Control of Enzyme Activity in Organic Media by Solid-State Acid−Base Buffers

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The behavior of enzymes is strongly dependent on the protonation state of their ionizable groups. In low-water media,[1] the initial protonation state of enzymes is set by the pH of the aqueous medium from which they are dried,[2] but may be controlled subsequently by appropriate "organic-phase buffers".[3] Here, we identify a new versatile method of control based on "solid-state buffers". These consist of pairs of crystalline solids that can be interconverted by exchange of H^+ and another ion. Each pair sets, at equilibrium, a characteristic potential for the appropriate acid−base exchange, quantifiable as a ratio or product of thermodynamic activities of ions. The concept was demonstrated by adding insoluble mixtures of zwitterionic amino acids and their hydrochloride salts to a suspension of immobilized subtilisin in hexane or toluene. As predicted, the rate of enzyme-catalyzed transesterification depends on the choice of solid buffer pair, here decreasing in the sequence: Lys and Lys·HCl, Arg and Arg·HCl, Lys·HCl and Lys·2HCl. Importantly, the rates were independent of both the initial aqueous pH to which the immobilized enzyme was adjusted and of the ratio of the solid species. The acid−base exchange potential of these solid-state buffers is theoretically solvent-independent, giving an important advantage over organic-phase buffers. Combined with their ease of use, this may lead to widespread applications for the control of protein ionization states in organic media.

Change in the protonation state of functional groups on a protein requires a balancing change in counterions. The high dielectric in aqueous solution means these counterions are free to move away from the protein. As a result, their identity often does not affect ionization; therefore, pH alone describes behavior. In low-water organic media, where dielectric is smaller, the counterions will be much more closely associated with the protein molecules. Hence, the protonation of protein groups will be controlled by the availability of both counterions and H^+. Hence, it is best[3c] to consider equilibria explicitly involving counterions, such as the following:

$$\text{Protein-NH}_3^+\text{Cl}^- \rightleftharpoons \text{Protein-NH}_2 + \text{H}^+ + \text{Cl}^- \quad (1)$$

$$\text{Protein-COOH} + \text{Na}^+ \rightleftharpoons \text{Protein-COO}^-\text{Na}^+ + \text{H}^+ \quad (2)$$

Equilibrium can be reached with other pairs of species able to exchange ions in the same way, such as Na^+A^- and HA dissolved in the organic phase.[4] The equilibria can be characterized by potentials for the exchange of ions, which can be presented as products or ratios of thermodynamic activities, $a_{H^+}a_{Cl^-}$ and a_{H^+}/a_{Na^+}.[5] Each protein group will have a characteristic value of the appropriate potential.

Another type of equilibrium that can exchange ions in the same way involves solid-phase species, e.g.[6]

$$\text{Lys·HCl}_{\text{cryst}} \rightleftharpoons \text{Lys}_{\text{cryst}} + \text{H}^+ + \text{Cl}^- \quad (3)$$

Such a pair of solids will generate a single characteristic value of $a_{H^+}a_{Cl^-}$ at equilibrium, since the crystalline solids will have a fixed thermodynamic activity, whatever their quantity. An alternative, equivalent picture is the generation of a fixed (extremely small) partial pressure of HCl gas. It is possible to think of a combination of reactions 1 and 3 as an equilibrium between the solid pair and protein amino groups:

$$\text{Protein-NH}_3^+\text{Cl}^- + \text{Lys}_{\text{cryst}} \rightleftharpoons \text{Protein-NH}_2 + \text{Lys·HCl}_{\text{cryst}} \quad (4)$$

Thus, the solid buffer pair will control the protonation state of the protein.

Figure 1 shows how subtilisin activity in hexane is affected by the pH of the aqueous solution in which the immobilized enzyme is washed before drying. Without other additions, the well-known phenomenon of "pH memory" is clearly observed. In contrast, in the presence of solid particles of Lys and Lys·HCl, there is little dependence on previous aqueous pH, consistent with the proposed buffering action. The rate is also around four times that in the optimal pH control. This latter effect is less obviously expected, but clearly valuable for applications. It probably reflects the fact that the enzyme activity will depend on at least two independent acid−base parameters, as introduced above. Adjusting the pH of the initial aqueous sodium phosphate buffer will principally affect a_{H^+}/a_{Na^+} (or pH − pNa) of the dried enzyme, and probably cannot bring $a_{H^+}a_{Cl^-}$ (or pH + pCl) into the range achieved with the Lys and Lys·HCl solid buffer. This is an example of how these two parameters can be independently manipulated,[7] so that the full acid−base behavior of the enzyme in low-water media is a function of both.

