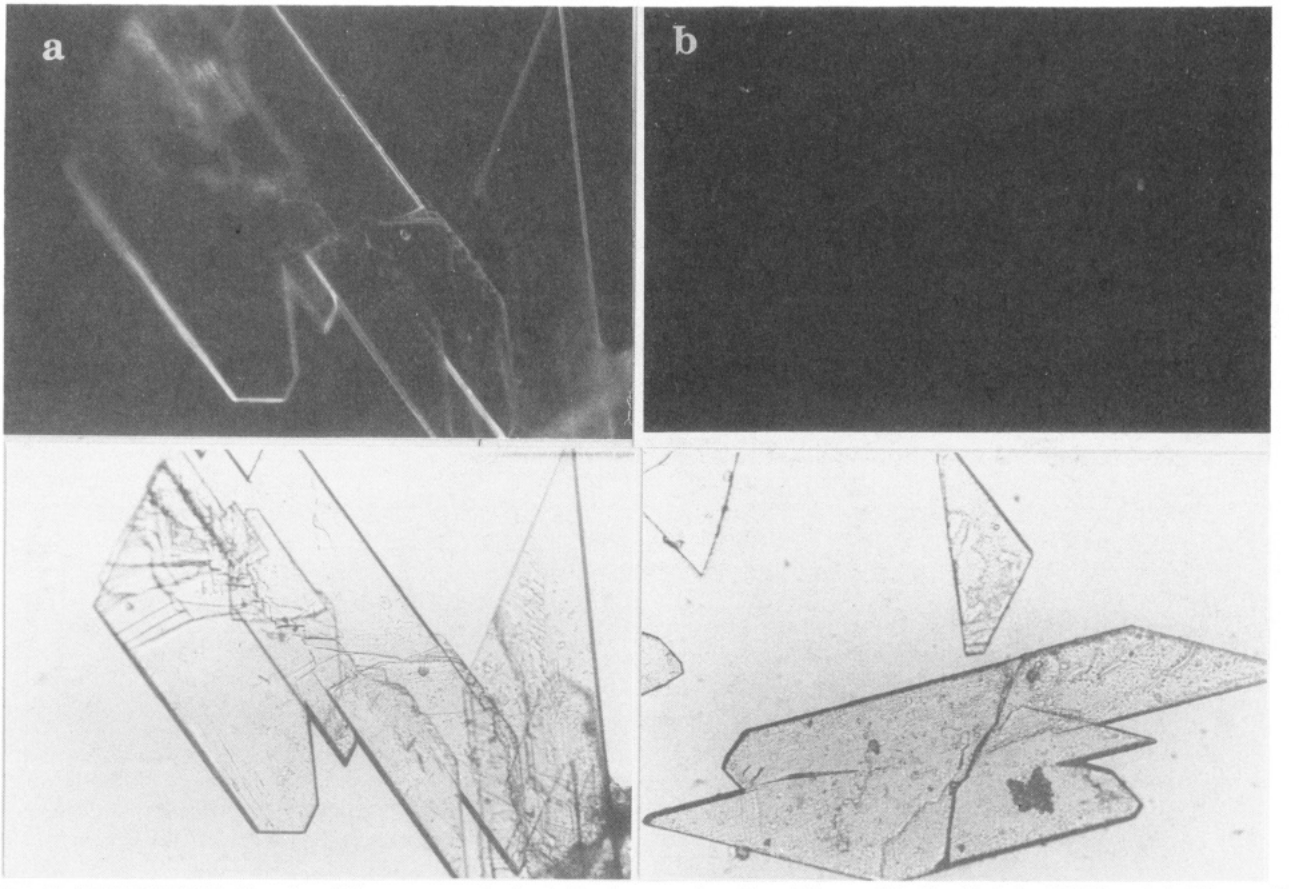

Figure 8. Bright field (bottom) and fluorescence (top) photomicrographs of brushite crystals incubated with 10 μg/mL fibronectin (a) or without fibronectin (b), and immunolabeled with anti-fibronectin antibodies and FITC–protein A.

calcium (*R*,*R*)-tartrate tetrahydrate and calcium fumarate trihydrate, fibronectin adsorption gradually decreased as the amount of lattice water molecules on the crystal surface increased.

