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Acid phosphatase interactions with organo-mineral complexes: influence on catalytic activity

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Abstract. The influence of montmorillonite intercalated with representatives of three major classes of biological molecules L-lysine (amino acid), α-D-glucose (carbohydrate) and rhamnolipid (lipid) on the catalytic activity of acid phosphatase was investigated. In comparison to pure clay, the presence of the organic intercalates preserves residual activity at extreme pH values of 3 and 11 and temperatures as low as 10 °C. Thermodynamic parameters of free energy, enthalpy and entropy, suggest that catalytic activity on the lysine and rhamnolipid intercalated surfaces is more spontaneous and favorable than that of pure clay. Michaelis-constants (K_m values) and maximum reaction velocities (V_{max} values) were determined and confirmed the enhancement of activity on the organo-mineral surfaces. The catalytic reaction product was measured as a function of time and the data fitted to equations describing the behavior of first and second order rates of reaction. All processes apart from the glucose-intercalated clay (second order) could be described by first order reactions. Catalytic activity was generally less on the glucose-mineral surface compared to the other organo-mineral surfaces and the pure clay. However, when all surfaces were saturated with acid phosphatase the glucose complex exhibited the highest level of catalytic activity.

Introduction

The adsorption of biological molecules to surfaces is central to a diverse range of natural and synthetic processes. For example, the inherent tendency for proteins to adsorb has implications for evolutionary scientists who study the role of montmorillonite in the catalytic formation of RNA (Ferris 2002). Controlled protein adsorption can be used in drug delivery systems and biosensors for *in vivo* monitoring of glucose in blood (Klein et al. 1979). Pharmaceutical companies, materials and food scientists all have an interest in the symbiotic and sometimes destructive relationship between biomolecules and sorbents. In soils, the interaction of inorganic minerals and organic fractions, including biomolecules, plays a significant part in ecological relationships. For instance, mycorrhizal associations, the symbiotic relationship between fungi and plant roots, plays an essential role in the

nitrogen and phosphorous uptake of a host plant. Extracellullar enzymes such as phosphatases and proteases, secreted by bacteria and fungi are important components in this process (Tarafdar and Marschner 1994). Enzymes have a high affinity for colloidal soil components ($<2\,\mu m$) and our knowledge of the fundamental chemistry and properties of enzymes and clay-sized minerals can be enhanced by the study of their interactions. In living cells, enzymes exist primarily in an immobilized state (Khanna et al. 1998). This state also exists in natural soil systems where immobilized enzymes play an important role in the transformations of natural and anthropogenic organic compounds (Tapp and Stotsky 1998). Clay minerals, with adsorptive and possibly catalytic capabilities, greatly influence biological and anthropogenic processes (Kelleher et al. 2002). The defined natural structure of clays may facilitate catalytic reactions between the enzyme and substrate through inert support and/or involvement in the reaction directly. Either way, the exact nature of the relationship remains unclear.

The activity of the immobilized enzyme is affected by factors that include the type of enzyme, temperature, the clay mineral, pH, concentration and nature of the buffer (Gianfreda et al. 1991). Clay minerals may protect enzymes against denaturation and preserve their activity while in other cases, inhibition of enzyme activity results from the association (Kobayashi and Aomine 1967). Immobilized enzymes generally become more resistant to microbial degradation and fluctuations in humidity and temperature (Skujins 1976). Enzymatic reactions in soil occur in highly heterogeneous environments compared to those encountered by intracellular enzymes (Nannipieri and Gianfreda 1998). As a result, adsorptive and kinetic behavior of extracellular enzymes will differ from those intracellular enzymes (McLaren and Packer 1970; Katchalski et al. 1971).

In this paper we investigate the influence of pure and intercalated montmorillonite on the catalytic activity of acid phosphatase. Three biomolecules representing three major classes of biological molecules: L-lysine (amino acid), α- D-glucose (carbohydrate) and rhamnolipid (lipid) were intercalated between the layers of a sodic montmorillonite. The aim of this study is not only to investigate the influence of organo-mineral complexes, but to provide insight into the influence of representatives of different classes of biological molecules on catalytic enzyme activity.

