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A MICROENVIRONMENTAL REDOX SHIFT AT A CHARGED SURFACE DETECTED BY PAPAIN ACTIVITY

A. J. BENESI[2] and A. D. McLAREN

Department of Soils and Plant Nutrition
University of California
Berkeley, California 94720

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The relative activity of an SH-enzyme, papain, is decreased by increasing the mole ratio of oxidizing disulfide to reducing thiol in solution. The same inverse relationship applies to papain adsorbed on charged clay particles, but electrostatic interactions among the charged particles and charged disulfides and thiols significantly shift the dependence. Papain activity thus reflects the microenvironmental redox potential in the vicinity of the charged particles. The redox pairs cystine dimethylester–cysteine ethylester, dithiodiglycolic acid–mercaptoacetic acid, and dithiodiglycol–mercaptoethanol were used in the assays. A special form of the Boltzmann distribution must be used to calculate mole ratios of ions of different charge near a charged interface.

INTRODUCTION

A charged interface attracts ions of unlike charge and repels ions of like charge. Wherever ions affect enzyme activity, the observed enzyme activity for enzymes adsorbed or immobilized at a charged interface can be different from the activity observed in homogeneous solution.

Previous studies with enzymes adjacent to charged interfaces were concerned with apparent differences in enzyme activity caused by accumulation or repulsion of a single species of ion (e.g., hydrogen ions, charged substrates or inhibitors) (1–4), and it was found that local concentrations can be adequately predicted by double-layer theory (1,2) or by Donnan theory (3,4).

In nature, however, enzymes are exposed simultaneously to many different ions and molecules that affect their activity, and differences between local and bulk ratios of different ions can be orders of magnitude larger than differences between local and bulk concentrations of a single ion.

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[2]Dr. Benesi's present address is Department of Chemistry, University of California, Santa Barbara, California 93106.

Interfacial potentials in the vicinity of most membrane enzymes and soil enzymes are of the order of −20 mV (5–7), and local concentrations of small cations are generally less than 10 times higher near the charged interface than in solution. With the same potential, ratios of activators and inhibitors of opposite sign can be different in the vicinity of an adsorbed or immobilized enzyme by a factor of nearly 100. Local electrostatic effects can thus be very important in regulation of enzyme activities in nature.

In terms of properties of enzymes near charged surfaces, the first effect observed was the influence of the local pH in the vicinity of the surface due to attraction of hydrogen ions (or hydroxide ions for positive surfaces) (1,2). The pH shift in activity dependence of adsorbed or immobilized enzymes is readily apparent in most cases.

A second fundamental property that can be different near a charged interface is the oxidation–reduction potential. For enzymes with active sites susceptible to reversible reaction, a local redox potential would be expected to shift relative enzyme activity compared with bulk activity if the oxidant and reductant reactants bear different charges.

An experimental investigation was conducted in order to test these expectations. Adsorbed papain activity was used as a microenvironmental redox electrode (1), since its activity depends on an SH group susceptible to reversible oxidation and reduction.

EXPERIMENTAL PROCEDURE

Papain was adsorbed on negatively charged kaolinite (titrated to pH 8 with KOH) to the extent of 1.0 mg/g clay (8). The pH optimum (6.9) was measured with a pH-stat by following rates of hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester (BAEE). Dithiothreitol (5 mM) and EDTA (0.5 mM) were used to activate papain. A corresponding experiment with soluble papain revealed a lower pH optimum (6.1), as expected (1). The cationic strengths ($\Sigma c_i^+(z_i^+)^2$) were constant at about 0.06, including the contribution of BAEE.

Under identical conditions, the turnover number and K_m for soluble papain activity on BAEE were determined at pH 6.1 as 7.3 sec^{-1} and 15 mM, respectively. With adsorbed papain at bulk pH 6.9, the values were 5.5 sec^{-1} and 2.1 mM.

Similar experiments were performed under nitrogen with soluble (at pH 6.1) and adsorbed (at pH 6.9) papain in the presence of mixtures of several thiols and disulfides in various mole ratios. The mixtures included: dithiodiglycol (DTDG, a neutral disulfide) and mercaptoethanol (ME, a neutral thiol); cystine dimethylester (CDME, a +2-charged disulfide) and cysteine ethylester (CEE, a +1-charged thiol); and dithiodiglycolic acid

(DTDGA, a −2-charged disulfide) and mercaptoacetic acid (MA, a −1-charged thiol). Mixtures of disulfide and thiol were prepared in various mole ratios with a sum concentration of 1 mM. Experiments were also carried out in which the sum concentrations of CEE and CDME were 0.1 mM and 10 mM.

