Acid–Base Control for Biocatalysis in Organic Media: New Solid-State Proton/Cation Buffers and an Indicator

Neil Harper, Mark Dolman, Barry D. Moore,* and Peter J. Halling*[a]

Abstract: Although great care is generally taken to buffer aqueous enzyme reactions, active control of acid–base conditions for biocatalysis in low-water media is rarely considered. Here we describe a new class of solid-state acid–base buffers suitable for use in organic media. The buffers, composed of a zwitterion and its sodium salt, are able to set and maintain the ionisation state of an enzyme by the exchange of H^+ and Na^+ ions. Surprisingly, equilibrium is established between the different solid components quickly enough to provide a practical means of controlling acid–base conditions during biocatalysed reactions. We developed an organosoluble chromoionophore indicator to screen the behaviour of possible buffer pairs and quantify their relative H^+/Na^+ exchange potential. The transesterification activity of an immobilised protease, subtilisin Carlsberg, was measured in toluene in the presence of a range of buffers. The large observed difference in rates showed good correlation with that expected from the measured exchange potentials. The maximum water activities accessible without formation of hydrates or solutions of the buffers are reported here. The indicator was also used to monitor, for the first time in situ, changes in the acid–base conditions of an enzyme-catalysed transesterification reaction in toluene. We found that even very minor amounts of an acidic by-product of hydrolysis were leading to protonation of the enzyme, resulting in rapid loss of activity. Addition of solid-state buffer was able to prevent this process, shortening reaction times and improving yields. Solid-state buffers offer a general and inexpensive way of precisely controlling acid–base conditions in organic solvents and thus also have potential applications outside of biocatalysis.

Keywords: buffers · enzyme catalysis · protonations · solid-state chemistry · solvent effects

Introduction

For many enzyme reactions, it is advantageous to use organic solvents as the reaction medium (for recent reviews, see ref. [1]). In aqueous systems, the ionisation state of the enzyme affects catalytic activity and stability, and is controlled by adjusting pH. In organic solvents, control of the ionisation state of the enzyme is also important. For example, it has been shown that the pH at which an enzyme is washed before drying and suspension in an organic solvent affects its activity in the solvent.[2] A similar pH dependence to that in aqueous solution is observed. However, in many reactions there are changes in the acid–base conditions due to, for example, acidic or basic reactants (e.g. ref. [3]), and so the "pH memory" effect described above is lost. Buffers have been added to the reaction mixture in order to maintain the ionisation state of the enzyme and hence control its activity. These buffers may either dissolved in the solvent[4–6] or present as an insoluble solid.[7]

In solvents of low dielectric, opposite charges interact more strongly as there is less shielding between them. Therefore counterions are more closely associated with their opposite charges and their presence must be taken into consideration in determining the ionisation state of an enzyme. Depending on the type of ionisable group on the enzyme, the availability of ions such as Na^+ or Cl^- in the system will be important, in addition to that of H^+.[8] For example, the ionisation state of carboxyl groups will be controlled by the ratio of thermodynamic activities of H^+ and Na^+ (written as a_{H^+}/a_{Na^+}). The relevant parameter for amino groups is the product of the activities of H^+ and Cl^- ($a_{H^+} \cdot a_{Cl^-}$).

In principle, an individual solid-state buffer will set a single value of one of the above ionisation parameters at equili-

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brium. In this paper, we describe the use of "zwitterionic biological buffers" to control a_{H^+}/a_{Na^+}. The solids added are the zwitterion and the corresponding Na^+ salt, as shown in Equation (1).

$$\underset{\text{general formula for zwitterion}}{{}^{+}NHR_2\cdots SO_3^-} + Na^+ \rightleftharpoons \underset{\text{sodium salt}}{NR_2\cdots SO_3^-Na^+} + H^+ \quad (1)$$

In the zwitterion the charge on the protonated amine group balances the negative charge on the deprotonated sulfonate or carboxyl group. The Na^+ salt is formed by deprotonation of the amine group in the zwitterion. This leaves a single negative charge on the sulfonate (or carboxyl) group, which is balanced by Na^+.

The acid–base strength is therefore determined by the amine group. Since both the zwitterion and the Na^+ salt are charged and contain polar groups, they tend to be insoluble in most organic solvents. Also, they are present as crystalline solids, so they have a fixed thermodynamic activity whatever their quantity. Looking at this another way, the ions are held in regular positions in the crystals, resulting in a fixed energy for H^+/Na^+ exchange. The buffer pair will thus control a fixed value of a_{H^+}/a_{Na^+} at equilibrium. Such buffers could therefore be used to control the ionisation state of carboxyl groups on enzymes in organic solvents, as shown in Equation (2).

$${}^{+}NHR_2\cdots SO_3^- + Enz{-}COO^-Na^+ \rightleftharpoons NR_2\cdots SO_3^-Na^+ + Enz{-}COOH \quad (2)$$

Here, we screen potential solid buffers that can control the ratio of the activities of H^+ and Na^+ by measuring the response of an organosoluble indicator to this parameter in the presence of the buffers. The indicator response was calibrated using organosoluble buffers known to control the activity of subtilisin Carlsberg in the appropriate range. Using this method we identify a series of solid buffers that set a_{H^+}/a_{Na^+} values over the whole range of enzyme activity. We also demonstrate that the indicator can be used to monitor changes in this parameter resulting from the production of acid during an enzyme reaction in organic solvent.