Materials and methods

Chemicals

Rhamnolipid was purchased from Jeneil Biosurfactant Co. LLC, Sauville, WI, USA (two major rhamnolipids are present, $C_{26}H_{48}O_9$ and $C_{32}H_{58}O_{13}$). Acid phosphatase [EC 3.1.3.2, Type I, from wheat germ, Sigma Chemical Co., St Louis, MO, USA] was used as received (molecular weight: $55{,}000 \pm 5000$, Verjee 1969). L-lysine, α-D-glucose and all other chemicals used were purchased from Sigma Chemical Co., St Louis, MO, USA.

Table 1. Physical properties of Wyoming montmorillonite. SSA is specific surface area and CEC is cation exchange capacity (Carter et al. 1986; Laird and Barak 1991; Dixon and White 1995).

Clay	Layer charge ($mol_c\,mol^{-1}$)	SSA ($m^2\,g^{-1}$)	d(0 0 1) spacing (Å)	CEC $cmol_c{}^{-1}kg$
Wyoming	−1.04	781.7	9.67	80

Table 2. Calculated and observed d(1 0 0) spacings for intercalated montmorillonite.

Biomolecule	Molecular weights	Diameter of molecule (Å)	d(0 0 1) spacing (Å)	
			Calculated	Observed
L-Lysine	149.19	3.85	13.51	13.14
Glucose	180.16	5.07	14.74	13.85
Rhamnolipid	504–650	7.00	16.67	17.65

Intercalated complexes

The clay mineral used in this study was montmorillonite from Crook, Wyoming. Pretreatment was carried out to remove soluble salts, carbonates, trace organic matter, and crystalline iron oxides using standard methods (Dixon and White 1995). The $<2\,\mu m$ fraction of the clay was then separated by sieving and centrifugation after dispersion in water. A sodium saturated clay fraction was prepared by washing three times in a 0.5 M NaCl solution, followed by repeated rinsing with deionized water. Clay properties, including cation exchange capacity (Dixon and White 1995), specific surface area (Carter et al. 1986), and layer charge (Laird et al. 1991) are presented in Table 1.

Intercalated complexes were prepared at room temperature by mixing 1 M glucose or L-lysine with 1 g of clay. The rhamnolipid intercalation complex was prepared by mixing a 25% aqueous solution of rhamnolipid with 1 g of clay. Properties and d(1 0 0) spacings of the intercalated complexes are presented in Table 2. To negate the influence of interlayer expansion due to water molecules, XRD analysis was carried out after the clays were dried at 110 °C.

Acid phosphatase activity measurements

Catalytic activity of both free (initial) and immobilized (residual) acid phosphatase was assayed with 1 ml, 6 mM *p*-nitophenylphosphate (*p*NPP) substrate, 1 ml enzyme (0.1 or $0.05\,mg\,ml^{-1}$) and 2 ml of 0.1 M sodium acetate buffer. Twenty milligrams of clay or intercalated clay was added to the sodium acetate buffer to study their influence on activity. After an incubation time of 2 h the substrate was added for 20 min after which the reaction was stopped by the addition of 1 ml of 1.0 M NaOH. The concentration of the reaction product, *p*-nitrophenol (*p*NP),

was measured spectrophotometrically at 405 nm (molar absorption coefficient 18.5 cm^{-1} mM^{-1}). Enzymatic units (U) are defined as the μmoles of *p*-nitrophenol produced by 1 ml of free or immobilized enzyme solution after 1 min.

