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Mechanisms of Protein Adsorption on Surfaces and Consequences for Extracellular Enzyme Activity in Soil

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I. INTRODUCTION

The interaction of proteins with surfaces is a phenomenon that occurs in numerous processes [1–5]. This arises from the strong affinity for the solid-liquid and liquid-gas interfaces of these biopolymers, which is a consequence of the flexibility of the polypeptide chain and, above all, of the diversity of the physicochemical properties of their constitutive monomers. The 20 amino acids have side chains that can be classified on a hydrophobicity scale as nonpolar to polar and on an electrical scale as negatively, neutrally, or positively charged. This variety is far greater than the physicochemical properties of the constitutive sugars of polysaccharides or the nucleotides of nucleic acids and explains the unique properties of proteins with regard to their adsorption.

These surface-active properties of proteins are important for understanding overall soil functions. Biogeochemical cycles of major elements (C, N, P, and S), rhizosphere phenomena, and some aspects of the degradation of xenobiotics are affected by protein adsorption [6–30]. Two properties of proteins are at the basis of this ecological importance. First, many of these proteins are enzymes, i.e., biological catalysts, and are involved in various chemical reactions in soils. Second, proteins have an average N content of 16% and are a main source of

this element when proteins are released in soil after death of the biota and lysis of membranes. Thus, in the soil nitrogen cycle, proteins can be both catalysts, such as proteases and amidases, and N-rich substrates.

The enzymes found in soil have several origins and mechanisms of release. The most important sources are microorganisms, both bacteria and fungi. Root exudates make lesser contributions and soil fauna even less. The specialization of microorganisms in the secretion of extracellular enzymes is well evidenced in mycorrhizal associations where the fungal symbionts secrete phosphatases [31] and proteases [32] to mobilize P and N from soil organic matter. As concerns the mechanism of release, the enzymes are either essential for the survival and growth of the microorganism and are actively secreted, or they are released in soil after the death of the organism and the lysis of membranes.

The main functions of extracellular enzymes are to transform insoluble substrates into soluble products and to decrease the structural complexity of the original substrates. Most of the chemical compounds released in soil after the death of organisms are in a polymeric form and are frequently either insoluble (e.g., cellulose, starch, chitin) or adsorbed on soil surfaces. As microorganisms have limited mobility in the pore system of soil, they need to secrete enzymes to reach these polymers and to hydrolyze them into compounds with lower molecular weight that can be desorbed and made soluble. These products of the enzyme reaction can diffuse in the water-filled part of the pore system, and a fraction can reach the microorganism producing the enzyme. The interception of the diffusing products is more efficient if a spatially scattered population of microorganisms is considered rather than a single isolated microorganism. The second effect is linked to the specificity of the membrane transport systems of organisms. Compared with the relatively small number of constitutive monomers of biopolymers (20 amino acids, approximately 10 relatively abundant monosaccharides, five nucleotide bases) or chemical groups important in nutrition that can be released from these polymers (e.g., orthophosphate, sulfate), the number of molecular conformations increases exponentially with the number of monomers present in the polymer. To avoid this exponential requirement for different specific membrane transport systems, the enzymatic hydrolysis of biopolymers into monomers or low-molecular-weight oligomers is a favored mechanism. As the biogeochemical cycles of C, N, P, and S always imply a transformation from a polymeric form to small simple chemical groups, it can be assumed that all biogeochemical cycles in soil depend at some stage on the action of extracellular enzymatic systems (cellulases, amylases, xylanases, proteases, amidases, nucleases, lipases, phosphatases, arylsulfatases, etc.).

From a historical point of view, soil scientists led the research on the interaction between proteins and colloidal surfaces from the 1930s to the 1960s [33–36]. In particular, McLaren and co-workers, as early as the 1950s [37–39], demonstrated two essential aspects that explained most of the behavior of enzymes adsorbed on electrically solid surfaces, i.e., the shift in pH for the optimal activity

of an adsorbed enzyme and the maximum in the adsorption of proteins at the isoelectric point (pI). However, they failed to reach the appropriate mechanistic explanation for these two observations, for reasons that will be explained later and that were related to a lack of understanding of the stability of protein structure in the scientific community at that time and to a misinterpretation of a thermodynamic parameter.

In the last 30 years, several new topics involving the interaction of proteins with surfaces have emerged, and the human and financial forces engaged in understanding this phenomenon have now shifted largely toward biotechnological processes and problems of human health. These new topics include immobilized enzyme reactors, solid-phase immunoassays, enzyme electrodes, biocompatibility of medical implants to avoid induction of thrombosis, and fouling of contact lenses and filtration membranes for water treatment. This list will, undoubtedly, become larger with time.

The aim of this chapter is to show that if the enzyme activity in soil is analyzed at a molecular level as the consequence of physicochemical interactions, a vast scientific literature becomes available to soil scientists. To achieve this, analogies must be drawn between various systems, and, therefore it is necessary to understand the nature of the forces involved. The new biotechnological and medical studies do not focus on surfaces or coating polymers that are relevant to soil science, such as clays, oxyhydroxides, or humic substances. A noticeable exception is the growing use of muscovite surfaces as a support for the study of polymers by techniques such as surface force apparatus or atomic force microscopy where atomically smooth surfaces are required. Because muscovite, a mica, is a phyllosilicate, it shares many surface properties with clay minerals.

Nevertheless, the long-term consequences of interactions with solid surfaces regarding genetic evolution toward enzyme structures better adapted for catalysis in the adsorbed state (i.e., not unfolding on surfaces) will remain specific to the biology of natural ecosystems, especially soils. In contrast, all the biotechnological and medical devices involving adsorption of proteins are man-made situations where the proteins used will never be in contact with the surface of interest under natural conditions, thereby allowing no expression of evolutionary phenomena. In the future, the topic of evolutionary adaptation of enzymes that are naturally in the presence of solid surfaces will probably attract a growing interest of scientists working in other fields than soil for its possible biotechnological applications.

II. FORCES AFFECTING ADSORPTION AND CONFORMATION OF PROTEINS ON SURFACES

The study of the effect of various physicochemical factors on the quantity of protein adsorbed on surfaces cannot be separated from the study of the conforma-

tion adopted by the proteins on these surfaces. As will be shown later, two reasons can be invoked for this joint approach. First, a modification toward a less structured conformation on the surface is itself a contribution to the driving forces of adsorption, as it increases the entropy of the system and thus decreases the Gibbs energy, a necessary condition for spontaneous adsorption. Second, and conversely, the modification of conformation often leads to an increase of the specific interfacial area of the surface of contact between the adsorbed proteins and the solids, which has an effect on the maximum quantity of protein adsorbed per unit of surface area. It is, thus, difficult to deduce the degree of affinity of proteins for surfaces from the plateau values of the isotherms of adsorption data. For example, a lower quantity of a protein adsorbed under physicochemical condition 1 than under physicochemical condition 2 does not necessarily mean (as is too often asserted) that the forces of interaction between the protein and the surface are lower under condition 1 than under condition 2, as higher forces of interaction under condition 1 could result in an unfolding and spreading of the protein on the surface and in a lower quantity of protein in the adsorbed monolayer.

In addition, the diversity of the physicochemical properties of both soil surfaces and proteins makes understanding their interactions difficult, as several types of interactions, either entropic or enthalpic, act concurrently because no surfaces or proteins can give rise to only one type of interaction. Moreover, as pointed out below in the discussion of the factors involved in the structural stability of proteins in solution, a third component, the solvent, is involved, and its contribution to the interaction is as important as the contributions of the surface and the protein themselves.

As a result of its inherent complexity, a thorough treatment of this topic should reflect the diversity of the approaches used. Thus, successively (1) theoretical studies, (2) methodological progress, and (3) models of adsorption based on experimental studies on different systems will be presented.

A. Theoretical Aspects

The adsorption of proteins at constant temperature and pressure results from various interactions with surfaces and always leads to a decrease of the Gibbs energy of the system, according to the second law of thermodynamics [1,4,5,40–42]. The Gibbs energy, G, depends on two properties of state, enthalpy, H, which is a measure of the potential energy (i.e., the energy that has to be supplied to separate the molecular constituents from one another), and entropy, S, which is related to the disorder of the system. Thus, during a spontaneous adsorption:

$$\Delta_{ads}G = \Delta_{ads}H - T\Delta_{ads}S < 0$$

with T being the absolute temperature and Δ_{ads} being the change in the thermodynamic functions resulting from adsorption.

The difficulty in the analysis of these processes arises from the fact that the enthalpic effects, related to intermolecular forces, and entropic effects, related to the spatial arrangements of molecules, are not totally independent. Intermolecular forces influence the distribution of molecules, and the potential energy is also dependent on the molecular structure of the system. Nevertheless, for ease of presentation, the enthalpic and entropic contributions to the adsorption of proteins on surfaces will be presented separately.

Enthalpic Effects

Coulombic Interactions

Proteins as well as most mineral surfaces carry electrical charges. Their net charge depends on the pH and the concentration of electrolytes in the surrounding solution due to ionization of chemical groups or specific adsorption of ions. Coulombic forces are very strong intermolecular forces, and may even be as strong as covalent bonds [43]. They are also long range, as these forces are inversely correlated with the square of distance, r, whereas the energy of interaction is inversely correlated with r, at least theoretically when only the electrical charges are considered. However, as the thermodynamic cost of an uncompensated electrical charge in even a small unit of volume is high, all electrical charges of a given sign are compensated by an equal number of electrical charges of the opposite sign, giving rise to a diffuse double layer model of repartition of counterions and co-ions in the surrounding solution. Thus, a screening of the interaction occurs, and the electric field in solution decreases rapidly, and in an exponential manner. The electrostatic interactions between proteins and surfaces can be analyzed as an overlap of their electrical double layers [1,5]. The electrostatic part of the Gibbs energy is given by the isothermal and isobaric work of charging the electrical double layer:

$$G_{el} = \int_0^{\sigma} \phi(\sigma) d\sigma$$

where ϕ is the variable electrostatic potential and σ is the variable surface density during the charging process.

Lenhoff and collaborators [44,45] have used an approach to adsorption where the probability of finding a protein in a particular orientation at a given distance from the surface follows a Boltzmann distribution with respect to the interaction energy. Because solvation and conformational effects are difficult to model, only electrostatic and Lifshitz–van der Waals interactions are considered. Thus, the calculations have been made with model proteins that are less susceptible to modifications of their conformation on adsorption, such as "hard" proteins with a low adiabatic compressibility (e.g., ribonuclease A, lysozyme, chymotrypsinogen A). In a further step to simplify the computer simulation, protein-protein

interactions have not been considered, and thus only the dilute region of the adsorption isotherm was studied to take into account only protein-surface interactions. From the atomic coordinates of the proteins, a triangulated boundary was calculated with point charges placed in locations where amino acids with ionizable lateral chains were found, or, alternatively, to reduce computational time, the equivalent sphere was considered. The electrostatic potential, ϕ_i, inside the protein is given by the Poisson equation:

$$\nabla^2\phi_i = -\rho_i/\varepsilon_i$$

with ∇^2 being the Laplace operator, ρ_i the charge distribution, and ε_i the dielectric permittivity of the protein interior whose value is taken as 2. The electrostatic potential outside the protein, ϕ_e, in a $z{:}z$ electrolyte, is given by the linearized Poisson-Boltzmann equation:

$$\nabla^2\phi_e = 2\, e^2\, z^2\, \rho_\infty\phi_e/\varepsilon_e\, kT$$

where e is the electronic charge, z the valency of the ion, ρ_∞ the bulk concentration of the electrolyte solution, k the Boltzmann constant, and T the absolute temperature. The dielectric permittivity, ε_e, of the electrolyte solution is taken as 80. A constant charge density, σ, is assumed at the boundary between the protein and the solution; thus, the normal potential gradient at the boundary is given by Gauss law:

$$\partial\phi_e/\partial x = -\sigma/\varepsilon_e$$

This set of equations allows the numerical calculation of the electrostatic potential at any location. In turn, knowledge of the values of the potential allows computation of the free-energy change associated with bringing the protein to a given position and orientation near the electrically charged surface. Either the Gibbs free energy, G, or the Helmholtz free energy, F, can be used, as they are the same in an uncompressible system.