Results and Discussion

Identifying potential solid-state acid–base buffers: The use of solid-state acid–base buffer pairs to fix the value of a_{H^+}/a_{Na^+} within an organic reaction medium has not been reported previously, and hence the first step was to find a method of screening potential compounds. Besides wishing to identify the correct range, we were concerned that factors such as slow exchange kinetics and poor reproducibility might limit the practical use of such buffers. As a starting point we set out to identify a series of buffers which set values of a_{H^+}/a_{Na^+} within the normal range for enzyme activity, targeting in particular subtilisin Carlsberg, a protease commonly used for biocatalysis in low water media. As a tool for screening it was first necessary to synthesise an organosoluble indicator which would respond to the appropriate range of a_{H^+}/a_{Na^+} values. The compound in Figure 1A was synthesised by coupling of a diazonium salt with a phenolic crown ether obtained by the published method.[9] The phenolic group, present within a crown ether ring, will tend to ion-pair with Na^+ when deprotonated, particularly in less polar solvents. Hence the equilibrium between the two forms of the indicator will involve exchange of H^+ and Na^+, and so will respond to a_{H^+}/a_{Na^+}. In order to measure the relative concentrations of the protonated and Na^+-complexed forms, the molecule was designed to give a chromophoric response depending on whether the phenolic group is protonated or Na^+-complexed. Measurement of absorbance will indicate changes in a_{H^+}/a_{Na^+}, and larger differences will be observable by eye as a colour change. Refer to Figure 1B for the UV/visible absorption spectrum of the two forms of the indicator. In toluene/1M PrOH λ_{max} of the yellow protonated indicator was 370 nm (absorbance coefficient of $2.17 \times 10^4\,M^{-1}\,cm^{-1}$) and that of the red/orange deprotonated (Na^+-complexed) form was 470 nm (absorbance coefficient of $2.89 \times 10^4\,M^{-1}\,cm^{-1}$). The higher absorbance coefficient at the longer wavelength is typical of phenolic ionisation. (Further information regarding operational use of the indicator is given in the experimental section.)

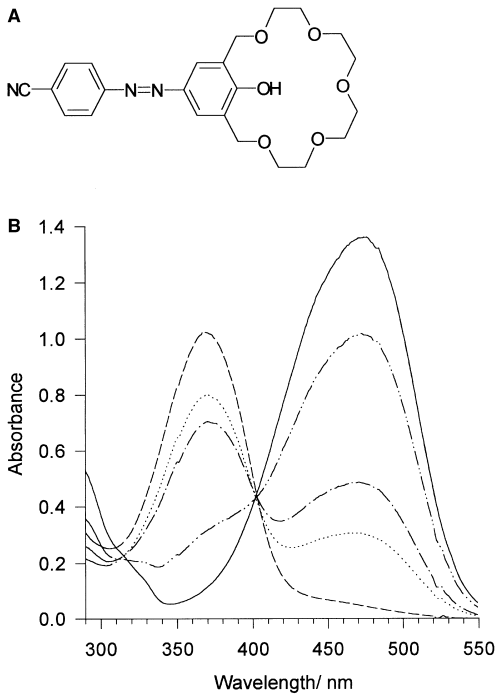

Figure 1. A. Structure of indicator. B. UV/visible spectrum of indicator in toluene/1M PrOH. The indicator (47 μM) in toluene/1M PrOH was equilibrated with a range of solid a_{H^+}/a_{Na^+} buffers to give ionisation states of the indicator between completely protonated (λ_{max} 370 nm) and completely deprotonated (λ_{max} 470 nm). The buffers were $NaHSO_4/Na_2SO_4$ (----), MOPS/Na^+ salt (····), TES/Na^+ salt (·–·–), HEPES/Na^+ salt (-···-) and glycine/Na^+ salt (——).

The indicator was tested for its suitability to screen for buffers that would control a_{H^+}/a_{Na^+} in the range of activity of subtilisin Carlsberg. This was done by equilibration with two organosoluble buffers used previously with this enzyme. The buffers were triphenylacetic acid and its sodium salt[4] and a polybenzyl ether dendritic acid and its sodium salt.[6] The indicator was equilibrated with buffers prepared over a range of concentration ratios of sodium salt to acid. In all cases the same solvent mixture, toluene containing 1M propan-1-ol, was

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used, since the nature of the solvent affects the equilibrium position of the indicator (see below).