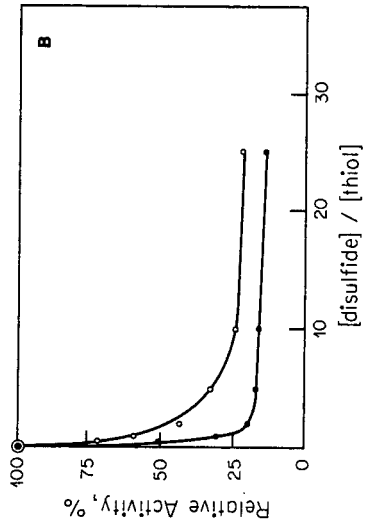
Among the enzyme assays, the criterion of thiol–disulfide exchange equilibrium with papain was a constancy of relative papain activity (relative to maximum activity observed in presence of pure thiol) with time. Equilibration times were less than 2 h for all assays. The solution phase consisted of phosphate buffer; 0.5 mM EDTA; 100, 20, or 4 mM BAEE; and the thiol–disulfide mixture. Reaction mixtures contained 0.5 mg papain either dissolved in solution or adsorbed on 0.5 g kaolinite. The ratio of moles of thiol + disulfide to papain was nearly 1,000, so that the initial mole ratio of disulfide to thiol was not changed significantly by exchange with papain.

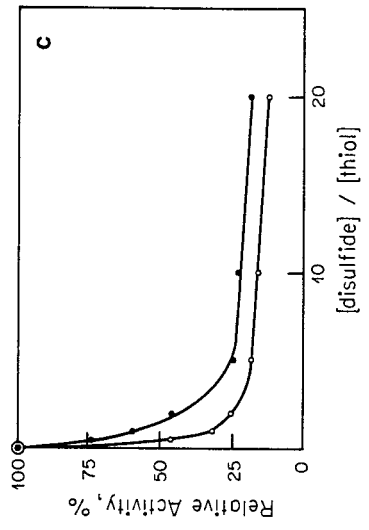
RESULTS AND DISCUSSION

The thiol group of cysteine 25 at the active site of papain participates in exchange with added thiol and disulfide (9). It was necessary to perform the redox experiments under nitrogen because reaction mixtures containing kaolinite and exposed to air rapidly lost enzyme activity, presumably due to oxidation of SH by O_2 (10). With soluble papain, the same maximum activity was observed with all reducing thiols at all concentrations of thiol and substrate. This finding held correspondingly true for adsorbed papain, although turnover numbers for adsorbed papain were 30% less than for soluble papain.

With the uncharged pair of redox compounds DTDG and ME, no difference in relative activity as a function of the ratio of these compounds was observed with soluble or adsorbed papain (Fig. 1A). Evidently the mole ratio of DTDG to ME was no different in the vicinity of adsorbed papain than in the vicinity of soluble papain, although increasing the ratio of disulfide to thiol reduces the amount of active reduced papain in both systems.

By contrast, the relative activity of papain adsorbed on kaolinite is considerably less in the presence of mixtures of CDME and CEE than is that of soluble papain (Fig. 1B), and is considerably higher than that of soluble papain in the presence of mixtures of DTDGA and MA (Fig. 1C). The dicationic and dianionic disulfides CDME and DTDGA are respectively more strongly attracted and repelled by the negatively charged kaolinite surface than are the monocationic and monoanionic thiols CEE and MA. Similar shifts were observed for mixtures of thiols and disulfides of opposite charges (e.g., −2 disulfide with +1 thiol), but analysis was complicated