For all experiments, three basic procedures (Leprince and Quiquampoix 1996) were used to study the effect of clay and intercalated clay on acid phosphatase catalytic activity. The first method involves the simple measurement of the free enzyme in solution. This value was then used for comparative analysis with immobilized enzyme activity. Secondly, the overall influence of the mineral and organo-mineral surfaces on enzyme activity was measured. Clay (20 mg) was added at the beginning of the incubation period and was then centrifuged out of solution ($30{,}000 \times g$ for 15 min) just before measurement of *p*-nitrophenol concentration. The third procedure is carried out to distinguish between the contribution of free and adsorbed enzyme to the overall activity value. It is an experimental method used to ensure that the value of immobilized activity actually applies to the activity on an immobilizing medium and not due to residual free activity in solution. The method is a simple adjustment to the second method whereby the clay is removed by centrifugation before the addition of the substrate. These procedures were carried out for all experiments and the activity due to free enzyme was subtracted from the overall immobilized activity in each case.

The influence of pH on the activity of both free and immobilized enzymes was carried out with a series of modified universal buffer solutions (Skujins et al. 1962) at 20 °C, with 6 mM *p*NPP in the pH range of 1–13.

The possibility that a decrease in catalytic activity could be due to the adsorption of the substrate, *p*NPP, to the clay rather than a decrease in catalytic activity was considered. After the test for catalytic product, *p*-nitrophenol, 0.5 ml acid phosphatase solution was added to the supernatant. An increase in the concentration of *p*-nitrophenol indicated, each time, that there was excess substrate in the solution. It was therefore concluded that a decrease in activity did not result from an unavailable substrate due to adsorption to the clay complex. The possible hydrolysis of *p*NPP by the mineral surface was also considered. Controls for each experiment were conducted without acid phosphatase and the presence of *p*-nitrophenol was analyzed. No independent hydrolysis was detected for any experiment.

The influence of buffers on the enzyme activity for each experiment was investigated by carrying out similar experiments (as close to the experimental conditions as possible) without a buffer. The influence of buffer was negligible.

Heat stability and thermodynamics

An activity-temperature profile was obtained from activity measurements at temperatures ranging from 10 to 60 °C at pH 5 (Fig. 3). Incubation time for these experiments was increased to 24 h to ensure equilibrium was reached.

The thermodynamic parameters for the catalytic process, Gibbs free energy (ΔG^0), enthalpy (ΔH^0) and entropy (ΔS^0) changes were calculated. Experimentally, the heat change (ΔH^0) resulting from activity can be determined in conjunction

with the Vant Hoff equation by carrying out activity assays at two or more separate temperatures (Segel 1975):

$$\Delta H^0 = R\left(\frac{T_1 T_2}{T_2 - T_1}\right) \ln \left(\frac{K_{eq2}}{K_{eq1}}\right) \quad (1)$$

where K_{eq} is the equilibrium constant ([product]/[substrate]) for the adsorption process at a specified temperature, T (R is the Gas constant, $8.3145\,JK^{-1}\,mol^{-1}$).

The change in surface free energy resulting from catalytic activity (ΔG^0) can be evaluated using:

$$\Delta G^0 = -RT \ln K_{eq} \quad (2)$$

Using a form of the equation for the Gibbs free energy change and its rearrangement leads to an estimation of the entropy change (ΔS^0) for the resulting catalytic process.

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (3)$$

$$\Delta S^0 = \frac{\Delta H^0 - \Delta G^0}{T} \quad (4)$$

Kinetic measurements

Kinetic parameters (V_{max} and K_m) were calculated for the free and immobilized enzyme from data generated from enzyme activity at six different substrate concentrations (1–6 mM *p*NPP) at 20° and pH 5. The kinetics were calculated based on the Michaelis–Menten model using the Lineweaver–Burk equation (Tabatabai 1982).

The order of the catalytic reactions was determined experimentally by measuring the concentration of substrate product (*p*NP) as a function of time. Substrate concentrations were obtained at time intervals of 2 min from 0 to 20 min and the order of the reaction was determined by comparing the fit of the data to equations describing the behavior of the reactions of various orders. A first order may be distinguished from a second order reaction by plotting ln[*p*NP] versus time and 1/[*p*NP] versus time and observing which, if any, of these plots is a straight line (Segel 1975; Voet and Voet 1995).