Figure 2 shows the relationship between the ratio of equilibrium concentration of the two forms of the indicator and that of the two forms of the buffer. Clearly, the indicator

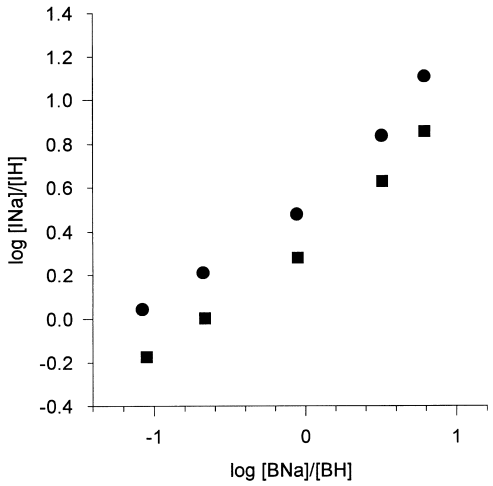

Figure 2. Equilibrium of indicator and known organosoluble buffers. Equilibrium concentration ratios are shown for indicator, deprotonated (INa) and protonated (IH) and for organic soluble buffer, sodium salt (BNa) and acid (BH). The buffer (1 mM) was dissolved in toluene/1M PrOH with a range of ratios of sodium salt to acid and equilibrated with the indicator (47 μM). The buffers were triphenylacetic acid/Na^+ salt (■) and the dendrimer acid/Na^+ salt (●).

responds in the range of a_{H^+}/a_{Na^+} controlled by the two known buffers: hence it is expected to be a useful tool for screening potential solid buffers for subtilisin Carlsberg. From Figure 2 it can be seen that the indicator is more acidic than the soluble buffers, since the ratio of concentration of deprotonated to protonated indicator is greater than one when the buffer ratio is equal to one. It can also be seen that of the two buffers the triphenylacetate is slightly more acidic since the indicator equilibrium lies further towards the protonated form.

Having established the indicator suitability, we next screened potential solid a_{H^+}/a_{Na^+} buffers. A selection of zwitterionic biological buffers (commercially available as both the zwitterion and sodium salt) were equilibrated with the indicator in toluene/propan-1-ol (1M). Figure 3 shows the indicator response as a function of the aqueous pK_a values of these buffers. Unexpectedly we found the general variation in fraction of deprotonated indicator relative to the *aqueous* buffer pK_a was similar to a normal aqueous titration curve. This was surprising because in the solid state the intrinsic acid–base strength of the ionisable groups (given by aqueous pK_a) is not the only factor which determines the potential for exchange of H^+ and Na^+ ions. This parameter will also depend on the difference in lattice energies between the crystalline solid zwitterion and its Na^+ salt. Indeed, given their large magnitudes, crystal energies might be expected to dominate exchange potentials, rendering aqueous pK_a inappropriate as a guide. The reasonable correlation with aqueous pK_a seen for many members of this buffer series indicates that here differences in crystal energies between the H^+ and Na^+ forms

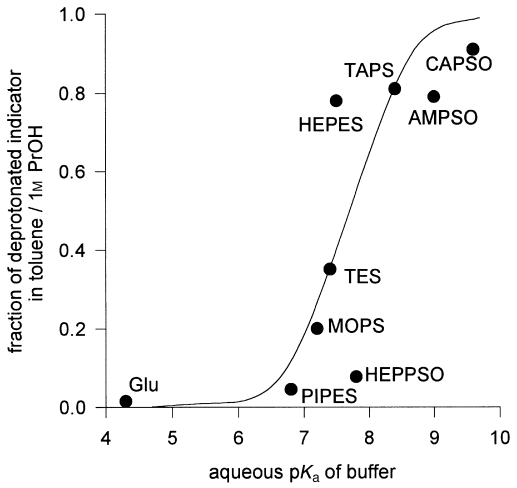

Figure 3. Response of indicator to solid-state buffers of various aqueous pK_a. The line shown is for a simple (e.g. aqueous) titration with pK_a 7.7. The following buffer pairs were equilibrated with the indicator (47 μM) in toluene/1M PrOH (pK_a values at 25 °C in brackets[11]): L-glutamic acid/monosodium salt (4.3), PIPES/monosodium salt (6.8), MOPS/sodium salt (7.2), TES/sodium salt (7.4), HEPES/sodium salt (7.5), HEPPSO/sodium salt (7.8), TAPS/sodium salt (8.4), AMPSO/sodium salt (9.0), CAPSO/sodium salt (9.6). Showing that equilibrium had been reached, it was found (with HEPES/sodium salt as buffer) that the same fraction of deprotonated indicator was obtained whatever form of the indicator was added initially. The reproducibility between duplicate measurements was better than 3%, except for PIPES (10%).

of the buffer must be relatively small. This may in part reflect the fact that these "biological buffers" have been designed such that both zwitterionic and salt forms are highly soluble in water. However, it can be seen from Figure 3 that, as anticipated, several of the buffers did behave anomalously and gave indicator responses considerably higher or lower than predicted from their aqueous pK_a. This shows the importance of being able to monitor a_{H^+}/a_{Na^+} in the organic solvent directly.

Further evidence of the unique nature of solid-state buffers was obtained from experiments varying the relative amounts of HEPES and its Na^+ salt. It was found that using molar ratios between 9:1 and 1:9 had no effect on the equilibrium indicator response (total amount of buffers constant at 740 μmol). Varying the amount of solid between 3.7 and 370 μmol of each form (at 1:1 ratio of zwitterion to salt) also had no significant effect on the position of equilibrium, although equilibrium was attained faster when more solid was present. These findings are exactly those expected for equilibria involving crystalline solids. Provided there is at least a trace of each form of the buffer, the amounts have no influence on the equilibrium position.