Another aspect, emphasized by Norde and collaborators [1,5,46,47], concerns the transfer of ions from the solution to the adsorbed layer of protein. This coadsorption has the effect of reducing the magnitude of the repulsion between like-charged parts of the protein and the surface in an interfacial region of low dielectric permittivity. Any cation in the solution can be transferred in principle, but Baron and Quiquampoix [48,49,49a] have shown, by a Fourier-transform infrared spectroscopy study of the pK_a of amino acids with a carboxyl side group (Glu and Asp), that mostly protons were transferred. This phenomenon is the origin of the pH change that occurs when a solution of protein and a suspension of clay at the same original pH and not buffered are mixed.

Lifshitz–van der Waals Interactions

In addition to electrostatic interactions, van der Waals forces act on all molecules, even if they are electrically neutral. These forces are composed of three different components whose energy of interaction decreases with distance, r, as r^{-6}. For this reason, they are considered short-range interactions. The main component is the dispersion (or London) forces, which originate from the instantaneous dipolar moment resulting from the fluctuation of the electrons around the nuclear protons in nonpolar molecules. The electric field that is created induces, by polarization, a dipole moment in nearby molecules, which, in turn, creates an instantaneous attractive interaction. The two other components of the van der Waals forces are the induction (or Debye) forces related to the interaction between a polar molecule and a nonpolar molecule and the orientation (or Keesom) forces related to the interaction between two polar molecules. With the exception of small, highly polar molecules, such as water, induction and orientation forces are less important than the dispersion forces [43].

The dispersion forces should be treated according to quantum mechanics, and the Lifshitz theory gives an accurate description. Usually, however, the simpler Hamaker approach, obtained by pairwise summation of dispersion energies between all interacting atoms, is used. The Gibbs energy of the London–van der Waals interaction between a spherical protein and a planar surface is:

$$\Delta_{ads}G_{vdW} = -(A_{132}/6)\,[(R/z) + (R/(2R + z)) + \ln\,(z/(2R + z))]$$

with R being the radius of the spherical protein, z the nearest distance between the protein and the planar surface, and A_{132} the Hamaker constant between the protein$_{(1)}$ and the surface$_{(2)}$ in the solvent$_{(3)}$. For $z \ll R$, the following approximation can be used:

$$\Delta_{ads}G_{vdW} \approx -A_{132}\,R/6z$$

When the Hamaker constant A_{132} is not known, its approximate value can be obtained from the following combining rules:

$$A_{132} \approx (A_{131}\,A_{232})^{1/2}$$

and

$$A_{132} \approx (A_{11}{}^{1/2} - A_{33}{}^{1/2})\,(A_{22}{}^{1/2} - A_{33}{}^{1/2})$$

where A_{xx} is the Hamaker constant for two bodies of material x interacting at short distance in the vacuum. For example, published values of Hamaker constants for materials of interest to enzyme activity in soil are the A_{131} value for bovine serum albumin in water [50], which is equal to $6.6\ 10^{-21}$ J, and the A_{232} value for mica in water [43], which is equal to $2.2\ 10^{-20}$ J. The calculated value of the Hamaker constant A_{132} of the interaction of bovine serum albumin with mica in water is,

thus, equal to 1.2 10^{-20} J from the previous combining rules. Hamaker constants are principally related to the number of atoms per unit volume. Therefore, as a first approximation, the value for bovine serum albumin will be very close to that of proteins that are densely packed and statistically composed of the same proportion of different atoms, and the value for mica very close to that of clays that have similar crystalline structures, such as montmorillonite and illite.

Computer Simulation of Enthalpic Effects

The modeling work of Roth and Lenhoff [45] is based solely on interactions of an enthalpic nature, as no effects of solvation or modification of the protein structure are taken into account. It is, thus, an interesting approach for comparisons between the results of experimental studies and theoretical investigations that consider proteins essentially as undeformable and neglecting the contribution of the solvent. In addition, these parameters are not taken into account in the interpretation of early studies on the adsorption of enzymes on clay minerals. The main results of this simulation approach can be divided into four classes.

The first class groups together conclusions that seem logical from what is expected from the enthalpic effects considered. These include the following: an important dependence of the electrostatic energy of interaction on the orientation of the protein; a large effect of the ionic strength of the solution, except when interacting proteins and surfaces bear a low electrical charge; the fact that van der Waals forces only act at short distances of separation; the effect of increasing size of the protein, which, for a constant net electrical charge, leads to decreasing electrostatic interaction and increasing van der Waals interaction.

The second class contains conclusions that seem reasonable but that would have been difficult to reach without this computer simulation approach. Among them, there is the effect of the dipole moment on the adsorption of proteins. For example, the incorporation of the dipole moment of 72.2 D (1 Debye unit = 3.336 10^{-30} C m) on the computation of the interaction energy of lysozyme with the surface has only a negligeable effect, whereas the 516 D dipole moment of chymotrypsinogen A has a very significant effect on the interaction energy.

The conclusions in the third class are surprising from an intuitive point of view, but they are sustained, in some cases, by experimental results. For example, oppositely charged bodies can experience electrostatic repulsion if their surface charge repartitions are mismatched and differ greatly in magnitude. Some results obtained in the chromatographic separation of proteins can be explained by this effect.

The fourth class contains conclusions that totally contradict all experimental studies, despite being logical if only enthalpic effects are considered. The principal conclusion in this class is that with a net zero charge on the protein, very low adsorption is expected on negatively charged surfaces. In contrast, most experimental studies show that maximum adsorption occurs at the isoelectric

point (pI) of proteins. As will be discussed later, this observation can be explained by experimentally demonstrated pH-dependent modifications of proteins interacting with surfaces. Thus, the contrast between results of computer simulations and experimental data emphasizes the limits of the purely enthalpic approach to explaining the adsorption of protein. Obviously, entropic effects, such as hydrophobic and solvation effects and, above all, molecular conformation effects, must be considered.

Entropic Effects

Hydrophobic Interactions and Solvation Effects

One of the most important factors responsible for the stability of proteins in solution is the shielding of amino acids with a hydrophobic side chain in the core of the protein from contact with water. This phenomenon contributes to the decrease of the Gibbs energy of the polypeptidic chain that accompanies its folding. The shielding originates from the "hydrophobic effect," which causes water molecules to establish more hydrogen bonds among themselves in the presence of a nonpolar group than in the presence of a polar group. Water molecules cannot form hydrogen bonds with nonpolar groups and, thus, rearrange themselves around nonpolar groups so as to maximize their mutual association by hydrogen bonds. This mechanism is so efficient that almost all the four hydrogen bonds of the tetrahedrally coordinated water molecule are satisfied when only 3–3.5 bonds, on average, are established in the bulk liquid water [43]. Because exposure of nonpolar groups to the polar solvent means an increased order of the surrounding water, i.e., a decrease in entropy, most nonpolar groups are actually shielded from contact with water in the core of the protein during the folding process.

It can be inferred from this mechanism that the presence of a surface with hydrophobic properties should perturb this spatial arrangement of nonpolar amino acids. Consequently, the adsorption of proteins on surfaces cannot be analyzed without taking into account the interaction of the water molecules of the suspension with both constituents [5,51]. Two hydrophilic entities interacting will tend to retain a hydrated layer between them, whereas two hydrophobic entities will expel water from their surface of interaction. This phenomenon leads to an increase in the entropy of the released water molecules, which decreases the Gibbs energy of the system and promotes adsorption. Thus, the contribution to the energy of interaction of both the proteins and the surfaces of adsorption is dependent on their relative hydrophobicity [5,52].

Proteins with low molecular mass have a higher proportion of their water-accessible external surface composed of nonpolar amino acids [5], which could be related to the higher surface-to-volume ratio of small proteins that makes the burial of nonpolar amino acids in the core of the proteins entropically more diffi-

cult. However, the relation between the size of a protein and its hydrophobicity is not straightforward. For example, Lu et al. [53] have calculated the repartition of hydrophobic amino acids on four proteins: lysozyme, trypsin, immunoglobulin F_{ab}, and hemoglobulin, with a total number of amino acids of 130, 223, 435, and 574, respectively; the percentage of hydrophobic amino acids was 36.2, 34.5, 36.8, and 48.1%, the percentage of all amino acids on the surface was 35.4, 26.0, 16.8, and 16.2%, and the percentage of hydrophobic amino acids on the surface was 13.0, 15.5, 9.6, and 28.0%. Thus, the effect of the size of the protein on the surface-to-volume ratio can be counterbalanced by the proportion of hydrophobic amino acids in the primary structure, as shown by the high surface hydrophobicity of hemoglobin. In addition, as pointed out by Norde [1,4,5], the surface hydrophobicity of proteins is not the only factor involved in hydrophobic interactions of proteins with surfaces, as internal nonpolar amino acids can be exposed to solid surfaces as the result of rearrangement of the protein structure after adsorption. Thus, even large proteins with relatively few hydrophobic patches on their surface can be susceptible to hydrophobic interactions with surfaces.

As with proteins, the effect of the balance between the hydrophobicity and hydrophilicity of surfaces is also complex. One difficulty is linked to the fact that the variation in hydrophobicity between two surfaces is often associated with a modification of their surface chemistry, which results in changes in such properties as the electric charge density or steric interactions if they are made of grafted organic polymers. Because of this, clays provide more interesting models of adsorption surfaces than the organic polymeric surfaces used in most studies. Clays are composed of a structural framework composed of primarily silica and alumina sheets. The electric charge of their basal surfaces originates from isomorphic substitutions inside their crystalline structures, which do not affect the composition of the external atomic layer. In the same type of phyllosilicate structure, surface properties, which depend on the extent of isomorphic substitutions, can range from hydrophobic properties for pyrophyllite to hydrophilic properties for montmorillonite. The origin and the consequences of this phenomenon for protein adsorption will be discussed later.

A second problem is associated with the fact that hydrophobicity/hydrophilicity is originally and basically linked to the affinity of water for surfaces, but this parameter is used for more complex situations [54,55]. Often, a simple macroscopic measurement, such as contact angles of water or other liquids on the surface, is used to deduce hydrophobicity. When complex polymers and surfaces (i.e., amphiphilic, amphoteric, with sorbed ions, or with polymeric tails extending in the solution) are present, their interaction can result in phenomena that are not expected from logical deductions from classical macroscopic measurements of hydrophobicity/hydrophilicity. For example, Staunton and Quiquampoix [56] showed that the adsorption of more hydrophobic proteins relative to more hydrophilic ones could be enhanced on the hydrophilic surfaces of swell-

ing clays. They compared the adsorption on montmorillonite of bovine serum albumin (BSA) with a methylated derivative of this protein. The reductive methylation treatment of the lysine groups did not change their pK_a, and thus, at a given pH, the methylated BSA and the native BSA should have the same electrostatic interactions with the clay surface. However, the hydrophobic derivative of BSA had a higher affinity for the hydrophilic clay surface than the native BSA, with the phenomenon being more pronounced above the pI of the protein. The explanation offered is that the adsorption of proteins is accompanied by the exchange of charge-compensating cations on the clay, which are the true hydrophilic centers, leaving a hydrophobic siloxane layer [57]. Thus, the montmorillonite surface exhibits "hydrophilic properties" for molecules whose adsorption does not result in the removal of charge-compensating cations but in "hydrophobic properties" for molecules that replace the charge-compensating cations.