All experiments were minimally run in triplicate, to ensure reproducibility and accuracy and all activity values were reproducible within ±5 activity units (U). Polypropylene tubes with water substituted for acid phosphatase but including all other reactants and clays were used as blanks.

Equilibrium adsorption isotherm

Clay suspensions ranging from 0 to 4 mg were prepared in 20 ml of aqueous solution with a 0.1 M sodium acetate buffer solution [pH 5]. Acid phosphatase

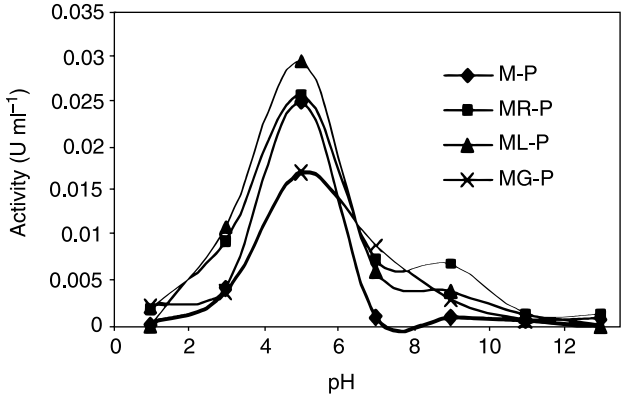

Figure 1. Effect of pH on the catalytic activity of acid phosphatase in association with pure and intercalated clays at 20 °C. [M-P = unmodified montmorillonite and AP (acid phosphatase); MR-P = montmorillonite intercalated with rhamnolipid and AP; ML-P = montmorillonite intercalated with Lysine and AP; MG-P = montmorillonite intercalated with glucose and AP.]

(100 mg protein 1) was added to a polypropylene tube. The final mixture was gently shaken for 2 days at 21 °C. After centrifugation at 20,000×g, the enzyme adsorbed was calculated from the difference between the concentration of an acid phosphatase-only control and the final concentration of the enzyme in the aqueous phase after mixing with the clay. All experiments were run in triplicate to ensure enzyme assay reproducibility and accuracy. Enzyme protein concentration was determined spectrophotometrically at 280 nm.

Results and discussion

pH effects

The results presented Figure 1 report the effect pH has on the activity of the enzyme in association with the pure clay and its intercalated complexes. Within the pH profile studied, neither the pure clay nor its intercalated complexes have an effect on the optimal activity pH. As expected, at all pH values studied, the presence of the montmorillonite results in a large decrease in the activity of acid phosphatase (M-P). At the pH of optimal activity, pH 5, the presence of the clay leads to a residual activity (percentage of free enzyme activity) of 15.3. Higher activities were observed for both the ML-P (clay–lysine) and MR-P (clay–rhamnolipid) complexes (18.22 and 15.8%, respectively). Interaction between the MG-P (clay–glucose) complex and acid phosphatase resulted in the lowest residual activity (10.59%). As the buffered solution became either more acidic or more alkaline overall activity levels dropped for the enzyme on its own and with clay amendments. Figure 2 outlines the remaining activity percentage, on each clay complex (immobilized

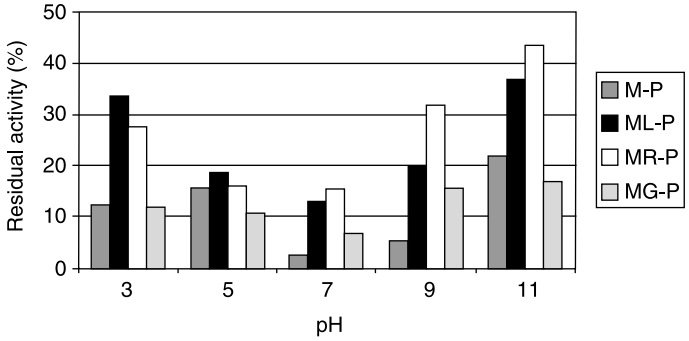

Figure 2. Residual activity, relative to free enzyme activity, at various pH levels at 20 °C. [M-P = unmodified montmorillonite and AP (acid phosphatase); MR-P = montmorillonite intercalated with rhamnolipid and AP; ML-P = montmorillonite intercalated with Lysine and AP; MG-P = montmorillonite intercalated with glucose and AP.]