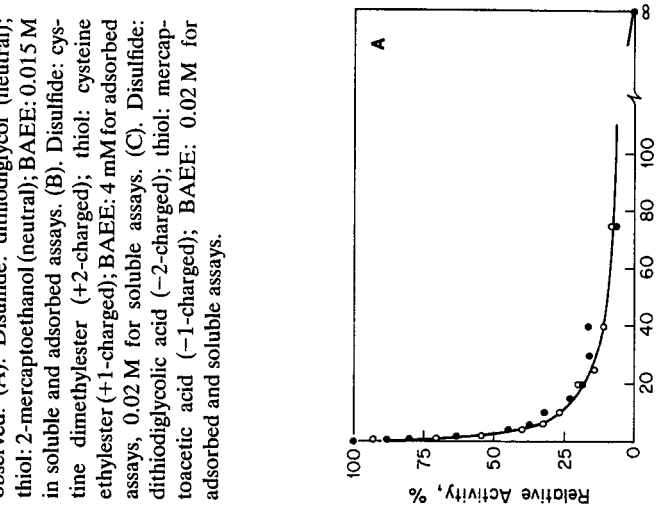


FIG. 1A–C. Soluble (○) and adsorbed (●) papain activity as a function of the initial mole ratio of disulfide to thiol with sum concentration of thiol plus disulfide equal to 1 mM. Activities are plotted as percentage of respective maximum activity observed. (A). Disulfide: dithiodiglycol (neutral); thiol: 2-mercaptoethanol (neutral); BAEE: 0.015 M in soluble and adsorbed assays. (B). Disulfide: cystine dimethylester (+2-charged); thiol: cysteine ethylester (+1-charged); BAEE: 4 mM for adsorbed assays, 0.02 M for soluble assays. (C). Disulfide: dithiodiglycolic acid (−2-charged); thiol: mercaptoacetic acid (−1-charged); BAEE: 0.02 M for adsorbed and soluble assays.

because species bearing different charges are formed during thiol–disulfide exchange.

After taking into account effects of local substrate and hydrogen ion concentrations on relative papain activity, it was calculated from the observed papain activity dependence that the mole ratio of CDME to CEE in the vicinity of adsorbed papain was 3.5 times the mole ratio in the vicinity of soluble papain. The mole ratio of DTDGA to MA was calculated for adsorbed papain as 3.3 times less than that for soluble papain. The difference in local redox potentials for adsorbed and soluble papain can be calculated from these values as about ±30 mV, depending on whether the oxidizing disulfide or reducing thiol has the more positive charge.

A clear dependence of the "redox shift" on total sum concentration of disulfide + thiol was observed. At CDME + CEE = 1 mM, the mole ratio of CDME to CEE for adsorbed papain was 3.5 times that for soluble papain. For CDME + CEE = 0.1 mM, the mole ratio for adsorbed papain was only 2.0 times that for soluble papain; for CDME + CEE = 10 mM, the mole ratio of CDME to CEE was calculated to be 6.9 times that for soluble papain. In an experiment in which 10 mM $MgCl_2$ was added to mixtures containing CDME + CEE = 1 mM, the mole ratio of CDME to CEE for adsorbed papain was 2.7 times that observed for soluble papain. The reason for the latter finding is that increased cationic strength damps out the surface potential of negatively charged kaolinite. The other findings make clear that it is not the bulk mole ratios of charged enzyme regulators that determine the activity of an enzyme near a charged interface, but rather the ratios of regulatory mole fractions of the total bulk concentrations of ions of the same charge as the regulators (see the following equation).

Assuming a Boltzmann distribution, the local ratio of regulator A to regulator B in the vicinity of an enzyme near a charged interface is given by:

$$\frac{A_{enz}}{B_{enz}} = \frac{\chi_A[\sum c(z_A)]}{\chi_B[\sum c(z_B)]} \exp[(z_B - z_A)e\varphi_{enz}/kT]$$

where:

φ_{enz} = potential in vicinity of enzyme

$\frac{A_{enz}}{B_{enz}}$ = mole ratio of regulator A to regulator B in vicinity of an enzyme at a charged interface

e = proton charge

z_A = charge of regulator A

z_B = charge of regulator B

$\sum c(z_A)$ = total bulk concentration of ions of same charge as regulator A

$\sum c(z_B)$ = total bulk concentration of ions of same charge as regulator B

χ_A = mole fraction of $\sum c(z_A)$ that is regulator A

χ_B = mole fraction of $\sum c(z_B)$ that is regulator B

In summary, the redox potential near a charged interface can be significantly different than in the bulk solution, and this difference can affect enzymes that are sensitive to redox changes. The Boltzmann distribution can be used to predict the ratio of ions of different charge (in this case ions that determine the local redox potential) near a charged surface. Since non-regulatory ions may constitute a significant mole fraction of the ions of any given charge, it is necessary to express the ratio of two ion concentrations in terms of their mole fractions of the total concentration of their respective charges.

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