Other potential solid buffers tested did not seem to buffer in the range appropriate for subtilisin Carlsberg. Essentially completely protonated indicator was found for tartaric acid/mono-Na^+ salt, ascorbic acid/Na^+ salt, NaH_2PO_4/Na_2HPO_4 and $NaHSO_4/Na_2SO_4$. The indicator was almost completely deprotonated in the cases of $NaHCO_3/Na_2CO_3$, Na_2HPO_4/Na_3PO_4 and glycine/Na^+ salt.

Enzyme reaction in the presence of solid buffers: The range of indicator responses in equilibrium with the above solid buffers

(Figure 3) is wider than that obtained with the organosoluble buffers (Figure 2). These organosoluble buffers are known to affect substantially the catalytic activity of subtilisin Carlsberg by controlling its ionisation state.[4, 6] Therefore an even wider range of activity would be expected when the solid-state buffers are used to control enzyme reactions in organic solvent. To verify this, the solid buffers were tested in the subtilisin-catalysed transesterification of Ac-Tyr-OEt with PrOH in toluene at a_w 0.53.

Figure 4 shows the initial rates for the enzyme found in the presence of the different buffer pairs. These are plotted against the fraction of deprotonated indicator in equilibrium

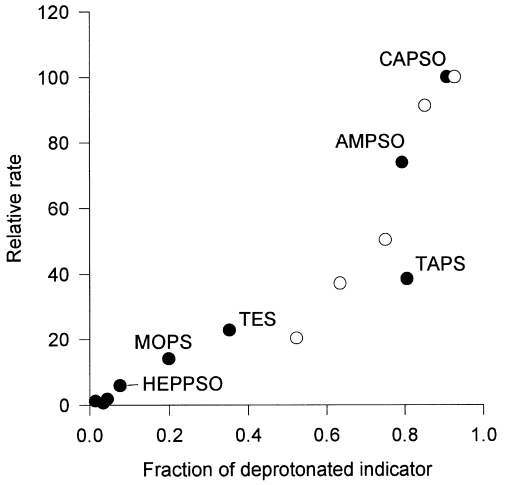

Figure 4. Graph of relative initial rate, obtained with solid buffers (●) and the organosoluble dendrimer buffer (○), against the fraction of deprotonated indicator (from separate equilibrium with buffers). The dendrimer was dissolved in toluene with a range of molar ratio of acid to sodium salt (9:1 to 1:9) and rates obtained for the transesterification of Bz-Tyr-OEt with PrOH catalysed by immobilised subtilisin Carlsberg in toluene at a_w 0.53 (for experimental details, see ref. [6]). The rates thus obtained were correlated with the indicator response to the buffer ratios in toluene/1M PrOH (see Figure 2). The solid buffers listed in Figure 3 were used in the transesterification of Ac-Tyr-OEt with PrOH in toluene at a_w 0.53. The three unlabelled points represent L-glutamic acid/monosodium salt, control (no added buffer) and PIPES/monosodium salt (in order of increasing fraction of deprotonated indicator). The rates thus obtained were correlated with the indicator response to the buffers in toluene/1M PrOH. Reproducibility between duplicate rate measurements was better than 20% except for TAPS (25%) and MOPS (50%). The maximum rates (100%) were 8.68 nmol h^{-1} mg^{-1} catalyst in the experiments with the dendrimer buffer, and 201 nmol h^{-1} mg^{-1} catalyst with the solid buffers. The difference is largely due to the use of different substrates: an experiment with Bz-Tyr-OEt in the presence of CAPSO/Na^+ salt gave a rate of 18.8 nmol h^{-1} mg^{-1}. The remaining twofold difference is probably due to batch variations in the immobilised subtilisin.

with the same buffer, to give a measure of a_{H^+}/a_{Na^+}. As expected for the solid buffers, there was a large increase (greater than 80-fold) in the reaction rate between the minimum rate obtained with L-glutamic acid/Na^+ salt (2.38 nmol h^{-1} mg^{-1}) and the maximum with CAPSO/Na^+ salt (201 nmol h^{-1} mg^{-1}). Figure 4 also shows rates obtained with the soluble dendrimer buffer, plotted on the same basis (i.e. against the indicator response to the appropriate buffer ratio). The general trend is clearly the same, as expected if both types of buffer control a_{H^+}/a_{Na^+}, and the indicator and enzyme respond to this. There remain some slight anomalies to explain, in particular the lower than expected rate with TAPS and its Na^+ salt.