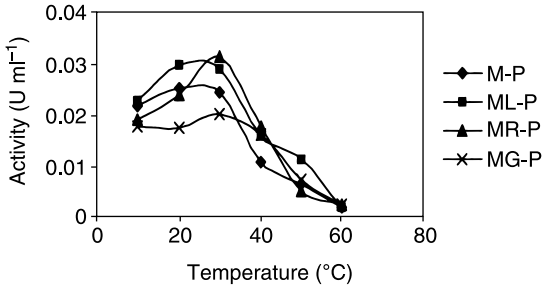

Figure 3. Temperature stability of acid phosphatase associated with pure and intercalated montmorillonite at pH 5. [M-P = unmodified montmorillonite and AP (acid phosphatase); MR-P = montmorillonite intercalated with rhamnolipid and AP; ML-P = montmorillonite intercalated with Lysine and AP; MG-P = montmorillonite intercalated with glucose and AP.]

enzyme), as a function of the 100% activity of the free enzyme at that pH (initial activity). The percentage of initial activity that survived these harsher pH conditions was higher for both the lysine and rhamnolipid amended clays and less so for the pure clay or glucose modified clay. The presence of these organic molecules appears to have stabilized the remaining activity at extreme pH's.

Heat stability and thermodynamics

The optimum temperatures for acid phosphatase activity immobilized by the pure clay and the organo-mineral complexes are shown in Figure 3. As can be seen from Figure 3, of the temperatures studied, the optimum temperature for all four complexes is from 20 to 30 °C. There is no significant increase in temperature stability

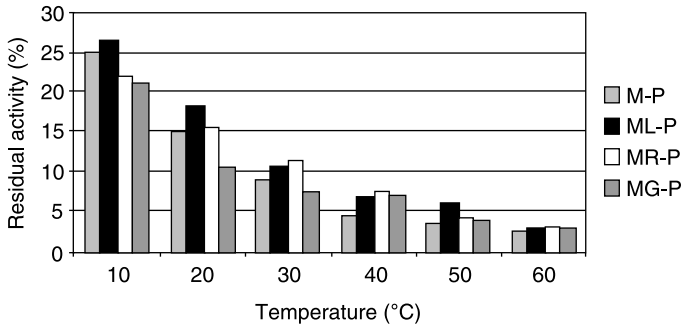

Figure 4. Residual activity, relative to free enzyme activity, at various temperatures at pH 5. [M-P = unmodified montmorillonite and AP (acid phosphatase); MR-P = montmorillonite intercalated with rhamnolipid and AP; ML-P = montmorillonite intercalated with Lysine and AP; MG-P = montmorillonite intercalated with glucose and AP.]

Table 3. Values of thermodynamic parameters associated with activity of free, pure and intercalated clay. Acid phosphatase on pure clay (M-P), lysine intercalated clay (ML0P), rhamnolipid intercalated clay (MR-P) and glucose intercalated clay (MG-P).