We note that the observed differences cannot be (solely) attributed to the nature of the ions composing the crystals. Fibronectin showed a drastically different affinity for the different faces of the same crystal having the same molecular composition, most notably the {010}, in contrast to the {*h*0*l*} faces of brushite.

Although results are reported here for fibronectin only, we have reasons to believe that they may well be relevant to a variety of globular proteins. Fibronectin is a large protein, composed of a series of tightly folded globular repeats, interconnected by flexible segments. At the functional level, FN contains a series of domains through which it interacts both with the cell surface and with different ECM molecules.[28] Moreover FN activity is usually manifested when it is immobilized on a surface.[5] It is conceivable that upon adsorption the protein changes its conformation, presumably spreading and flattening because of interdomain, rather than intradomain, modifications.[7] The variability of the nature and organization of the groups exposed at the protein surface together with the large size lead us to believe that specific interactions should not be the driving force to adsorption. This is in contrast to other cases studied previously in relation to biomineralization and pathological crystallizations.[41,42,33,32] In these instances, specific recognition takes place between structured repetitive motifs on the crystal and protein surfaces. Surface recognition is often an integral part of the protein function (or malfunction) and is thus specific to each system. Fibronectin on the contrary is supposed to be a common intermediary to cell adhesion to a variety of different substrates. Although fibronectin obviously has its distinct surface chemistry, we shall regard it, at this stage, as a large adhesive biopolymer with general characteristics that may be at least partially common to many other proteins.

Water molecules that are part of a crystal lattice are strongly bound to the crystal surfaces due to a tight net of hydrogen bonds holding the molecules in well-defined positions. In contrast, bulk water of hydration is loosely bound to the surface by virtue of transient electrostatic or polar forces or by hydrogen bonds and is more easily exchangeable. Although hydration water effectively screens charges in solution, charged residues on crystal and protein surfaces are known to generate electrical potentials that extend many angstroms out in the solution.[43–46]

Calcite is a good example for this effect. As it contains no lattice water, it represents one extreme of our scale of hydrated structures. In calcite, a high level of protein adsorption is indicated both by the strong effect on crystal growth and by the high fluorescence, obtained by direct binding assay as well as by immunofluorescence surface labeling.

The presence of other proteins during the pFN binding to calcite significantly lowered the levels of fluorescence,

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confirming that adsorption of pFN does not occur via fibronectin-specific sites on the crystal faces but is subject to competition for general sites over the whole surface. We assume that this binding is mainly driven by dense charge on the calcite surface. We note that the evidence reported here still does not provide any information as to whether individual molecules adsorbed homogeneously over the entire surface or cluster in concentrated patches, in single or multilayers.

In calcium (*R,R*)-tartrate tetrahydrate crystals both the {011} and {101} faces adsorb fibronectin, though at levels significantly lower than those observed for calcite, as indicated both by the fluorescence experiments and by the relative effects on crystal growth. Both faces of tartrate contain water, but in a different organization. On the {011} faces, water molecules are organized in channels parallel to the *a* axis and lying on the face. Also at the surface sites that are not directly covered by water, very little charge emerges. Of the tartrate molecule, only the hydroxyl groups are directly exposed. The carboxylates lie almost parallel to the plane of the face. In contrast, on the {101} faces the water molecules are organized in deep pools. The tartrate molecules emerge at the surface with one carboxylate perpendicular and one oblique to the face. The calcium cation is chelated by one hydroxyl and one carboxylate of each tartrate molecule and is not directly exposed at the surface of either face. In conclusion, the {101} faces will expose a much more charged surface to an approaching protein molecule than will the {011} faces. We have observed that proteins in general, and fibronectin in particular, show a higher affinity for the {101} faces.