In these experiments, the solid enzyme preparation was not preequilibrated with the solid-state buffers. Because of this, a lag time was generally observed before the straight line "initial" reaction rate was established. The duration of the lag was dependent on the type and amount of buffer and varied from 20–180 min. This reflects both the kinetics of exchange of H^+ and Na^+ between the crystal surfaces and their diffusion to and from enzyme molecules located in the pores of the support. When the same solid-state buffers were used in acetonitrile, no lag in the initial rate was observed (Partridge et al., unpublished), presumably as a result of improved solvation of cations by such a polar solvent. It is interesting to note that for the class of solid buffers that control $a_{H^+} \cdot a_{Cl^-}$ there was also no observed lag, even in hexane.[7] This difference may be due to the fact that such buffers can exchange HCl as a neutral molecule that is expected to be slightly soluble in hexane. To maximise reproducibility with solid-state buffers that control a_{H^+}/a_{Na^+} in nonpolar media, they should be preequilibrated with the insoluble biocatalyst preparation in the organic solvent for 2–3 h before the reactants are added.

Because of the hygroscopic nature of the biological buffers, the maximum a_w used in these studies was 0.53. As can be seen in Table 1, above this value many of the Na^+ salts were found

Table 1. Water uptake by solid buffer salts over a range of a_w.[a]

	Highest a_w with probably only H_2O adsorption[b]	Hydrates formed and a_w range	Limits on a_w for saturated solution[c]
Na^+ salts[d]			
AMPSO	0.75	no evidence	0.75–0.98
CAPSO[e]	0.53	2 H_2O; 0.65–0.85	0.85–0.98
HEPES	0.23	2 H_2O; 0.53	0.53–0.58
HEPPSO	0.75	no clear evidence	0.85–0.98
MOPS	0.23	2 H_2O; 0.53–0.75	0.75–0.85
PIPES	0.23	1 H_2O; 0.53–0.75	0.85–0.98
TAPS	0.58	no evidence	0.58–0.65
K^+ salts			
HEPES	0.58	no evidence	0.58–0.65

[a] The salts were dried (at 105 °C) and then equilibrated at room temperature over the appropriate saturated salt solution at fixed a_w (at 0.23, 0.53, 0.58, 0.65, 0.69, 0.75, 0.85 and 0.98). The water content was then compared with that expected in the hydrate. [b] The water content is below that found in the monohydrate. [c] At the lower a_w in the range there was no liquid phase; at the higher a_w a solution started to form. For sodium L-glutamate this range was 0.85–0.98 and for the TES Na salt it was 0.75–0.85. [d] Certain zwitterions (HEPES, MOPS, PIPES and TES) adsorbed H_2O to less than monohydrate level at a_w 0.53. [e] Equilibration was not carried out at a_w 0.58.

to go into solution on equilibration through the vapour phase with appropriate aqueous saturated salt solutions. The data also show that hydrates of some of the salts had formed at a_w 0.53. This will affect the acid–base behaviour of the buffers, since the H_2O will be involved in the ionisation equilibrium, as shown in Equation (3), where B represents the solid buffer. The equilibrium constant for this reaction is given by Equation (4). It follows that changing a_w will lead to changes

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$$B^- Na^+ \cdot H_2O + H^+ \rightleftharpoons B^\pm + Na^+ + H_2O \quad (3)$$

$$K = a_{Na^+} \cdot a_w / a_{H^+} \quad (4)$$

in the value of a_{H^+}/a_{Na^+} set by the buffer. At fixed a_w, as used in our reactions, the same value of a_{H^+}/a_{Na^+} will be obtained each time, but this may differ somewhat from that obtained under anhydrous conditions.

Detection of changes in a_{H^+}/a_{Na^+} during enzyme reactions: In addition to screening for new a_{H^+}/a_{Na^+} buffers, the indicator can also detect changes in a_{H^+}/a_{Na^+} during enzyme reactions in organic solvents. It is commonly suggested that changes in acid–base conditions may affect enzyme activity in organic solvents. A change in pH of an inaccessible aqueous phase during enzyme-catalysed esterification and ester hydrolysis was demonstrated using hydrophobic fluorescein derivatives as indicators in pentan-3-one.[10] Our indicator was used to show changes in a_{H^+}/a_{Na^+} during transesterification of Ac-Tyr-OEt with propan-1-ol catalysed by subtilisin Carlsberg in toluene. The acid–base conditions can change owing to a side reaction in which water competes with propanol as a nucleophile to produce Ac-Tyr-OH.

The amount of acid produced depends on the water activity at which the reaction takes place. The above reaction was therefore carried out at two a_w levels, 0.53 and 0.85. In order to ensure the indicator would all start off in the deprotonated form, the enzyme preparation was first prewashed with sodium phosphate of pH 9.9. This will initially leave the enzyme in a protonation state corresponding to a pH higher than optimum, but this is expected to change during the reaction. Following washing and equilibration at the correct a_w, the enzyme was suspended in solvent containing the indicator. The substrate was then added to the solvent and the response of the indicator measured periodically. Figure 5 shows the relationship between the fraction of deprotonated indicator and the reaction progress at a_w 0.53. (Controls showed the presence of the indicator had no significant effect on this reaction progress.) The HPLC started to detect acid (approx. 25 μM) only after 20 h. However, the fraction of deprotonated indicator initially fell rapidly and it is deduced that this was due to low levels of Ac-Tyr-OH being produced. Since the reaction was not buffered the reaction rate also fell quickly even though the conversion to product was still low. This is because at higher a_{H^+}/a_{Na^+} values the active site of subtilisin will become protonated and the catalytic efficiency will drop. Consistent with this interpretation, in the reaction at a_w 0.85, both the fraction of deprotonated indicator and reaction rate fell even more rapidly (results not shown), due to the faster rate of formation of the acid at higher a_w. This demonstrates the utility of the indicator: it is able to detect practically important and previously unquantified changes in a_{H^+}/a_{Na^+} occurring during an unbuffered enzyme reaction in a nonpolar organic solvent.