Enzyme/complex	ΔG^0 (20 °C) (kJ mol^{-1})	ΔH^0 (kJ mol^{-1})	ΔS^0 (JK^{-1} mol^{-1})
Free AP	11.02	41.81	−37.55
M-P	15.57	4.08	−53.10
ML-P	15.15	6.70	−51.57
MR-P	15.70	17.70	−53.48
MG-P	16.42	4.70	−55.39

for any of the organo-mineral complexes although the lysine modified clay displays higher activity values at extreme temperatures than montmorillonite on its own. At 60 °C the enzyme is denatured and is not protected by any of organo-mineral complexes. As with pH analysis, presentation of remaining activity on the clay complexes relative to the free enzyme at a given temperature produces some interesting results (Figure 4). As the temperature increases it can be seen that the organo-mineral complexes retain a slightly higher activity than found with pure montmorillonite as was shown in Figure 3. However, we can now also see that the percentage relative activity retained on all surfaces is higher at low temperatures (10 °C). In other words, a much higher percentage of the original free enzyme activity is retained on the clay surfaces in cold conditions rather than elevated temperatures. In nature, we are much more likely to have temperatures of 10 °C than 50–60 °C and it would appear that natural systems have mechanisms to adapt to these conditions.

Thermodynamic concepts can be used in transition-state theory to characterize chemical and enzyme catalyzed reactions. The positive standard enthalpy change values (ΔH^0) in Table 3 indicates that the enzymatic activity is an endothermic process. The enthalpy of the immobilized enzyme on both pure and intercalated

clay is much less than that of the free enzyme. This would suggest that adsorption onto these complexes enhances the ability of the enzyme to catalyze a reaction due to the need for less energy to reach the transition state. The lower value for ΔH^0 obtained for immobilized enzymes indicates that the energy barrier for electron transfer has been reduced in comparison to that of the free enzyme. It should be pointed out that pure montmorillonite has the lowest ΔH^0 value. Positive ΔG^0 values reflect the nonspontaneous character of the main reaction, which is the hydrolysis of *p*NPP to *p*NP, and the need for an enzyme to facilitate the reaction.

The entropy of activation is a measure of the inherent possibility of reaching the transition-state (ΔS^0) notwithstanding energy requirements. The more negative the value of ΔS^0, the more precise the conformation and controlled approach the reacting molecules must have (Cornish-Bowden 1979). The value obtained for ΔS^0 for the free enzyme is higher than those for the immobilized enzymes. This would indicate, as expected, that the catalytic reaction is more favorable for the free enzyme. The decrease in relative flexibility of the immobilized enzyme would account for the observed decrease in ΔS^0. The conformational mobility of an enzyme in water would be expected to increase relative to enzymes adsorbed onto a surface and this is reflected in the ΔS^0 values. The slight differences between the pure clay and organo-clay complexes reflects the overall situation where the lysine and rhamnolipid modified clays appear to facilitate higher catalytic activity. The apparent contradiction in the interpretation of the ΔH^0 and ΔS^0 values may be because they are compensative changes promoted by the new surroundings of the enzyme. The observed decrease in ΔH^0 may indicate that the active site of acid phosphatase is preferentially stabilized on the mineral surface while decreases in ΔS^0 reflect structural changes and a more compact structure with reduced flexibility.

Enzyme kinetics and reaction order

The kinetic properties of enzymatic reactions can be altered due to enzyme–mineral interactions (Boyd and Mortland 1990). The Michaelis–Menten equation is the basic equation of enzyme kinetics. The kinetics of free and immobilized acid phosphatase followed the Michaelis–Menten model. The kinetic parameters (K_m and V_{max}) were calculated using the Lineweaver–Burk equation. The Michaelis constant, K_m is the substrate concentration at which the reaction is half maximized (Voet and Voet 1995). A small value for K_m indicates that an enzyme can reach maximal catalytic activity at low substrate concentration. The K_m will therefore also be influenced by the effect a mineral or organo-mineral will have on the distribution of the enzyme and its substrate and consequent availability of active sites. The K_m value for the free enzyme in this study is slightly higher than those of the immobilized complexes which is in contrast to similar work including Huang et al. (1995) and Makboul and Ottow (1979). The charge of the clay–enzyme complex, relative to the substrate charge may have an effect on this parameter when considering that the free enzyme may have a different charge, which may attract or repulse the substrate which in turn may require

Table 4. First order rate constants and first and second order correlation co-efficient values for different enzyme associated complexes.