A computer calculation of the effect of the energies involved in solvation interactions on adsorption of proteins on several polymeric surfaces with different hydrophobicities has been performed by Lu et al. [53]. From the X-ray crystallographic structures of the proteins, they simulated all different possible rotations and brought the proteins in contact with the surface. The solvation interaction energies were calculated, assuming that they were the same as the free energy of transfer of both amino acids and polymer surfaces from water to a nonpolar solvent. The results showed that if the total adsorption energies, i.e., the sum of van der Waals and solvation components, is always negative, the solvation component showed greater variations regarding the nature of the polymeric surface. For the three hydrophobic surfaces (polystyrene, polypropylene, polyethylene) studied, the solvation interaction energies were negative for all proteins studied, indicating a positive contribution to adsorption. However, for the two hydrophilic surfaces (polyHEMA, polyvinyl alcohol) the solvation interaction energies were positive, thus opposing the adsorption of the proteins, even though the stronger van der Waals contribution was in favor of adsorption.

Modifications in Molecular Structure

The interaction of proteins with surfaces is a more complex phenomenon than the interaction of ions or small rigid molecules with surfaces. This increase in complexity cannot be fully described by the basic aspect of adsorption, i.e., the geometric presence of the macromolecule in the vicinity of a plane, because it can lead to modifications of conformation of the interacting proteins. As previously discussed, several types of forces can develop at interfaces, of either enthalpic (electrostatic, van der Waals) or entropic (hydrophobic) nature, and the energy of interaction can be sufficiently intense, with respect to the marginal stability of the three-dimensional structure of proteins in solution, to lead to structural modifications [1,2,5,58]. However, an added difficulty derives from the fact that a modification in the conformation of a protein on a surface is not only a conse-

quence of driving forces promoting adsorption, but it is itself a driving force of adsorption of an entropic nature.

The entropic contribution to adsorption that results from a modification of the conformation of a protein is essentially related to an increase of the rotational freedom of the peptide bonds engaged in secondary structures, such α-helices and β-sheets. These ordered secondary structures represent an important part of the densely packed hydrophobic core of proteins. When a protein is adsorbed on a surface, internal hydrophobic amino acids can reach more external positions in contact with the surface, as the amino acids remain shielded from contact with the water molecules of the surrounding solvent phase. The breaking of hydrogen bonds that maintain the peptide chain in a given conformation, which is associated with a decrease of ordered secondary structures, results in an increase of conformational entropy.

According to Norde and co-workers [4,5], the gain of conformational entropy, S_{conf}, that accompanies this process can be calculated from the assumption that four different conformations are possible for peptide units in random structures as compared with only one in α-helices and β-sheets. Thus:

$$\Delta_{ads} S_{conf} = R \ln 4^n$$

where R is the molar gas constant and n is the number of peptide units involved in the transfer from an ordered secondary structure to a random secondary structure.

B. Experimental Studies with Model Systems

Experimental Evidence for Changes in the Structure of Adsorbed Proteins

As indicated above, modifications in conformation are an important parameter to consider in the adsorption of proteins. These modifications need to be evaluated to explain the variation in the adsorption of proteins on different surfaces and with differences in the physicochemical properties of the solvent phase, such as pH or ionic strength, as the entropic effect linked to these structural changes is itself a factor of adsorption. These three-dimensional changes will, obviously, affect the biological activity of the proteins. Some of the observed consequences of this phenomenon include decreased antigen binding of antibodies in solid-phase immunoassays or decreased enzyme activity, either in reactors or naturally in soils after adsorption on mineral surfaces.

A major difficulty in studying the conformation of adsorbed proteins is related to the fact that the only two efficient techniques for the determination of the tertiary structure are not adapted to adsorption studies. X-ray diffraction requires the preparation of protein crystals, a condition that is not possible with adsorbed proteins. Nuclear magnetic resonance (NMR) spectroscopy, owing to

line-broadening effects, is restricted to proteins with a molecular mass below 20,000 daltons in solution. As the line broadening is related to the rate of molecular tumbling, which averages the anisotropies to zero, adsorption on the surface of large particles will drastically slow the rotational movement, thereby making the determination of the molecular structure of even small adsorbed proteins impossible.

Even if future progress in solid-state NMR spectroscopy makes possible the determination of the structure of an adsorbed protein, it will probably be of no help. As Horbett and Brash [59] have pointed out in a thorough literature survey, proteins are probably adsorbed in multiple states. If this concept is correct, the spectroscopic signals available for structural information will originate from a range of molecules with different conformations.

The practical consequence of these considerations is the inability to determine the tertiary structure of an adsorbed protein by the physical methods presently available, with little hope of improvement in the forseeable future. Thus, only lower levels of structural information on the adsorbed proteins can be obtained. Several techniques exist with which information on the following parameters can be obtained: (1) the secondary structure, (2) the specific interfacial area occupied by adsorbed proteins, and (3) the thickness of the adsorbed layer.

Modification in Secondary Structures

Knowledge of the changes in the repartition of the different secondary structures on adsorption enables not only the possibility to deduce the occurrence of a modification of conformation, but also the direct calculation of the component of the Gibbs energy of adsorption that is linked to this parameter. Circular dichroism [60–63] and infrared spectroscopy [26,48,49,49a] are among the best-suited techniques to investigate secondary structure of proteins.

Circular dichroism has, nevertheless, limitations in turbid suspensions because of light-scattering effects and, thus, has been restricted to studies of adsorption on ultrafine particles with a diameter below 30 nm [61,64–66]. Such particles are only slightly larger than the proteins themselves and are not, for this reason, an ideal model of adsorption surfaces, if only for reasons of the radius of curvature.

Fourier-transform infrared spectroscopy (FTIR) does not have this disadvantage and can be applied to more turbid samples. Baron and Quiquampoix [26,48,49a] have studied the changes in the secondary structure of bovine serum albumin after adsorption on montmorillonite by FTIR spectroscopy. The decomposition of the amide I signal was obtained by acquisition of the frequencies of the different components from second derivative, deconvolution, and computed curvature of the transmission spectra, followed by a least squares iterative fitting with fixed bandwidth and Lorentzian/Gaussian profile for the calculation of the different intensities. The following attributions were made: 1681 cm^{-1} to non-

hydrogen-bonded peptide units in nonpolar domains; 1672 cm^{-1} to non-hydrogen-bonded peptide units in polar domains; 1662 cm^{-1} to peptide units present in slightly disordered α-helices arranged in bundles at the clay surface; 1655 cm^{-1} to classical α-helices; 1639 cm^{-1} to nonordered peptide units associated with water; 1633 cm^{-1} to extended strands forming intramolecular β-domains; and 1619 cm^{-1} to extended β-strands associated by intermolecular hydrogen bonds between adjacent molecules on the clay surface. An important loss, about 25%, of ordered secondary structures of the BSA was observed after adsorption on montmorillonite, which showed the extent of the modification of conformation and the importance of the entropic contribution to adsorption, which was estimated to be -500 kJ mol^{-1}.

Interfacial Area of Protein-Surface Contact

The area of the solid that is covered by the protein is an important parameter in the calculation of the energy of interaction for both direct reasons, such as the work of dehydration of the interface during the process of adsorption, and indirect reasons, such as the lateral electrostatic repulsions between adsorbed proteins, which can increase when the degree of coverage of the solid increases. It can also provide information on the unfolding processes of proteins on the surfaces. The simultaneous knowledge of the quantity of adsorbed protein, the coverage of the surface of the solid, and the total number of adsorbed layers allow the calculation of the specific area of solid occupied by a single protein molecule. These parameters have been obtained by Quiquampoix and Ratcliffe [67] for the adsorption of BSA on montmorillonite using ^{31}P NMR spectroscopy. The method is based on the fact that proteins displace the charge-compensating cations from the clay surfaces. If a small quantity of the paramagnetic cation, Mn^{2+}, is added to a clay suspension containing orthophosphate in solution, an equilibrium of adsorption of the cation is established. The Mn^{2+} paramagnetic cations in solution interact with the orthophosphate molecules, decreasing their spin-spin relaxation time, which results in a linear increase of the line width of the orthophosphate peak in the NMR spectrum. The interesting aspect of this system is that the Mn^{2+} cations adsorbed on the clay surface do not contribute at all to this broadening effect. Thus, when the protein is introduced to the clay suspension, its adsorption will perturb the Mn^{2+} adsorption equilibrium, and the quantity of released Mn^{2+} can be detected by its line-broadening effect on orthophosphate. From the proportion of released Mn^{2+}, the surface coverage of the clay surface by the protein can be deduced, and the interfacial area of contact between a single protein molecule and the clay surface can be calculated. This method allows measurement of the specific interfacial area at different surface coverages and different pH values. It can also be used to distinguish between monolayer and multilayer adsorption, as only the first adsorbed layer of protein is involved in the cation-exchange mechanism.

Thickness of the Protein Layer

This parameter is less useful than the two former parameters, because it is not directly related to the energy of the interacting components, unless it is coupled with force-distance measurements. Nevertheless, it enables information to be gained on possible unfolding of the proteins on the surfaces when the layer thickness is less than the smallest of the X-ray diffraction crystallographic dimensions and on multilayer structures when the thickness is more than the largest crystallographic dimension of the protein. However, results obtained often fall between these two limits, and conclusions cannot be reached unambiguously without additional experimental approaches.

A large range of experimental techniques enable the measurement of the thickness of an adsorbed protein layer. The best known by soil scientists is the measurement of the X-ray basal spacing of clay-protein complexes [11,27,68,69]. Denaturation of the proteins, as well as complex layer structures of proteins with a native structure, has been inferred from these X-ray diffraction data. Nevertheless, great care should be taken in the interpretation of these data. Larsson and Siffert [70] have shown, by comparing X-ray diffraction data with elasticity and X-ray photoelectron spectroscopy data, that the intercalation of montmorillonite by lysozyme results from an association of dispersed, protein-coated clay platelets, rather than by a lateral diffusion of the protein between two layers of montmorillonite. Thus, how these complexes are prepared can influence the layer thickness measured.

Other techniques include ellipsometry [63,71] and reflectometry [72,73], which are based on changes in the state of polarization of reflected light at an interface, and neutron scattering [74], which provides information on the structure of the adsorbed layer from the reflectivity of neutrons when the scattering length density of the solvent matches that of the sorbent surface. The surface force apparatus [75–81] enables the measurement of the forces versus distance between layers of proteins adsorbed on mica surfaces with a resolution of 0.1 nm and a force sensitivity of 10^{-8} N. Thus, it can be used to infer the thickness of an adsorbed layer [82–86]. Atomic force microscopy can also be used to obtain the thickness of adsorbed proteins and the degree of surface coverage.