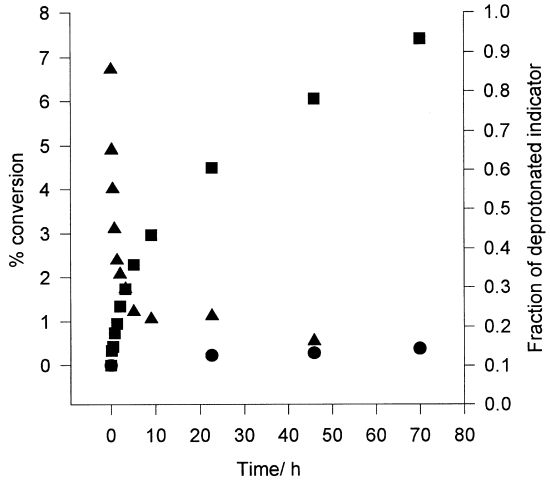

Figure 5. Correlation between reaction progress (conversion to Ac-Tyr-OPr (■) and Ac-Tyr-OH (●)) and response of indicator (▲) during the transesterification of Ac-Tyr-OEt with PrOH in toluene at a_w 0.53, catalysed by immobilised subtilisin Carlsberg, prewashed in sodium phosphate buffer (pH 9.9). The response of the indicator at the start of the reaction is that in equilibrium with the enzyme before addition of substrate.

In order to show the benefits of buffering, the same reaction was carried out in the presence of the solid a_{H^+}/a_{Na^+} buffer, CAPSO/Na^+ salt (at a_w 0.53). In this case the response of the indicator was more or less constant during the reaction. (The fraction of deprotonated indicator only fell from 0.90 to 0.86 over 93 h.) After 93 h, 90 μM Ac-Tyr-OH was detected at a level much higher than the level of acid causing the indicator to be predominantly protonated in the unbuffered reaction (Figure 5). This clearly demonstrates the ability of the solid-state buffers to maintain a constant a_{H^+}/a_{Na^+} during enzyme reactions in nonpolar solvents.

Conclusion

New solid buffers which control a_{H^+}/a_{Na^+} are able to control the ionisation state of subtilisin Carlsberg in toluene. Suitable solid-state buffer pairs were found by equilibration with an organosoluble indicator and comparison of their performance with the response to known organosoluble buffers. The enzyme reaction rate obtained in the presence of these solid buffers correlated with the response of the indicator. The indicator also detected changes in a_{H^+}/a_{Na^+} due to acid produced during an enzyme-catalysed reaction. These solid-state buffers should be useful for controlling the ionisation state of other enzymes in organic solvents. Some of the buffer pairs tested that are more acidic or basic than required with subtilisin Carlsberg may prove useful with other enzymes. If these buffers are used to tune the protonation state of an enzyme in an organic medium, it is no longer necessary to carry out lyophilisation or immobilisation at a whole series of different pH values to find the optimum.

Experimental Section

Materials: Most chemicals were of analytical grade and were obtained from Aldrich or Sigma and Merck. All solvents were of HPLC grade. *p*-Cyanoaniline and triphenylacetic acid were obtained from Aldrich. The following Sigma compounds, along with their sodium salts, were used as solid buffers: 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfon-

ic acid (CAPSO), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-hydroxypropanesulfonic acid) (HEPPSO), 3-(*N*-morpholino)propanesulfonic acid (MOPS), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES).

Other solid buffer compounds were L-glutamic acid (Fluka) and sodium L-glutamate (Aldrich). Na_2SO_4, tartaric acid, Na_2HPO_4, $Na_3PO_4 \cdot 12H_2O$, glycine and $NaHCO_3$ were supplied by Merck. $NaHSO_4$, NaH_2PO_4 and Na_2CO_3 were supplied by Aldrich. Sodium tartrate (monohydrate) was supplied by Fluka and sodium glycinate (monohydrate) was supplied by Sigma. The substrate for enzyme reactions, *N*-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt), was obtained from Sigma. The UV/visible spectra of the indicator were recorded on a Beckman DU 7500 polydiode array UV/visible spectrophotometer.