We varied the proportions of Lys and Lys·HCl between 10% and 90%, keeping the total buffer weight constant. The enzymic reaction rate changed by less than 10% (data not shown), as expected for equilibria involving crystalline solids. The use of different solid pairs did affect enzyme activity substantially, however (Table 1). The three pairs listed are all interconverted by the addition or removal of H^+ and Cl^- (or HCl) and should each give a characteristic value of $a_{H^+}a_{Cl^-}$ (or pH + pCl). This value will in turn control the equilibrium protonation state of basic groups in the protein and, hence, its enzymic activity. The solids were chosen to be zwitterionic, to minimize any solubility in the organic phase. Though the addition of Arg and Arg·

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(3) (a) Blackwood, A. D.; Curran, L. J.; Moore, B. D.; Halling, P. J. *Biochim. Biophys. Acta* **1994**, *1206*, 161−165. (b) Xu, K.; Klibanov, A. M. *J. Am. Chem. Soc.* **1996**, *118*, 9815−9819. (c) Halling, P. J.; Blackwood, A. D.; Moore, B. D. *Ann. NY Acad. Sci.* **1996**, *799*, 251−256.

(4) Compound A is an acid sufficiently hydrophobic that even its ion pair salts are organic soluble.

(5) An alternative presentation is as pH + pCl and pH − pNa.

(6) A basic amino-acid such as Lys has, of course, three ionizable groups. The crystalline solid written Lys is a zwitterion with one charged amino and carboxyl. Lys·HCl has both amino groups protonated, balanced by the carboxyl and a Cl^- ion.

(7) Subject to a maximum ratio of $a_{H^+}a_{Cl^-}$ to a_{H^+}/a_{Na^+} set by the precipitation of solid NaCl.

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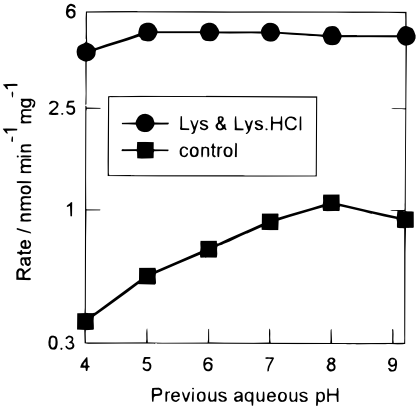

Figure 1. Solid-state acid−base buffer overcomes the effect of previous aqueous pH. Subtilisin Carlsberg was immobilized by covalent attachment to PolyHipe beads, as described.[3a] The beads were washed in 20 mM aqueous sodium phosphate buffer of the given pH before air drying. The catalyst (15 mg) and the organic phase (5 mL of hexane with 20 mM Ac-Phe-OEt and 1 M PrOH) were separately pre-equilibrated to thermodynamic water activity of 0.87. They were then shaken together at 22 °C, with periodic samples analyzed by GLC. The initial rate was determined as the linear increase in Ac-Phe-OPr concentration over 24 h or so, and normalized to the total weight of the immobilized enzyme preparation. Where shown, 100 mg each of Lys and Lys·HCl were added at the start of the reaction. (The same rates were obtained if these solids were present during water activity pre-equilibration.) All points represent at least two replicates in close agreement. The effect, and others reported in this paper, have also been demonstrated with two different batches of immobilized enzyme.

Table 1. Effects of Different Types of Solid-Phase Acid−Base Buffer[a]

	subtilisin activity (nmol min^{-1} mg^{-1})		equilibrated [DBU·HCl]/[DBU]
salts added	in hexane	in toluene	in MPO
Lys + Lys·HCl	5.1	0.80	0.28
Arg + Arg·HCl	1.3	0.25	0.42
Lys·HCl + Lys·2HCl	0.005	0.005	>60
none	1.0	0.31	

[a] Enzymic reactions as for Figure 1. Aqueous washing pH was 8. To relate to protonation of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 1.2 g of each of the pair of solids was shaken with 60 mL of 10 mM DBU in 4-methylpentan-2-one (MPO; methyl isobutyl ketone). Samples were removed and centrifuged, and 3 mL of liquid phase was mixed with 3 mL of MeOH, 2 mL of H_2O, and methyl red indicator and then titrated with 0.1 M aqueous HCl. Changes in titre from the initial DBU solution were monitored over time and reached steady values after 5 h (20 h for Lys·HCl and Lys·2HCl).