Principal Models of the Effect of pH on Interaction of Proteins with Solid Surfaces

For soil biochemical reactions, the pH of the soil solution is an important parameter, and the effect of this parameter on the interactions of proteins with surfaces will be discussed. To simplify the vast amount of information published on this subject, the results obtained will be classified according to the interpretations given by the authors of the most general observation, i.e., the fact that the maximum adsorption of a protein on an electrically charged surface occurs often

around the pI of the protein [1,5,39]. This approach is directly related to the interpretation of another general observation, i.e., the pH shift of the optimum catalytic activity of enzymes adsorbed on electrically charged surfaces, as will be discussed later. Three models of the effect of pH on protein adsorption have been proposed.

Symmetric Model with Lateral Repulsions

The term "symmetric" is used for this model and the next, as the same type of mechanism is assumed to cause the decreased adsorption of the protein above, as well as below, its pI. In the first model, it is assumed that the decreasing amount of proteins adsorbed is caused by lateral electrostatic repulsions between the proteins. At the pI, the electrostatic interactions between the adsorbed proteins are minimal. Thus, a compact layer of protein on the surface can be formed. Below the pI, proteins bear a net positive charge, and above the pI, they bear a net negative charge. The electrostatic lateral repulsion between the like-charged molecules decreases their density on the surface [87,88]. This model considers that protein-protein interactions have a more important role than protein-surface interactions and that the modifications of conformation on the surface are very limited. Su et al. [74] studied the adsorption of lysozyme on silica by neutron reflection, and they deduced the structure of the adsorbed layer of protein at different pH values. They interpreted their results according to this model.

Symmetric Model with Modifications of Conformation

The difference between this and the former model is that the decreasing quantity of adsorbed proteins is assumed to be caused by a decreasing structural stability of the adsorbed protein. The origin is again the electric charge of the protein, but the mechanism is less direct. When a protein is at a pH distant from that of its pI, its net electric charge, positive or negative, decreases its structural stability. When exposed under these conditions to surface forces, the proteins undergo an unfolding more easily than at the pI. These structural changes result in a higher area of surface occupied by a single protein and, thus, a lesser quantity can be adsorbed. This model has been developed by Norde and collaborators on various systems [89–104]. The main observation in favor of this model is the indirect evidence of a more unfolded state at a pH other than at the pI, as shown by the dependence on temperature of the initial slope of the adsorption isotherms and by differential scanning calorimetry.

Asymmetric Model with Both Modifications of Conformation and Surface Repulsion

The third model does not assume similar reasons for the lower adsorption at pH values above and below the pI. In this model, the protein-surface interactions have the most important role. For an electronegative surface, a positively charged

protein below its pI unfolds under the strong electrostatic attraction with the surface, and above its pI, the electrostatic repulsion between a negatively charged protein and a negatively charged surface prevents adsorption. Quiquampoix and Ratcliffe [67] used the previously mentioned ^{31}P NMR spectroscopical method of the exchange of paramagnetic Mn^{2+} ions on a montmorillonite surface by BSA to show an example of this model. At a pH above the pI, the maximum amount of protein adsorbed and the amount of Mn^{2+} exchanged decreased in exactly the same proportions, indicating repulsive interactions as the pH increased. At a pH below the pI, the maximum amount of protein adsorbed also decreased, but in this case, there was no variation in the quantity of Mn^{2+} exchanged, which remained constant. This can be explained by a progressive unfolding of the protein on the surface as the pH decreased, and thus, a lower quantity of protein covered the same surface area. As will be shown later, this model is also the best to explain the pH shift of the maximum catalytic activity of adsorbed enzymes.

III. CONSEQUENCES OF INTERFACIAL INTERACTIONS ON ENZYME ACTIVITY IN SOIL

A. Parameters of Enzyme Activity Affected by Adsorption

The interaction of enzymes with surfaces of clay minerals can affect several parameters of enzyme kinetics, decreasing the velocity of the reaction [105–116]. The maximum velocity, V_{max}, and the Michaelis constant, K_m, can change; usually V_{max} decreases and K_m increases. The stability of the adsorbed enzymes against different factors, such as temperature and biodegradation [116–118], has also been studied. Another important effect is the shift of the optimal catalytic activity to higher pH values when enzymes are adsorbed on negatively charged surfaces [119–122]. Because this phenomenon is intimately related to the mechanism of interaction of proteins with surfaces, the latter effect will be primarily discussed.

B. Proposed Models of Interaction

Four different mechanisms can be invoked to explain differences between the catalytic activity of enzymes adsorbed on surfaces and of free enzymes in solution. Two mechanisms are based on properties of the microenvironment of the enzymes, either a static modification of local concentrations of ions, which can cause an interfacial pH effect, or a dynamic effect resulting from a limitation of the diffusion of the substrate toward the surface in the Nernst diffusion layer. Two other mechanisms are not based on the microenvironment but on the enzyme itself, either an orientation effect of the enzyme relative to the surface, which could mask the active site of the enzyme, or an alteration of the structure of the enzyme as the result of surface forces [123,124].

Interfacial pH Effects

The hypothesis most frequently invoked to explain the pH shift in the optimal catalytic activity when an enzyme is adsorbed on an electrically charged surface is the difference in pH between the bulk of the solution (pH_b) and the liquid layer near the solid surface (pH_s) [38,125–127]. The difference is the result of the repartition of the counterions in the vicinity of a charged surface as described in the double diffuse layer theory. As the electric charge of a particle has to be exactly compensated by counterions, some of the counterions are adsorbed on the surface (the Stern layer) and the remaining extend further in a diffuse atmosphere as the result of thermal agitation (the Gouy-Chapman layer). The consequence of the double diffuse layer is an increased concentration of cations, including protons, on an electronegative surface, such as that on clay. Thus, the active site of an adsorbed enzyme on a clay surface could be at a pH lower than that measured in the bulk of the solution (e.g., with a glass pH electrode). Consequently, the apparent pH_b for maximal activity of a bound enzyme appears to be higher than that of a free enzyme, which has lower catalytic activity in the presence of a lower pH_s. Although this difference between pH_b and pH_s explains the pH shift of the catalytic activity of adsorbed enzymes [119,120,122,128], this hypothesis of an interfacial pH effect is being increasingly contested. Some important consequences of this model have not been experimentally verified, and a more thorough examination of its thermodynamic basis is required.

Effect of Ionic Strength on pH Shifts

Despite the fact that a pH surface effect based on the double diffuse layer theory should be sensitive to the ionic strength of the suspension, there have been few studies on the effect of this parameter on the catalytic activity of enzymes adsorbed on clays. Theng [14] attributed this lack of studies to the difficulty associated with the modification of the amount of enzyme bound on surfaces when the ionic strength is varied.

The work of Katchalski and co-workers [128,129] on a covalent polyanionic derivative of trypsin, although not strictly related to adsorption on surfaces, is often invoked to confirm the double diffuse layer theory on the pH shift. In this study, at an ionic strength of $5\ 10^{-3}$ M, the polyanionic derivative had its optimal activity shifted by 2.5 pH units to the alkaline side relative to the free native trypsin. When the ionic strength was increased from $5\ 10^{-3}$ M to 1 M, the pH shift was reduced to 0.5 pH unit. A decrease of the pH shift with an increase in the ionic strength is qualitatively expected from double layer theory. However, the results were normalized to the maximal values obtained at each ionic strength, and the absolute values were not given. As will be emphasized

later, normalized values imply a loss of information, which can mask elements in favor of an alternative explanation.

Leprince and Quiquampoix [130] provided values of the catalytic activity measurements of an extracellular acid phosphatase from an ectomycorrhizal fungus in solution or adsorbed on montmorillonite. Care was taken to correct for the effect of the ionic strength on the adsorption of the enzyme. The optimal pH for the activity of the enzyme in solution remained constant at pH 5.5 when the ionic strength was varied from 20 to 500 mM. In the adsorbed state, the phosphatase had no measurable catalytic activity at an ionic strength of 20 mM, an optimal activity at pH 6.5 at an ionic strength of 40 mM, and the optimal activity remained constant at pH 6 and ionic strength 90–500 mM. This persistence of a pH shift at such a high ionic strength I was not expected, as the thickness of the electric diffuse layer, as calculated by the Debye length, was (in nm) $\kappa^{-1} = 0.304/I^{1/2}$ for the 1:1 electrolyte used in this study. Thus, at an ionic strength of 500 mM, the Debye length should be 0.43 nm, i.e., roughly one-tenth of the size expected for this enzyme with a molecular mass of 51,000 Da. The active site of the phosphatase must be either outside or within the electric diffuse layer at low ionic strength. In the first case, there is no reason for the interfacial pH effect to cause a persistence of a pH shift when the ionic strength is increased to 500 mM. In the latter case, with the active site oriented toward the mineral surface, no catalytic activity should be expected for steric reasons, whatever the ionic strength, which is not what was observed.

Values of the Activity of Enzymes Adsorbed Versus in Solution

An interfacial pH effect resulting from the diffuse double layer of electronegative surfaces should imply a translation of the catalytic activity curve of the adsorbed enzyme on the alkaline side of the pH axis relative to the curve of the enzyme in solution [127]. Thus, at alkaline values, the catalytic activity should be higher in the adsorbed state than in solution. When the measurements are normalized to their maximum values, this is what is apparently often observed. However, when absolute values are given, this theoretical prediction is never observed, as the catalytic activity of an adsorbed enzyme is always lower than that of an enzyme in solution, even on the alkaline side.

For example, McLaren and Estermann [38] showed that the normalized values of the catalytic activity of α-chymotrypsin adsorbed on kaolinite were higher than for the enzyme in solution between pH 9 and 10. Quiquampoix et al. [26] performed a similar experiment with α-chymotrypsin adsorbed on montmorillonite rather than on kaolinite, and they observed the same effect with normalized values between pH 9 and 10. However, they also presented the absolute values from which the normalized values were calculated, and the absolute values appeared to be lower in this pH range. When absolute values were given, the

curve describing the catalytic activity of adsorbed enzymes versus pH was always included in the envelope of the curve of the enzyme in solution, and the apparent pH shift appeared to be the result of a pronounced decrease of catalytic activity at lower pH.

Irreversible Aspects of Variations in pH on the Activity of Adsorbed Enzymes

Another important aspect that mitigates against the surface pH explanation of the pH shift is the observation of irreversible effects that affect the activity of adsorbed enzymes when the pH at which the reaction proceeds is changed relative to the pH at which the adsorption occured. Quiquampoix [131] showed this effect on the activity of a β-D-glucosidase adsorbed on montmorillonite: the enzyme was first incubated with the clay at the pH of adsorption for 2 h (pH_{ads}), then the pH was changed (pH_{react}), and after 2 h to reach a new equilibrium, the catalytic activity was measured at the same pH (pH_{react}). For a given pH_{react}, the catalytic activity decreased when the pH_{ads} was decreased. At a pH_{ads} of 3.6, no catalytic activity could be detected over the entire range of pH_{react}, despite a complete stability of the enzyme in solution when exposed to these changes in pH. This behavior would not be expected if the effect of surface pH was involved, as the 2 h of equilibration should be sufficient to reach a new repartition of the protons in the double diffuse layer. Thus, no lasting effect of pH_{ads} should have been observed if an interfacial pH effect was involved, contrary to the experimental observation.