In contrast, we have found that the adhesive behavior of A6 epithelial cells toward the two types of calcium (*R,R*)-tartrate tetrahydrate faces is drastically different.[47] Cells massively and rapidly adhered to the {011} faces. This adhesion was followed, however, by cell death within 1 to 3 days. Cell adhesion to the {101} faces, on the other hand was very slow and only after 24 h was cell attachment and spreading apparent. Following further incubations, the cells continued to develop normally. We do not fully understand at this stage the mechanisms that underlay these distinct behaviors of cells and proteins. It is however apparent that cell adhesion is not subjected to the same rules as protein adsorption but is surprisingly highly sensitive to surface organization. In particular, the massive attachment to {011} is probably not mediated by surface-attached matrix proteins. It may result from direct membrane–crystal interactions, mediated either by the abundant sugar moieties on the cell surface or by specific receptor–crystal binding. On the other hand, cell attachment to the {101} faces appears to be dependent on surface conditioning with "adhesive proteins". It is nevertheless noteworthy that effective conditioning was obtained only after a 24 h preincubation of the crystals with complete medium.

Fibronectin adsorption on calcium fumarate trihydrate crystals follows a pattern different from that of other crystals. Preferential adsorption on the {011} faces is indicated by the change in morphology of the protein-affected crystals. A comparison between the affected {011} and the unaffected {110} faces supports the notion that protein adsorption is correlated to the amount of charge directly exposed at the surface. In general, the {011} faces expose more charged groups than {110}, due to both carboxylates and partially ionized water. The {010} faces are also charged. The immunofluorescence experiments show homogeneous distribution of low fluorescence on both face types, {010} and {011}. We conclude that the specific effect manifested by the change in morphology is probably related to the amplification of a kinetic mechanism, possibly due to adsorption of very low protein amounts on few active growth sites. Such an effect would not be revealed by immunofluorescence.

In brushite, the pattern of pFN adsorption is clear-cut; no fibronectin adsorption is detected on the large plate face covered by a continuous layer of structured water, but pFN adsorption occurs on the side faces that expose substantial charge density of both phosphates and calcium. We note that this is not exclusive for FN. Other proteins, the acidic glycoproteins extracted from mollusk shells, also show similar behavior.[48] Furthermore, in different crystals containing hydrated layers within the lattice, as octacalcium phosphate, similar selectivity was observed; namely, proteins that are adsorbed at specific motifs on certain crystal faces are not adsorbed at other crystal faces where the same motifs are screened by the hydrated layer.[49] Thus the effect of crystal-bound water in preventing protein adsorption to charged surfaces is not limited to this particular system.

The overall result of this study may appear, at first sight, paradoxical. Proteins, which are normally stabilized in solution by a tightly bound hydration layer, do not bind to hydrated surfaces. One should however consider that both hydration layers of the proteins and of the substrate, are more structured than the bulk molecules of liquid water. Upon protein adsorption, some of this water must be removed or/and rearranged, a process that requires energy. The tighter the water is bound to the surface, the higher is the energy cost, proportional to the amount of molecules to be rearranged. As a more general conclusion, water is a strong competitor for binding "hydrophilic" groups, and different hydrophilic surfaces routinely used as substrates for protein adsorption may contain different amounts of bound water that must be replaced. Although this concept is not new, the obvious advantage in the use of crystal surfaces as substrates is that for the first time these energies can be quantified, surface potentials can be calculated, and the importance of specific interactions can be evaluated at the molecular level.

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Supplementary Material Available: New refinement of the structure of calcium (*R,R*)-tartrate tetrahydrate, including crystal data, fractional atomic coordinates and thermal parameters, anisotropic temperature factors, and bond lengths and distances (4 pages). Ordering information is given on any current masthead page.

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