HCl does not greatly affect the initial rate, it did have a noticeable effect on the full course of the reaction. With added solid-state buffers, the decline in rate at longer reaction times was much less pronounced than in the control reaction (data not shown). This effect probably indicates that, without buffering, some unfavorable change in acid−base conditions at longer times. Small amounts of acidic Ac-Phe, from a hydrolytic side reaction, may be responsible.

Table 1 shows that the solid buffers have comparable effects on the activity of the same enzyme in toluene. Since we predict the value of $a_{H^+}a_{Cl^-}$ generated by a given solid buffer pair should be independent of the solvent (assuming the use of solvent-independent standard states), this is encouraging. A pair found optimal for a particular enzyme should be valid for all low-polarity solvents in which ion-pairing remains complete, as supported by our data (Table 1). In contrast, the effective acidity of buffers *dissolved* in the bulk organic phase will alter because of differing solvation effects.

There should also be a relationship between the different values of $a_{H^+}a_{Cl^-}$ generated by these solid-state buffers and the positions of other equilibria. Table 1 shows the effects of the solid buffers on the equilibrium between 1.8-diazabicyclo[5.4.0]-undec-7-ene (DBU) and its hydrochloride ion pair in homogeneous organic solution. We have previously used such DBU-based buffers to control the activity of subtilisin in pentan-3-one.[3c] The effective basicity parallels the catalytic activity obtained with each pair, suggesting that the optimal value of $a_{H^+}a_{Cl^-}$ for this enzyme is equal to or smaller than that in equilibrium with Lys and Lys·HCl.[8]

It should be noted that these effects are quite different from the effects of co-drying salts and buffers with the enzyme preparations.[9] Addition of solid KCl to our reaction system had no effect on the rate.[10] It is interesting however, that solid-state acid−base buffer pairs may also be generated inadvertently during drying of enzymes from buffered aqueous solutions. This may be one reason for the observed dependence of activity in organic media on the type of buffer used,[2b,c] as well as previous aqueous pH.

As explained, thermodynamics indicates that the solid pairs used should fix the protein protonation state at equilibrium. Less expected is the finding that the effect on the enzyme and its catalytic activity is rapid. To test this more clearly, we performed a reaction with a high enzyme concentration of 36 g L^{-1}, so the reaction progress could be followed over a shorter time period. With the addition of solid Lys and Lys·HCl, no lag period in the progress curve could be detected, allowing us to set an upper limit of 3 min for the equilibration time. Achievement of equilibrium requires that HCl (or its component ions) be exchanged between the solid buffers and the suspended particles of immobilized enzyme. This might occur via dissolution in the organic liquid phase, though solubilities in hexane will be small, as will the vapor pressure of HCl. It is possible that trace impurities such as organic amines transport HCl bound as an ion pair. An alternative possibility is transfer on direct contact between particles in the shaken suspension. We tested this mechanism in experiments where such contact is reduced or prevented. The particles of Lys and Lys·HCl were separated from the enzyme by a no. 0 glass frit or encapsulation with a polyamide membrane. The rates observed were still substantially faster than the control (2.9 and 2.1 times, respectively), though slower than with direct addition.

Finally, we demonstrated the potential of solid-state buffers to enhance the rate with freeze-dried enzyme powders. Lys and Lys·HCl were tested with subtilisin in the classic "straight from the bottle" form. The rate with added buffer was more than doubled.

In conclusion, we have demonstrated that suitable pairs of crystalline solids can control acid−base conditions in low-water organic media and, hence, the catalytic activity of enzymes. This method seems to have considerable potential. Further development will require the identification of solid pairs able to generate values of $a_{H^+}a_{Cl^-}$ covering the full range of interest.[11] A separate series will be needed to control the complementary parameter a_{H^+}/a_{Na^+}.

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(8) Note that Arg binds HCl slightly less strongly than Lys in the solid state. The relevant protonation in Arg is presumably of the α-amino group, and crystal interactions will play an important role in each case.

(9) For example, see: Khmelnitsky, Y. L.; Welch, S. H.; Clark, D. S.; Dordick, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 2647−2648 and ref 2d.

(10) Khmelnitsky et al.[9] also found that co-drying with the enzyme was essential to observe any effect. Note also that our experiments are performed with immobilized subtilisin, where enzyme makes up only a small portion of the solid particle.

(11) Some salts that might be used in buffer pairs also form hydrates at the water activities used for enzymic reactions, which will complicate their application.