Thermodynamic Considerations on the Electrochemical Potential

It is not clear that a diffuse double layer should, from a theoretical point of view, affect the optimum pH of an adsorbed enzyme. It has been pointed out by several authors [26,132–135] that although the concentration of protons is different near an electrically charged surface and in the bulk of the solution, their electrochemical potentials must, nevertheless, be identical, as they are in thermodynamic equilibrium. This assumption on the equality of the electrochemical potentials of the ions, regardless of their distance from the surface, is central to the Maxwell-Boltzmann equation, which, along with the Poisson equation, forms the basis of the Gouy-Chapman double diffuse layer theory. Because the electrochemical potential is the molar Gibbs energy of the reactants, it determines the work available for a reaction at constant temperature and pressure. Thus, a higher proton activity near a surface does not mean a higher reactivity of these protons, as their electrochemical potential is the same as in the bulk of the suspension. A confusion has apparently been established between reactions in solution, where the activity of the solutes is the only variable parameter determining the chemical potential, and the reaction near electrically charged surfaces, where an electrostatic term has to be added to the activity parameter. In such systems, only the electrochemical potential has to be considered.

The experimental and theoretical points raised above should convince soil scientists that despite its continuing popularity, the interfacial pH explanation is, at best, a minor factor in the explanation for the pH shift of the optimal catalytic activity of adsorbed enzymes. More probably, it is a totally inadequate explanation.

Diffusion-Limited Reactions

A decrease of the apparent enzyme activity could result from limitations in the diffusion of substrates toward adsorbed enzymes [136–139]. The concentration of substrate is probably lower near the surface of an adsorbed enzyme than in the bulk of the solution, as the result of its consumption by the adsorbed enzyme. A concentration gradient will thus be established. If diffusion is slow with respect to substrate consumption, a steady state will eventually be established such that the rate of diffusion of the substrate in the unstirred layer (Nernst diffusion layer) equals the consumption rate of the substrate. This mechanism will not be treated in depth for two reasons. First, a high density of an enzyme on the surface has to be attained to enable sufficient consumption of the substrate to counterbalance its diffusion toward the surface. This is unlikely in most soil conditions where enzymatic activity is usually low with respect to the large surface area of adsorbent surfaces.

Second, as the rate of consumption of the substrate is the only factor that depends on the enzyme in this process, the rate should not cause a shift in the pH of the optimal catalytic activity. The enzyme activity versus pH follow a bell-shaped curve, and thus, there are always two pH values, below and above the optimal pH for activity, where the enzyme activity is similar, and, consequently, the decrease in catalytic activity as a result of a limitation in diffusion of the substrate should be the same. The expected effect should, thus, be symmetrical with respect to the optimal pH, and with no pH shift. This applies well for the conditions under which the enzyme activity in soils is usually measured, i.e., with a substrate concentration much greater than the Michaelis constant. However, under the conditions that normally exist in natural soil, the substrate concentration may be lower than the Michaelis constant. If the pH dependence of the Michaelis constant is different from the pH dependence of the maximal velocity of the enzyme reaction, a shift in pH can be expected. Nevertheless, this pH shift would depend only on the intrinsic properties of the enzyme, and no relation between the pH shift and the sign of the electrical charge of the surface of adsorption is expected.

Orientation Effects

Another possible factor that could decrease the catalytic activity of an enzyme after adsorption is an orientation of the active site toward the surface [140]. Few studies have been devoted to the occurrence of this mechanism. Before giving

examples of cases where this mechanism may be involved, it should be pointed out that this mechanism cannot explain the general observation of an alkaline pH shift in the optimal activity of enzymes adsorbed on electronegatively charged surfaces.

A preferential orientation of a given face of the enzyme on an electronegative surface is expected if a higher density of positively charged amino acids is found on this face. Four cases can be distinguished. If the more positively charged face of the enzyme remains the same over the entire range of pH studied, either a complete inhibition of the enzyme could be expected, if the active site is localized on this face, or no reduction of enzyme activity should be observed if the catalytic site is on the opposite, less positive face. This could be the case if the density of Lys and Asp, whose pK_a values are above pH 10, is particularly important. If the more positively charged face changes when the pH is increased, an alkaline shift of the optimal enzyme activity should be observed if the active site is on this face, and, conversely, an acidic shift should be observed if the active site is on the opposite face. This can be the case if His, with a pK_a of 6, is involved or if there is a dissimetry in the repartition of neutral amino acids and amino acids with a carboxylic side chain, such as Glu and Asp. Neutral amino acids remain neutral whatever the pH, but Glu and Asp with a pK_a of 4 will change from electroneutrality to electronegativity when the pH increases. Thus, a more positively charged face at low pH can lose this electrical charge property at higher pH if it is enriched in Asp and Glu residues.

In addition to these four cases, a variety of intermediate situations, where the active site is neither completely masked by the surface of adsorption nor completely exposed to the solvent, can be expected. Thus, orientation of the active site on the surface cannot be the major mechanism involved in the modification of the catalytic activity of enzymes adsorbed on clays, as it cannot explain the generality of the alkaline pH shift. On the other hand, it would be surprising if this mechanism does not have a role to a certain extent. In particular, it is likely for enzymes composed of proteins defined by Norde [1,5] as "hard" proteins, for which a pH-dependent modification of conformation is less likely than for "soft" proteins, and for enzymes with a large dipolar moment that are more likely to be adsorbed with a preferential orientation.

Such a case has been described by Lee and Belfort [140] for a ribonuclease A during adsorption on mica. Ribonuclease A is considered by Norde as a hard protein [1,5]. In addition, Yoon and Lenhoff [44] have calculated a relatively large dipole moment of 461 D of its charge distribution at pH 7 with the positive end pointing toward the region of the active site. The effect of pH has not been studied by Lee and Belfort, but reorientation of the enzyme on the surface has been followed over time by combining both surface force-distance measurements and enzyme activity assays. The catalytic activity of the ribonuclease adsorbed

on mica increased linearly with the square root of the time from 16% to 78% relative to equal amounts of enzyme in solution over a period of 1–24 h. Corrections for the amount of adsorbed enzyme were made by measurement of the mean refractive index by multiple-beam interferometry along with the force-distance measurements, and the adsorbed amounts varied only from 1.7 to 2.5 mg/m^2. Over the same period, the force-distance measurements allowed determination of the thickness of the protein layer, and the thickness increased linearly with the square root of the time from a size matching the smallest axis of the ribonuclease (taken from crystallographic data and assuming a hydration of one water layer) after 1 h to a size equal to the longest axis after 24 h. These data, along with the square root time dependence of these phenomena, strongly suggest a translational diffusion process leading to molecular reorientation of the ribonuclease from an initial side-on adsorption state with the active site in the positive electrostatic potential region facing the mica surface, followed by a progressive "standing up" of the enzyme, which exposes the active site to the solvent and, hence, to the substrate.

Another example is the pH dependence of the adsorption of α-chymotrypsin on montmorillonite, the study of which has been performed by a comparison of the activity of the adsorbed enzyme and its secondary structure determined by FTIR [140a]. In comparison with BSA, a soft protein, the changes in the secondary structure of α-chymotrypsin (241 amino acids) were minor on adsorption and suggested only 10–15 peptide units in the peripheral domains of the protein. Thus, it is unlikely that a modification of conformation could explain the observed decrease of the catalytic activity after adsorption when the pH was decreased from 9 to 7, with a complete absence of activity below pH 7. On the other hand, an orientational effect could explain this effect, as the active site of α-chymotrypsin is located in a domain where two His and one amine end chain will lead to an increase of the positive charge below pH 7, allowing the enzyme to adsorb with the active site facing the clay surface.

pH-Dependent Modifications of Conformation

In contrast to the three preceding models, all of which assume that the enzymes retain the same conformation in the adsorbed state and in solution, another model is based on a pH-dependent unfolding of the enzyme on the surface. Among the different models of interaction of proteins with surfaces versus pH discussed in the preceding section, the asymmetric model is better suited than the symmetric models to explain a pH shift in the activity of the adsorbed enzyme. Indeed, the symmetric model with lateral repulsions assumes a stable structure of the protein on adsorption. Thus, no effect on enzyme activity should be observed, except if, in some cases, there is an orientation effect. The symmetric model with modifications of conformation could explain the decreased activity of enzymes on surfaces

but not its pH shift, as the structural rearrangement of the protein occurs at pH values below and above the pI, i.e., on both the acid and alkaline sides of the pH activity curve of the enzyme.

Only the asymmetric model, with both modifications of conformation and surface repulsions, can explain the pH shift in the maximal catalytic activity of adsorbed enzymes. Different enzyme systems studied by Quiquampoix and coworkers [26,49,130,131,141,142] can be interpreted according to this model. The interactions between the enzymes and the mineral surfaces at different pH values were studied using three procedures. Procedure A is a measurement of the enzyme activity in the absence of adsorbent surfaces. Procedure B is a measurement of the enzyme activity in the presence of the adsorbent surface. Because in procedure B, all enzyme molecules are not necessarily adsorbed, procedure C is designed to determine the catalytic activity of the nonadsorbed fraction in procedure B. It is a measurement of the enzyme activity in the supernatant after centrifugation of the enzyme-adsorbent surface suspension. If A, B, and C are the values of the catalytic activity measured by the respective procedures, useful parameters may be calculated. The first is F, the proportion of nonadsorbed enzyme:

$$F = C/A$$

And the second is the relative activity, R, which is defined by the ratio of the catalytic activity resulting from the enzyme fraction adsorbed (B − C) and that of an equal quantity of enzyme in solution (A − C):

$$R = (B - C)/(A - C)$$

These two parameters are well suited to a physicochemical analysis of the interaction of enzymes with solid surfaces, as F is inversely related to the affinity for the surface and R is a structure-related parameter.

The data obtained with the β-D-glucosidase of *Aspergillus niger* [142], which has a pI of 4.0, and montmorillonite provide a good example. At a pH above the pI, the nonadsorbed fraction, F, increases progressively with pH until no enzyme is adsorbed. This shows the electrostatic repulsions between the net negatively charged enzyme and the electronegative clay surface. In contrast, when the pH decreases below the pI, the relative activity, R, of the enzyme decreases as the result of a progressive unfolding caused by the electrostatic attractions between the electronegative surface and the positively charged enzyme in this range of pH. The pH dependence of these parameters related to the affinity for the surface and the protein structure is similar to results obtained for the adsorption of BSA on montmorillonite.

C. Irreversibility Aspects

According to Norde [1,5,143], adsorption of proteins on surfaces is a quasi-irreversible phenomenon, and the use of the Langmuir equation to describe adsorp-

tion isotherms should be proscribed. When after adsorption at a given concentration of protein the system is diluted, little or no desorption is usually observed, in contrast to what would be expected in a reversible phenomenon [144]. An example of irreversible effects of pH changes on the activity of adsorbed enzyme has also been given in the discussion of the interfacial pH effect [131]. The reason is probably to be found in the addition of the different thermodynamic contributions to the adsorption process, which can act in a synergistic manner, each component amplifying the contribution of the other components. The example of the adsorption of BSA on montmorillonite can illustrate this point [56,67]. If the protein unfolds on adsorption on the clay surface, then the number of contact points with the surface increases, and there is also an increase in the rotational freedom of the polypeptide chain accompanying the decrease of the ordered secondary structure. After removal of the positively charged counterions by the positively charged amino acids of the protein, the hydrophobic amino acids in the core of the protein can interact with the hydrophobic siloxane surface of the tetrahedral layer of clays and remain shielded from contact with water in the surrounding solvent phase. This phenomenon can explain the observed irreversibility, as the energy necessary to return to the original conformation may be higher than the thermal energy available to the system.