Enzyme complex	First order plot of ln[*p*NPP] versus *t*, correlation co-efficient (R^2)	Second order plot of $1/[p\text{NPP}]$ versus *t*, correlation co-efficient (R^2)	First order rate constant $k(s^{-1})$
AP	0.9337	0.8301	2.4×10^{-2}
M-P	0.9537	0.9004	2.2×10^{-3}
ML-P	0.9585	0.8663	3.3×10^{-3}
MR-P	0.9238	0.8454	3.1×10^{-3}
MG-P	0.4223	0.9692	1.3×10^{-3}

more or less substrate to achieve optimum catalytic activity. Other properties that will also make the reaction more or less likely are steric hindrance, conformational changes and diffusional effects (Theng 1979). Apart from the glucose–clay complex, the differences in K_m values for the free and immobilized enzyme are not largely different indicating that the cumulative effect of electrostatic, steric and conformational changes due to the presence of clay and organic compounds have not affected access of enzyme to substrate.

The maximal velocity of a reaction V_{max} occurs at substrate concentrations wherein the enzyme is saturated. As would be expected, the free enzyme has a higher V_{max} value than the immobilized enzyme. In accordance with a higher ΔS^0, the free enzyme retains a more flexible conformation in solution that allows a higher turnover of substrate hydrolysis. Lysine and rhamnolipid altered clay have slightly higher velocities than the pure clay while the glucose complex has a lower value.

A study of the rates of reactions can contribute to knowledge of the mechanisms of the reaction. The rate of an enzymatic reaction is proportional to the frequency with which the enzyme and substrate simultaneously come together. The order of this reaction corresponds to the number molecules that simultaneously collide and of the orders possible, first order (unimolecular) and second order (bimolecular) are common. The reaction product, *p*NP was measured as a function of time and the data fit to equations describing the behavior of first and second order rates of reaction. As can be seen from Table 4, free acid phosphatase and all of the clay-organic complexes, except the glucose-clay complex, are more closely characterized as first order reactions. Again, the clay-glucose complex is anomalous in that the value of the correlation coefficient indicates that this enzymatic reaction cannot be described as first order. It is better described as second order where the variation of concentration of reactants with time is different from that in a first order reaction in that it is dependant on the initial concentration.

Clay saturation

Adsorption studies (isotherm with author) have indicated that the saturation or the plateau of an adsorption isotherm for acid phosphatase on the pure montmorillonite

Table 5. Kinetic parameters of free and immobilized acid phosphatase by Michaelis–Menten equation.

Enzyme complex	K_m (mmol ml^{-1})	V_{max} (μmol *p*NPP 50 mg^{-1} h^{-1})	V_{max}/K_m
AP	0.97	0.42	0.43
M-P	0.67	0.04	0.06
ML-P	0.56	0.05	0.09
MR-P	0.91	0.05	0.05
MG-P	0.31	0.035	0.11

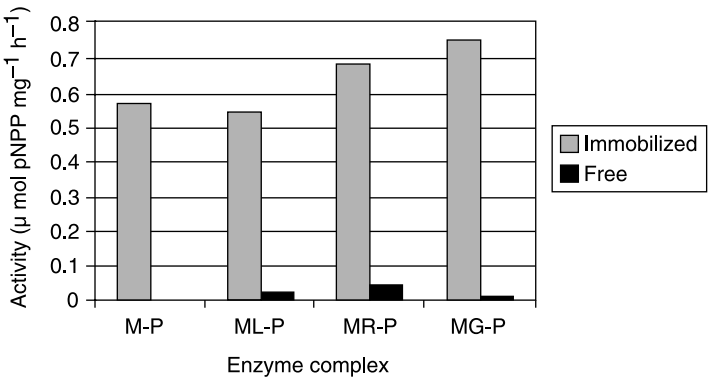

Figure 5. Free and immobilized activities of pure and intercalated clay saturated with acid phosphatase at 20 °C and pH 5. [M-P = unmodified montmorillonite and AP (acid phosphatase); MR-P = montmorillonite intercalated with rhamnolipid and AP; ML-P = montmorillonite intercalated with Lysine and AP; MG-P = montmorillonite intercalated with glucose and AP.]