Synthesis of indicator: *p*-Cyanoaniline (0.31 g, 2.53×10^{-3} mol) was dissolved in a solution of concentrated hydrochloric acid (0.60 mL) and water (10 mL). The solution was cooled to 0 °C, and sodium nitrite (0.17 g, 2.53×10^{-3} mol) was added; the solution was stirred for one hour and added to a solution of 2-hydroxy-1,3-xylyl-18-crown-5 (0.77 g, 2.47×10^{-3} mol) (compound **7** from ref. [9]) in 1M sodium hydroxide solution (20 mL) at 0 °C over 15 min and stirred at this temperature for a further hour. The solution was then extracted with dichloromethane (4×75 mL), and the organic extracts were combined and evacuated to dryness. The product was then purified on a silica column with a solvent gradient of dichloromethane (100%) to dichloromethane/ethanol (97%/3%) and evacuated to dryness to yield an orange solid (0.81 g, 74%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta =$ 3.73 (m, 16H, CH_2CH_2O), 4.78 (s, 4H, $ArCH_2O$), 7.77 (AB d, 2H, $J =$ 7.5 Hz, ArH), 7.82 (s, 2H, ArH), 7.92 (AB d, 2H, *J* 7.5 Hz, ArH), 8.71 (br s, 1H, OH); ^{13}C NMR (62.5 MHz, $CDCl_3$): $\delta = 69.17$, 69.44, 69.92, 70.16 (8C, CH_2CH_2O), 72.66 (2C, $ArCH_2O$), 112.97, 122.94, 125.19, 125.23, 126.81, 133.08, 145.35, 159.88 (12C, ArH), 207.48 (1C, ArCN); MS (70 eV, EI): *m*/*z* (%) = 102.0 (42) [$C_7H_4N^+$], 249.1 (31) [$C_{15}H_{11}N_3O^+$], 441.2 (100) [M^+]. Accurate mass found 441.18957; calcd for $C_{23}H_{27}N_3O_6$ 441.18999.

Use of indicator: Glassware containing stock solutions was washed in 1M HCl, deionised water and acetone (in that order) in order to minimise ionisation of the indicator. Because of the light-sensitive nature of the indicator, all solutions were stored in the dark. (When dissolved in pentan-3-one the indicator was particularly sensitive to effect of light: after several weeks a decrease in absorbance was observed in normal daylight.) The solvents were checked to ensure that there was no spectral overlap at the wavelengths of measurement.

Absorbance measurements: In most cases a 47 μM indicator solution was used and was added in the protonated form. The absorbance coefficient of the protonated form was obtained by equilibrating the indicator with an equimolar mixture of Na_2SO_4 and $NaHSO_4$ (present as solids in organic solvent). An equimolar mixture of glycine and its sodium salt (monohydrate) was used to give the completely deprotonated form. In both these cases 0.37 mmol of each component was added to indicator solution (4 mL). The mixture was shaken at 200 min^{-1} and samples were taken periodically until the absorbances remained constant, that is, equilibrium was reached. Solutions were filtered to remove suspended solid (0.2 μm PTFE, Whatman) prior to spectral measurement. It was assumed that the indicator was present completely in one form since no shoulder peaks were observed in the spectra. In subsequent experiments, the concentration of the two forms was calculated from the absorbances at the two λ_{max} peaks and the absorbance coefficients, taking into account the spectral overlap. In most experiments, samples removed for absorbance measurements were returned immediately to the equilibration or reaction mixture.

Properties of indicator

Selectivity for cation: NaOH and KOH solutions (both 10 mM) were prepared in deionised water, both with a pH of 12.0. Each of these solutions (5 mL) was added to 5 mL of protonated indicator (47 μM) in pentan-3-one. The mixture was shaken (at 200 min^{-1}) for 1 min, then the spectrum was obtained from the organic phase. This was repeated until equilibrium was reached.

Titration with KOEt and NaOEt: Solid KOH was added to absolute ethanol (2 mg mL^{-1}) to give KOEt. Its concentration was determined by titration against aqueous HCl, with bromothymol blue as indicator. After appropriate dilution, the KOEt was added in aliquots of 0.27 equiv to the indicator (initially protonated) in toluene. Shaking at 200 min^{-1} for 30 s was sufficient to reach equilibrium, after which the spectra were obtained. NaOEt was prepared in a similar way and was likewise added in portions of 0.27 equiv to the indicator in toluene.

Partitioning in biphasic system: The organic phase consisted of indicator (47 μM) in toluene or pentan-3-one, and the aqueous phase consisted of Na phosphate of pH 11.3 (0.05M). Equal volumes of the two phases were shaken together at 200 min^{-1} for 1 min; then the spectrum of the organic phase was obtained. This procedure was repeated until the system attained equilibrium. The distribution coefficient was determined from the ratio of the concentration of the deprotonated indicator in the organic phase to that in the aqueous phase, taking into account the different absorbance coefficients in different solvents.

Equilibration of indicator with organosoluble buffers: The buffers used were a polybenzyl ether dendritic acid and its sodium salt (for outline of synthesis, see ref. [6]), and triphenylacetic acid and its sodium salt. Sodium triphenylacetate was prepared as follows: NaOH (0.33 g, 8.25 mmol) was dissolved in absolute ethanol (70 mL), then added to triphenylacetic acid (1.02 g, 3.54 mmol); after agitation for 15 min, the ethanol was evaporated off and the sodium salt recrystallised from ethyl acetate. Solutions in toluene/propanol (1M) of the buffer acid (1 mM) were mixed in different proportions with solutions of the buffer salt (1 mM), both containing the indicator (47 μM), to give a range of molar ratios of the two forms of the buffer in equilibrium with the indicator. Shaking at 200 min^{-1} for 30 s was sufficient for equilibrium to be reached, after which the spectra were recorded.