D. Thermal Stability

The thermal stability of adsorbed enzymes is often described as being enhanced relative to that of the free enzymes [117]. However, as with studies on the optimal pH of adsorbed enzymes, results are often represented relative to the activity of the enzyme at the beginning of the thermal treatment. At this point, the adsorption process itself has been the cause of a modification of the conformation of the enzyme. Thus, the relative activity observed in thermal stability experiments can represent only a small part of the activity of the original free enzyme and can be not representative of the entire process. According to Steadman et al. [145], if the whole system is taken into account and not only the active enzyme fraction, proteins have a lower thermal stability when adsorbed than in solution. The differential scanning calorimetry and front surface fluorescence studies of Steadman et al. [145] were in good agreement with similar results obtained by Norde and co-workers [5].

E. Protective Effect of Organic Matter

This aspect will be only briefly treated, as it has been recently reviewed by Nannipieri et al. [146]. The case where the enzymes and the humic substances are covalently bound will not be discussed, because it is outside the scope of this review on the effect of noncovalent intermolecular forces. Nevertheless, some

experimental work has indicated that a purely physical phenomenon could be involved in the protection of enzyme activity in the presence of clay-humic complexes. This mechanism is based on the exchange of an adsorbed polymer by another polymer with a greater affinity for the surface [26,141]. A comparison between the effect on the catalytic activity of a β-D-glucosidase of a natural clay-humic complex and artificial clay-organic polymer complexes of different energies of interaction [141] has shown that the enzyme can displace organic polymers with a lower energy of interaction with the surface than the enzyme if, for example, the electric charge of the enzyme is positive. If conditions are less favorable for the displacement phenomenon, e.g., if the enzyme is negatively charged, the activity of the enzyme is protected from the denaturing effect of the mineral surface by the organic layer.

F. Selection Pressure of Adsorption Surfaces

An emerging topic in biotechnology is the industrial application of enzymes originating from extremophile microorganisms. These enzymes have stable structures when placed in media with extreme values of temperature, pH, or salinity [147–149]. As one of the main consequences of the interaction of proteins with surfaces is a modification of the conformation of the protein, the question arises as to whether the selection pressure of mineral surfaces has made the enzymes secreted by soil microorganisms more stable after adsorption than the enzymes whose natural environment is free of adsorbing surfaces. Few investigations have been devoted to this aspect of biological evolution in soil. Matumoto-Pintro and Quiquampoix [150] have compared the effect of interaction with clays of four phytases. For the two phytases (from wheat and *Suillus collinitus*) not naturally secreted in soil, the adsorption on clays led to a total inactivation of the enzyme activity, whereas the two other phytases, which were naturally secreted in soil (from *Aspergillus ficuum* and *Hebeloma cylindrosporum*), retained a significant catalytic activity in presence of clays. Thus, as with extremophile microorganisms, a physical constraint of the external medium could have exerted a selection pressure during evolution of soil microorganisms toward more stable enzymes.

IV. CONCLUSIONS

The adsorption of proteins on solid surfaces is a complex phenomenon. Both enthalpic (electrostatic and van der Waals forces) and entropic (hydrophobic interactions and molecular structural rearrangements) effects are involved, and these do not act independently. The subtle balance between the intrinsic stability of the protein in solution, the physicochemical properties of the solid surface, and the pH and ionic strength of the solvent phase will have important effects

on the state of the adsorbed enzyme. Accordingly, the effect of adsorption on mineral surfaces on the catalytic activity of extracellular enzymes in soil should be mainly interpreted as a change in the conformation of the enzyme rather than as a microenvironmental effect. In particular, it has been shown, from experimental observations as well as for theoretical reasons, that a surface pH effect is not an appropriate explanation for the shift in the pH of the optimal enzyme activity when adsorbed on clays. In contrast, an asymmetric model for the interpretation of the maximum adsorption of proteins at their pI offers a logical explanation for the shift in the pH of the optimal enzyme activity. At pH values below the pI of the enzyme, the electrostatic interactions between the positively charged enzymes and negatively charged clay surfaces lead to an unfolding of the enzymes. This explains both the decrease in enzyme activity and the lower amount of protein adsorbed at a pH below its pI, as the increase of the specific interfacial area between a single protein molecule and the solid surface allows less protein to be adsorbed at saturation. At a pH above the pI, the electrostatic interactions are repulsive between the net negatively charged enzymes and net negatively charged clays, which explains the decreasing amount of protein adsorbed when the pH increases and, thus, the increasing ability of extracellular enzymes to diffuse in the water-filled soil pore network. In addition, hydrophobic interactions are also implicated in the interaction of proteins with clays, even if the latter, such as smectites, have marked hydrophilic properties. This shows the interplay between different driving forces in adsorption, as the hydrophobic interactions with clays result from a primary electrostatic exchange of the hydrophilic counterions on the clay surface leaving a hydrophobic siloxane surface. The rearrangement of the protein structure on the clay surface is facilitated when hydrophobic amino acids come in contact with the hydrophobic siloxane layer, and remain shielded from the water molecules of the solvent. This rearrangement is accompanied by a decrease of ordered secondary structures, which implies an increase in the rotational freedom of the peptide bond. This increase in conformational entropy can be an important contribution to the decrease of the Gibbs energy of adsorption. The combination of all these different subprocesses, which can amplify synergistically their effects, is responsible for the quasi-irreversibility of adsorption and the modifications of the conformation of enzymes on clay surfaces. An interesting question arises about the possibility of a selection pressure of mineral surfaces in soil on the structural stability after adsorption of extracellular enzymes secreted by soil microorganisms.

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REFERENCES

1. Norde W. Adsorption of proteins from solution at the solid-liquid interface. Adv Colloid Interface Sci 25:267—340, 1986.

2. Norde W, J Lykiema. Protein adsorption and bacterial adhesion to solid surfaces: a colloid-chemical approach. Colloids Surfaces 38:1—13, 1989.

3. Suzawa T, H Shirahama. Adsorption of plasma proteins onto polymer lattices. Adv Colloid Interface Sci 35:139—172, 1991.

4. Norde W, J Lyklema. Why proteins prefer surfaces. J Biometer Sci Polymer Edn 2:183—202, 1991.

5. Haynes CA, W Norde. Globular proteins at solid/liquid interfaces. Colloids Surfaces B: Biointerfaces 2:517—566, 1994.

6. Armstrong DE, G Chesters. Properties of protein-bentonite complexes as influ enced by equilibration conditions. Soil Sci 98:39—52, 1964.

7. Mortland MM. Clay-organic complexes and interactions. Adv Agron 22:75—117, 1970.

8. Hailer RD, G Stotzky. Formation of clay-protein complexes. Soil Sci Soc Am Proc 35:383—389, 1971.

9. Ross DJ, BA McNeilly. Some influences of different soils and clay minerals on the activity of glucose oxidase. Soil Biol Biochem 4:9—18, 1972.

10. Albert iT, RD Hailer. Adsorption of lysozyme and ovalbumin by clay: effect of clay suspension pH and clay mineral type. Soil Sd 115:130—136, 1973.

11. Hailer RD, G Stotzky. X-ray diffraction, electron microscopy, electrophoretic mobility, and pH of some stable smectite-protein complexes. Soil Sci Soc Am Proc 37:116—123, 1973.

12. Kiss 5, M Dragan-Bularda, D Radulescu. Biological significance of enzymes accumulated in soil. Adv Agron 27:25—87, 1975.

13. Burns RG. Enzyme activity in soil: some theoretical and practical considerations. In: RG Burns, ed. Soil Enzymes. New York: Academic Press, 1978, pp 295—340.

14. Theng BKG. Proteins and enzymes. In: BKG Theng, ed. Formation and Properties of Clay-Polymer Complexes. Amsterdam: Elsevier, 1979, pp 157—226.

15. Bums RG. Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biol Biochem 14:423—427, 1982.

16. Stotzky G, RG Burns. The soil environment: clay-humus-microbe interactions. In: RG Bums, JH Slater, eds. Experimental Microbial Ecology. Oxford: Blackwell Scientific Publications, 1982, pp 105—133.

17. Frankenberger WT, WA Dick. Relationships between enzyme activities and microbial growth and activity indices in soil. Soil Sci Soc Am J 47:945—951, 1983.

18. Bums RG. Interaction of enzymes with soil mineral and organic colloids. In: PM Huang, M Schnitzer, eds. Interactions of Soil Minerals with Natural Organics and Microbes. Madison, WI: Soil Science Society of America, 1986, pp 429—451.

19. Stotzky G. Influence of soil mineral colloids on metabolic processes, growth, adhesion, and ecology of microbes and viruses. In: PM Huang, M Schnitzer, eds. Inter actions of Soil Minerals with Natural Organics and Microbes. Madison, WI: Soil Science Society of America, 1986, pp 305—428.

20. Fusi P, GG Ristori, L Calamai, G Stotzky. Adsorption and binding of protein on "clean" (homoionic) and "dirty" (coated with Fe oxyhydroxides) montmorillon ite, illite and kaolinite. Soil Biol Biochem 21:911—920, 1989.

21. Boyd SA, MM Mortland. Enzyme interactions with clays and clay-organic matter complexes. In: JM Bollag, G Stotzky, eds. Soil Biochemistry 6. New York: Marcel Dekker, 1990, pp 79—122.

22. Nannipien P. JM Bollag. Use of enzymes to detoxify pesticide-contaminated soils and waters. J Environ Qual 20:510—517, 1991.

23. Bollag JM. Decontaminating soil with enzymes: an in situ method using phenolic and anilic compounds. Environ Sci Technol 26:1876—1881, 1992.

24. Lähdesmiiki P, R Piispanen. Soil enzymology: role of protective colloid systems in the preservation of exoenzyme activities in soil. Soil Biol Biochem 24:1173— 1177, 1992.

25. Gianfreda L, JM Bollag. 1994. Effect of soils on the behavior of immobilized enzymes. Soil Sci Soc Am J 58:1672—1681, 1992.

26. Quiquampoix H, J Abadie, MH Baron, F Leprince, PT Matumoto-Pintro, RG Ratcliffe, S Staunton. Mechanisms and consequences of protein adsorption on soil mineral surfaces. In: TA Horbett, JL Brash, eds. Proteins at Interfaces II, ACS Symposium Series 602. Washington, DC: American Chemical Society, 1995, pp 321—333.

27. Violante A, A De Cristofaro, MA Rao, L Gianfreda. Physicochemical properties of protein-smectite and protein-Al(OH) complexes. Clay Miner 30:325— 336, 1995.

28. Ruggiero P, J Dee, JM Bollag. Soil as a catalytic system. In: G Stotzky, JM. Bollag, eds. Soil Biochemistry 9. New York: Marcel Dekker, 1996, pp 79—122.

29. Tabatabai MA, M Fu. Extraction of enzymes from soils. In: G Stotzky, JM Bollag, eds. Soil Biochemistry 7. New York: Marcel Dekker, 1996, pp 197—227.

30. Gianfreda L, JM Bollag. Influence of natural and anthropogenic factors on enzyme activity in soil. In: 0 Stotzky, JM Bollag, eds. Soil Biochemistry 9. New York: Marcel Dekker, 1996, pp 123—193.

31. Mousain D, P Matumoto-Pintro, H Quiquampoix. Le role des mycorhizes dans la nutrition phosphatée des arbres forestiers. Rev For Fr 49 (n° sp):67—81, 1997.

32. Plassard C, M Chalot, B Botton, F Martin. Le rôle des ectomycorhizes dans la nutrition azotée des arbres forestiers. Rev For Fr 49 (n° sp):82—98, 1997.