occurs at 630 mg g^{-1} Table 5. Therefore, 630 mg of acid phosphatase will adsorb onto a gram of pure clay and no more. In theory, if this amount is adsorbed on the pure clay, there should be no activity due to free enzyme as all of the enzyme is now associated with the clay. If this maximum concentration of enzyme interacts with a similar amount of intercalated clay it would be expected that less adsorption would take place due the presence of intercalated organic molecules and activity due to free enzyme should exist. Also, if all of the clays are saturated, an experiment like this may help explain the role of the interlayer region on the activity of an enzyme adsorbed on a mineral surface. Figure 5 outlines activity on the clay surface and free enzyme activity in solution for a clay/enzyme ratio resulting in a saturated pure clay. No free enzyme activity was detected for the pure clay solution indicating that all of the enzyme has been adsorbed. Residual free enzyme activity was detected for the three intercalated clays indicating that these clays cannot adsorb all of the enzyme at this concentration due to an already occupied interlayer region. What is interesting about this result is that we know that the pure clay has adsorbed more of the enzyme than the other organo-mineral complexes but two of the intercalated clays still exhibit higher immobilized enzyme activities. It would appear from this result that enzyme adsorbed in the interlayer region of the clay is inhibited from enzymatic activity and

that the ability to catalyze reactions is limited to the enzyme adsorbed on the outer surface where its active site can come into contact and react with the substrate. Drapeau (1974) determined that the external surface of montmorillonite comprises approximately 6% of the total surface area. Since the pure clay retains a residual 15% of the original enzyme activity it is reasonable to presume that all of this activity is on the outer surface while the enzyme in the interlayer remains inactive. It is also interesting that, in this instance, the clays intercalated with glucose and rhamnolipid exhibit more activity than the pure clay even though we know that there are fewer enzyme molecules adsorbed onto these clays. Intercalation of montmorillonite will reduce its surface area but the sites where the enzyme are active (external surface) are available and there is also an increase in external surface area due to intercalation. This increased external surface may facilitate more activity by simply providing extra sites for the enzyme to attach to on the outer surface. The behavior of the glucose complex was anomalous from a number of perspectives: (1) pH experiments indicate that activity on the glucose complex is generally less than that on the other organo-minerals; (2) Enzyme kinetics revealed that the rate of activity is slower and less likely; (3) Reaction kinetics followed second rate order characteristics as opposed to first order for all other systems and (4) Thermodynamic values suggest that catalytic activity on the glucose-clay surface is the least spontaneous and favorable of all reaction conditions. Nonetheless, when clay was saturated with enzyme molecules, the glucose complex exhibited the most activity of clay–organic complexes and pure clay.

The fact that the activity on the glucose–clay complex can be described as a second order reaction would suggest that not all enzyme active sites are available for catalytic activity. There could be many causes of this including the non-productive binding of the substrate to the enzyme caused by changes in the crystal structure of the enzyme induced by glucose. The presence of the glucose–clay complex may result in destabilization of the substrate or desolvation of the enzyme and/or substrate. These situations could also apply to the lysine and rhamnolipid complexes. For the saturated clay environment this kind of explanation does help to explain the extra activity on the glucose–clay complex. Table 2 shows the basal spacing for all clay complexes and the extra activity could be explained in terms of extra external surface area of glucose compared to lysine. Rhamnolipid is a much larger molecule and may not have as many binding sites available for the enzyme to adsorb to, especially considering its dipolar nature.

Throughout all the experiments the presence of intercalated organic molecules enhanced enzyme activity compared to pure clay. Significantly, activity appeared to be buffered against cooler temperatures and extreme pH conditions.

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