33. Mattson S. The laws of soil colloidal behavior. VII. Proteins and proteinated complexes. Soil Sci 23:41—72, 1932.

34. Ensminger LE, JE Gieseking. The adsorption of proteins by montmorillonitic clays. Soil Sci 48:467—47 1, 1939.

35. Ensminger LE, JE Gieseking. The adsorption of proteins by montmonllonitic clays and its effect on base exchange capacity. Soil Sci 51:125—132, 1941.

36. Mortland MM, JE Gieseking. The influence of clay minerals on the enzymatic hydrolysis of organic phosphorus compounds. Soil Sci Soc Am Proc 16:10—13, 1952.

37. McLaren AD. The adsorption and reactions of enzymes and proteins on kaolinite. J Phys Chem 58:129—137, 1954.

38. McLaren AD, EF Estermann. Influence of pH on the activity of chymotrypsin at a solid-liquid interface. Arch Biochem Biophys 68:157—160, 1957.

39. McLaren AD, GH Peterson, I Barshad. The adsorption and reactions of enzymes and proteins on clay minerals. IV. Kaolinite and montmorillonite. Soil Sci Soc Am Proc 22:239—244, 1958.

40. Norde W, J Lykiema. Thermodynamics of protein adsorption. Theory with special reference to the adsorption of human plasma albumin and bovine pancreas ribo nuclase at polystyrene surfaces. J Colloid Interface Sci 71:350—366, 1979.

41. Lyklema i. Proteins at solid-liquid interfaces. A colloid-chemical review. Colloids Surfaces 10:33—42, 1984.

42. Norde W. Energy and entropy of protein adsorption. J Dispers Sci Technol 13: 363—377, 1992.

43. Israelachvili J. Intermolecular and Surface Forces. 2nd ed. London: Academic Press, 1991.

44. Yoon Bi, AM Lenhoff. Computation of the electrostatic energy between a protein and a charged surface. J Phys Chem 96:3130—3134, 1992.

45. Roth CM, AM Lenhoff. Electrostatic and van der Waals contributions to protein adsorption: computation of equilibrium constants. Langmuir 9:962—972, 1993.

46. Van Dulm P, W Norde, J Lyklema. Ion participation in protein adsorption at solid surfaces. J Colloid Interface Sci 82:77—82, 1981.

47. Norde W. Ion participation in protein adsorption at solid surfaces. Colloids Surfaces 10:21—31, 1984.

48. Baron MH, H Quiquampoix. Conformational changes of bovine serum albumin on adsorption on montmorillonite: a FTIR study. In: T Theophanides, J Anastassopou Lou, N Fotopoulos, eds. Fifth International Conference on the Spectroscopy of Bio logical Molecules. Dordrecht: Kiuwer Academic Press, 1993, pp. 109—111.

49. Quiquampoix H, S Staunton, MH Baron, RG Ratcliffe. Interpretation of the pH dependence of protein adsorption on clay mineral surfaces and its relevance to the understanding of extracellular enzyme activity in soil. Colloids Surfaces A: Physicochem Eng Aspects 75:85—93, 1993.

49a. Servagent-Noinville S, M Revault, H Quiquampoix, MH Baron. Conformational changes of bovine serum albumin induced by adsorption on different clay surfaces: FTIR analysis. J Colloid Interface Sci. In press.

50. Nir S. Van der Waals interactions between surfaces of biological interest. Prog Surf Sci 8:1—58, 1976.

51. Lee SH, E Ruckenstein. Adsorption of proteins onto polymeric surfaces of different hydrophilicities—a case study with bovine serum albumin. J Colloid Interface Sci 125:365—379, 1988.

52. Bowen WR, DT Hughes. Ion exchange of proteins: A microcalorimetric study of the adsorption of bovine serum albumin on anion-exchange materials. i Colloid Interface Sci 158:395—402, 1993.

53. Lu DR, SJ Lee, K Park. Calculation of solvation interaction energies for protein adsorption on polymer surfaces. J Biomater Sci Polymer Edn 3:127—147, 1991.

54. Van Oss Ci, RF Giese. The hydrophilicity and hydrophobicity of clay minerals. Clays Clay Miner 43:474—477, 1995.

55. Vogler EA. Structure and reactivity of water at biomaterial surfaces. Adv Colloid Interface Sci 74:69—117, 1998.

56. Staunton S, H Quiquampoix. Adsorption and conformation of bovine serum albumin on montmorillonite: modification of the balance between hydrophobic and electrostatic interactions by protein methylation and pH variation. J Colloid Interface Sci 166:89—94, 1994.

57. Chassin P. C Jouany, H Quiquampoix. Measurement of the surface free energy of calcium-montmorillonite. Clay Miner 21:899—907, 1986.

58. Lundström I. Models of protein adsorption on solid surfaces. Prog Colloid polymerSci 70:76—82, 1985.

59. Horbett TA, JL Brash. Proteins at interfaces: current issues and future prospects. In: JL Brash, TA Horbett, eds. Proteins at Interfaces, ACS Symposium Series 343. Washington DC: American Chemical Society, 1987, pp 1—33.

60. Geluk MA, W Norde, HKAI Van Kalsbeek, K Van't Riet. Adsorption of lipase from Candida rugosa on cellulose and its influence on lipolytic activity. Enzyme Microb Technol 14:748—754, 1992.

61. Kondo A, K Higashitani. Adsorption of model proteins with wide variation in molecular properties on colloidal particles. i Colloid Interface Sci 150:344—351, 1992.

62. Norde W, JP Favier. Structure of adsorbed and desorbed proteins. Colloids Surfaces 64:87—93, 1992.

63. Wu H, Y Fan, i Sheng, SF Sui. Induction of changes in the secondary structure of globular proteins by a hydrophobic surface. Eur Biophys J 22:201—205, 1993.

64. Kondo A, F Murakami, K Higashitani. Circular dichroism studies on conformational changes in protein molecules upon adsorption on ultrafine polystyrene particles. Biotechnol Bioeng 40:889—894, 1992.

65. Kondo A, F Murakami, M Kawagoe, K Higashitani. Kinetic and circular dichroism of enzymes adsorbed on ultrafine silica particles. AppI Microbiol Biotechnol 39: 726—731, 1993.

66. Tian M, WK Lee, MK Bothwell, J McGuire. Structural stability effects on adsorption of bacteriophage T4 lysozyme to colloidal silica. J Colloid Interface Sci 200: 146—154, 1998.

67. Quiquampoix H, RG Ratcliffe. A 31 NMR study of the adsorption of bovine serum albumin on montmorillonite using phosphate and the paramagnetic cation Mn modification of conformation with pH. i Colloid Interface Sci 148:343—352, 1992.

68. Morgan HW, CT Corke. Adsorption, desorption and activity of glucose oxidase on selected clay species. Can J Microbiol 22:684—693, 1976.

69. Garwood GA, MM Mortland, Ti Pinnavala. Immobilization of glucose oxidase on montmorillonite clay: hydrophobic and ionic modes of binding. i Mol Cat 22:153— 163, 1983.

70. Larsson N, B Siffert. Formation of lysozyme-containing crystals of montmorillonite. .1 Colloid Interface Sci 93:424—431, 1983.

71. Arwin H. Spectroscopic ellipsometry and biology: recent developments and challenges. Thin Solid Films 313—314:764—774, 1998.

72. Norde W, F MacRitchie, G Nowicka, J Lykiema. Protein adsorption at solid-liquid interfaces: reversibility and conformational aspects. I Colloid Interface Sci 112: 447—456, 1986.

73. Schaaf P, P Dejardin. Structural changes within an adsorbed fibrinogen layer during the adsorption process: a study by scanning angle reflectometry. Colloids Surfaces 31:89—103, 1988.

74. Su Ti, JR Lu, BK Thomas, ZF Cui, J Penfold. The effect of solution pH on the structure of lysozyme layers adsorbed at the silica-water interface studied by neutron reflection. Langmuir 14:438—445, 1998.

75. Afshar T, AL Bailey, PF Luckham, W MacNaughtan, D Chapman. Forces between proteins and model polypeptides adsorbed on mica surfaces. Biochim Biophys Acta 915:101—111, 1987.

76. Gallinet JP, B Gauthier-Manuel. Adsorption-desorption of serum albumin on bare mica surfaces. Colloids Surfaces 68:189—193, 1992.

77. Luckham PF, BA de L Costello. Recent developments in the measurement of inter-particle forces. Adv Colloid Interface Sci 44:183—240, 1993.

78. Blomberg E, PM Claesson, RD Tolton. Short-range interaction between adsorbed layers of human serum albumin. J Colloid Interface Sci 166:427—436, 1994.

79. Blomberg E, PM Claesson, JC Froberg, RD Tilton. Interaction between adsorbed layers of lysozyme studied with the surface force technique. Langmuir 10:2325— 2334, 1994.

80. Nylander T, NM Wahlgren. Forces between adsorbed layers of J Langmuir 13:6219—6225, 1997.

81. Froberg JC, I Arnebrant, J McGuire, PM Claesson. Effect of structural stability on the characteristics of adsorbed layers of T4 lysozyme. Langmuir 14:456—462, 1998.

82. Eppel SJ, FR Zypman, RE Marchant. Probing the resolution limits and tip interac tions of atomic force microscopy in the study of globular proteins. Langmuir 9: 2281—2288, 1993.

83. Batey AM, PK Leavitt, CA Siedlecki, BJ Tyler, PA Suci, RE Marchant, GG Geesey. Adsorption of adhesive proteins from the marine mussel, Mytilus edulis, on polymer films in the hydrated state using angle dependent X-ray photoelectron spectroscopy and atomic force microscopy. Langmuir 13:5702—5710, 1997.

84. Ta TC, MT Sykes, MT McDermott. Real-time observation of plasma protein film formation on well-defined surfaces with scanning force microscopy. Langmuir 14: 2435—2443, 1998.

85. Waner MJ, M Gilchrist, M Schlindler, M Dantus. Imaging the molecular dimen sions and oligomerization of proteins at liquid/solid interfaces. J Phys Chem B 102:1649—1657, 1998.

86. Bowen WR, N Hilal, RW Lovitt, CJ Wright. Direct measurements of interactions between protein layers using an atomic force microscope. J Colloid Interface Sci 197:348—352, 1998.

87. Duinhoven 5, R Poort, G Van der Voet, WGM Agterof, W Norde, J Lykiema. Driving forces for enzyme adsorption at solid-liquid interfaces. 1. The serine prote ase savinase. J Colloid Interface Sci 170:340—350, 1995.

88. Duinhoven S, R Poort, G Van der Voet, WGM Agterof, W Norde, J Lyklema. Driving forces for enzyme adsorption at solid-liquid interfaces. 2. The fungal lipase lipolase. J Colloid Interface Sci 170:351—357, 1995.

89. Norde W, J Lyklema. The adsorption of human plasma albumin and bovine pan creas ribonuclease at negatively charged polystyrene surfaces I. Adsorption iso therms. Effects of charge, ionic strength, and temperature. J Colloid Interface Sci 66:257—265, 1978.

90. Norde W, J Lykiema. The adsorption of human plasma albumin and bovine pancreas ribonuclease at negatively charged polystyrene surfaces. II. Hydrogen ion titrations. J Colloid Interface Sci 66:266—276, 1978.

91. Norde W, J Lyklema. The adsorption of human plasma albumin and bovine pancreas ribonuclease at negatively charged polystyrene surfaces. III. Electrophoresis. J Colloid Interface Sci 66:277—284, 1978.

92. Norde W, J Lyklema. The adsorption of human plasma albumin and bovine pancreas ribonuclease at negatively charged polystyrene surfaces. IV. The charge distribution in the adsorbed state. J Colloid Interface Sci 66:285—294, 1978.

93. Norde W, J Lyklema. The adsorption of human plasma albumin and bovine pancreas ribonuclease at negatively charged polystyrene surfaces. V. Microcalorimetry. J Colloid Interface Sci 66:295—302, 1978.

94. Koutsoukos PG. CA Mumrne-Young, W Norde, J Lykiema. Effect of the nature of the substrate on the adsorption of human plasma albumin. Colloids Surfaces 5: 93—104, 1982.

95. Koutsoukos PG, W Norde, J Lyklema. Protein adsorption on hematite (a-Fe surfaces. J Colloid Interface Sci 95:385—397, 1983.

96. Van DuIm P. W Norde. The adsorption of human plasma albumin on solid surfaces, with special attention to the kinetic aspects. J Colloid Interface Sci 91:248—255, 1983.

97. Arai T, W Norde. The behavior of some proteins at solid-liquid interfaces 1. Ad sorption from single protein solutions. Colloids Surfaces 51:1—15, 1990.

98. Shirahama H, J Lyklema, W Norde. Comparative protein adsorption in model sys tems. J Colloid Interface Sci 139:177—187, 1990.

99. Elgersma AV, RU Zsom, W Norde, J Lyklema. The adsorption of bovine serum albumin on positively and negatively charged polystyrene lattices. J Colloid Inter face Sci 138:145—156, 1990.

100. Norde W, ACI Anusiem. Adsorption, desorption and re-adsorption of proteins on solid surfaces. Colloids Surfaces 66:73—80, 1992.

101. Bos MA, Z Shervani, AC! Anusiem, M Giesbers, W Norde, JM Klejin. Influence of the electric potential of the interface on the adsorption of proteins. Colloids Surfaces B 3:91—100, 1994.

102. Haynes CA, E Sliwinsky, W Norde. Structural and electrostatic properties of globular proteins at a polystyrene-water interface. J Colloid Interface Sci 164:394—409, 1994.

103. Maste MCL, W Norde, AJWG Visser. Adsorption-induced conformational changes in the serine proteinase savinase: a tryptophan fluorescence and circular dichroism study. J Colloid Interface Sci 196:224—230, 1997.

104. Zoungrana T, GH Findenegg, W Norde. Structure, stability and activity of adsorbed enzymes. J Colloid Interface Sci 190:437—448, 1997.

105. Ramirez-Martinez JR, AD McLaren. Some factors influencing the determination of phosphatase activity in native soils and in soils sterilized by irridiation. Enzymologia 31:23—38, 1966.

106. Kobayashi Y, S Aomine. Mechanism of inhibitory effect of allophane and montmorillonite on some enzymes. Soil Sci Plant Nutr 13:189—194, 1967.

107. Haskaa G. Influence of clay minerals on sorption of bacteriolytic enzymes. Microb Ecol 1:234—245, 1975.

108. Makboul HE, JCG Ottow. Michaelis constant (Km) of acid phosphatase as affected by montmorillonite and kaolinite clay minerals. Microb Ecol 5:207—213, 1979.

109. Makboul HE, JCG Ottow. Alkaline phosphatase activity and Michaelis constant in the presence of different clay minerals. Soil Sci 128:129—135, 1979.

110. Makboul HE, JCG Ottow. Clay minerals and the Michaelis constant of urease. Soil Biol Biochem 11:683—686, 1979.

111. Haskaa G. Activity of bacteriolytic enzymes adsorbed to clays. Microb Ecol 7: 331—341, 1981.

112. Pflug W. Effect of clay minerals on the activity of polysaccharide cleaving soil enzymes. Z Pflanzenernaehr Bodenkd 145:493—502, 1982.

113. Ottow JCG, HE Makboul, JC Munch. Effect of pedogenic clay minerals on the kinetics (Km and V of alkaline and acid phosphatases. Z Pflanzenernaehr Bo denkd 146:3—12, 1983.

114. Ross DJ. Invertase and amylase activities as influenced by clay minerals, soil clay fractions and topsoils under grassland. Soil Biol Biochem 15:287—293, 1983.

115. Makboul HE, JCG Ottow. Kinetic behavior of alkaline phosphatase desorbed from different clay minerals. Z Pflanzenernaehr Bodenkd 147:497—501, 1984.

116. Gianfreda L, MA Rao, A Violante. Invertase (effects of montmo rillonite, Al-hydroxide and Al(OH), complex on activity and ki netic properties. Soil Biol Biochem 23:581—587, 1991.

117. Nannipieri P, B Ceccanti, C Conti, D Bianchi. Hydrolases extracted from soil: their properties and activities. Soil Biol Biochem 14:257—263, 1982.

118. Sarkar JM, A Leonowicz, JM Bollag. Immobilization of enzymes on clays and soils. Soil Biol Biochem 21:223—230, 1989.

119. McLaren AD. Concerning the pH dependence of enzyme reactions on cells, partic ulates and in solution. Science 125:697, 1957.

120. Monsan P. G Durand. Preparation d'invertase insolubilisée par fixation sur benton ite. FEBS Lett 16:39—42, 1971.

121. Skujini J, A Pukite, AD McLaren. Adsorption and activity of chitinase on kaolinite. Soil Biol Biochem 6:179—182, 1974.

122. Douzou P, P Maurel. Ionic control of biochemical reactions. Trends Biochem Sci 2:14—17, 1977.

123. Sandwick RK, KJ Schray. The inactivation of enzymes upon interaction with a hydrophobic latex surface. J Colloid Interface Sci 115:130—138, 1987.

124. Sandwick RK, KJ Schray. Conformational states of enzymes bound to surfaces. J Colloid Interface Sci 121:1—12, 1988.

125. Durand G. Modification de l'activité de l'uréase en presence de bentonite. CR Acad Sci 259:3397—3400, 1964.

126. Aliev RA, VS Gusev, DG Zvyagintsev. Influence of adsorbents on the optimum pH of catalase. Vestn Moskov Univ Serie 6 Biol Pochvoved 31:67—70, 1976.

127. Douzou P, GA Petsko. Proteins at work: "stop-action" pictures at subzero temper atures. Adv Protein Chem 36:245—361, 1984.

128. Levin Y, M Pecht, L Goldstein, E Katchaiski. A water-insoluble polyanionic derivative of trypsin. I. Preparation and properties. Biochemistry 3:1905—1913, 1964.

129. Goldstein L, Y Levin, E Katchalski. A water-insoluble polyanionic derivative of trypsin. II. Effect of the polyelectrolyte carrier on the kinetic behaviour of the bound trypsin. Biochemistry 3:1913—1919, 1964.

130. Leprince F, H Quiquampoix. Extracellular enzyme activity in soil: effect of pH and ionic strength on the interaction with montmorillonite of two acid phosphatases secreted by the ectomycorrhizal fungus Hebeloma cylindrosporum. Eur J Soil Sci 47:511—522, 1996.

131. Quiquampoix H. A stepwise approach to the understanding of extracellular enzyme activity in soil. I. Effect of electrostatic interactions on the conformation of a D on different mineral surfaces. Biochimie 69:753—763, 1987.

132. Rouxhet PG. Immobilization of biocatalysts and interfacial chemistry. In: H Okada, A Tanaka, HW Blanch, eds. Annals of the New York Academy of Sciences, Vol 613, Enzyme Engineering 10. New York: New York Academy of Sciences, 1990, pp 265—278.

133. Rouxhet PG, N Mozes. The micro-environment of immobilized cells: critical as sessment of the influence of surfaces and local concentrations. In: JAM de Bont, J Visser, B Mattiasson, J Trampor, eds. Physiology of Immobilized cells. Amster dam: Elsevier, 1990, pp 343—354.

134. Van Loosdrecht MCM, J Lykiema, W Norde, MB Zehnder. Influence of interfaces on microbial activity. Microbiol Rev 54:75—87, 1990.

135. Fletcher M. The physiological activity of bacteria attached to solid surfaces. Adv Microb Physiol 32:53—85, 1991.

136. Konecny J, W Voser. Effects of carrier morphology and buffer diffusion on the expression of enzymic activity. Biochim Biophys Acta 485:367—378, 1977.

137. Arrio-Dupont M, ii Béchet. Diffusion limited kinetics of immobilised myosin AT Pase. Biochimie 71:833—838, 1989.

138. Bille V, D Plainchamp, S Lavielle, G Chassaing, J Remacles. Effect of the microen vironment on the kinetic properties of immobilized enzymes. Eur J Biochem 180: 41—47, 1989.

139. Ku CA, BB Lentrichia. Effects of diffusion limitation on binding of soluble ligand to avidin-coupled latex particles. J Colloid Interface Sci 132:578—584, 1989.

140. Lee CS, G Belfort. Changing activity of ribonuclease A during adsorption: a molec ular explanation. Proc Natl Acad Sci USA 86:8392—8396, 1989.

140a Baron MH, M Revault, S Servagent-Noinville, J Abadie, H Quiquampoix. Chymo trypsin adsorption on montmorillonite: enzymatic activity and kinetic ETIR struc tural analysis. J Colloid Interface Sci 214:319—332, 1999.

141. Quiquampoix H. A stepwise approach to the understanding of extracellular enzyme activity in soil. II. Competitive effects on the adsorption of a -D-glucosidase in mixed mineral or organo-mineral systems. Biochimie 69:765—771, 1987.

142. Quiquampoix H, P Chassin, RG Ratcliffe. Enzyme activity and cation exchange as tools for the study of the conformation of proteins adsorbed on mineral surfaces. Prog Colloid Polym Sci 79:59—63, 1989.

143. Norde W, CH Haynes. Reversibility and the mechanism of protein adsorption. In: TA Horbett, JL Brash, eds. Proteins at Interfaces II, ACS Symposium Series 602. Washington, DC: American Chemical Society, 1995, pp 26—40.

144. Harter RD. Effect of exchange cations and solution ionic strength on formation and stability of smectite-protein complexes. Soil Sci 120:174—181, 1973.

145. Steadman BL, KC Thompson, CR Middaugh, K Matsuno, S Vrona, EQ Lawson, RV Lewis. The effects of surface adsorption on the thermal stability of proteins. Biotechnol Bioeng 40:8—15, 1992.

146. Nannipieri P, P Sequi, P Fusi. Humus and enzyme activity. In: A Piccolo, ed. Humic Substances in Terrestrial Ecosystems. Amsterdam: Elsevier Science By, 1996, pp 293—328.

147. Saiki RK, DH Gelfand, S Stoffel, Si Scharf, R Higuchi, GT Horn, KB Mullis, HA Erlich. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487—491, 1988.

148. Jaenicke R. Protein stability and molecular adaptation to extreme conditions. Eur J Biochem 202:715—728, 1991.

149. Govardhan CP, AL Margolin. Extremozymes for industry—from nature and by design. Chem Indust 17:689—693, 1995.

150. Matumoto-Pintro PT, H Quiquampoix. La phase solide des sols comme contrainte au fonctionnement des enzymes sécrétées par les microorganismes: comparaison de phytases intra et extracellulaires. In: B Baleux, M Desmazeaud, C Divies, F Gendre, R Moletta, eds. Microbiologie Industrielle et Environment. Paris: Société Française de Microbiologie, 1997, pp 195—204.