The absorption coefficients were obtained by the usual method, but since there was spectral overlap between the dendrimer buffer (both forms) and indicator (at 370 nm), the absorbance of the buffer (equal for both forms) at 370 nm was subtracted from the absorbance in the presence of the indicator. A correction was also made for the change in buffer ratio due to reaction with the added protonated indicator.

Equilibration of indicator with solid-state buffers: Buffer pairs made up of 0.37 mmol of each form of the buffer (as supplied) were added to 47 μM indicator (4 mL) in organic solvent. The mixtures were shaken at 200 min^{-1} and samples taken every 15 min until equilibrated (the response of the indicator, determined by absorbance measurement, remained the same). The time required varied from 10 min to 2.5 h shaking.

Further properties of indicator: Several further experiments were conducted to investigate the chemical properties of the indicator relevant to its application in enzyme reactions in organic solvents.

Selectivity for cation: When the indicator in pentan-3-one was equilibrated with an aqueous phase containing KOH or NaOH at fixed metal ion concentration and pH, the response was the same (fraction of deprotonated indicator was 0.77). Therefore, under these conditions there is no selectivity between K^+ and Na^+. The responsiveness of the indicator to the ratio a_{H^+}/a_{K^+} as well as a_{H^+}/a_{Na^+} means that it can also be used to screen buffers which control a_{H^+}/a_{K^+}, for example HEPES and its K^+ salt. The larger ionic radius of K^+ means that it could potentially complex with two indicator molecules. However, protonated indicator was found to react with one equivalent of sodium or potassium ethoxide, confirming that a 1:1 complex was formed in both cases.

Partitioning behaviour: The deprotonated form was shown to partition into the aqueous phase from the organic phase, to an extent dependent on the solvent. When the indicator in organic solvent was equilibrated with an aqueous phase at pH 11.3 and sodium ion concentration of 0.1M, the distribution coefficient of the deprotonated form (concentration in organic relative to aqueous solution) was 0.0927 in toluene and 63.4 in pentan-3-one. When PrOH (1M) was added to toluene the value was 1.29. Thus, as expected, increasing the polarity of the organic phase reduced the extent of partitioning of the deprotonated indicator.

Effect of solvent and water activity on indicator (a_w): In principle, solid-state buffers set a_{H^+}/a_{Na^+} independently of the nature of solvent. This is because the crystalline solid phases involved in the ion exchange equilibrium remain the same whatever the external medium. Thus, if a solid buffer is equilibrated with the indicator in different solvents, differences in response should only reflect the effect of solvent on the ionisation and complexation of the indicator.

Table 2 shows how the nature of the solvent affects the equilibrium position of the indicator with HEPES/Na^+ salt. In a nonpolar solvent such as

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Table 2. Effect of the solvent on the position of equilibrium of the indicator and the λ_{max} of the deprotonated form.[a]

Solvent	No PrOH added		PrOH (1M) added	
	Deprotonated fraction	λ_{max} of deprotonated indicator [nm]	Deprotonated fraction	λ_{max} of deprotonated indicator [nm]
toluene	0.006	484	0.78	470
tert-butyl methyl ether	0.11	490	0.75	480
pentan-3-one	0.39	500	0.85	490

[a] The indicator was equilibrated within HEPES/Na^+ salt. Absorbance coefficients were obtained for each solvent in the presence and absence of PrOH.

toluene, the indicator was almost completely in the protonated form. Change to more polar solvents such as *tert*-butyl methyl ether and pentan-3-one, or addition of propan-1-ol (1M), shifted the equilibrium toward the deprotonated form. From Table 2 it can also be seen that the λ_{max} of the deprotonated form is solvent-dependent, changing by as much as 30 nm depending on the solvent (the λ_{max} of the protonated form varied only by 6 nm). The λ_{max} of the deprotonated form increased with solvent polarity, indicating more favourable solvation of the excited state in polar solvents. Interestingly, addition of propan-1-ol shifted λ_{max} of the deprotonated form in the opposite direction, and increasing water content had the same effect (see below). A possible explanation is preferential solvation of the ground state by hydrogen bonding.

Just as with solvent changes, the potential of the crystalline solids to exchange ions should not be affected by water activity (provided no hydrate is formed). Thus, for a given buffer pair, the indicator response should reflect the effect of a_w on the indicator. To determine this, HEPES and its K^+ salt were equilibrated with the indicator in pentan-3-one at a_w values of 0, 0.17, 0.33 and 0.53. Surprisingly the deprotonated fraction remained almost constant with values of 0.71, 0.68, 0.68 and 0.71, respectively. This was unexpected since addition of propan-1-ol (1M) to pentan-3-one resulted in a significant change in indicator response (see Table 2). The difference must arise because water solvates the protonated and ion-paired forms similarly so that the equilibrium is little altered.

Enzyme reaction using solid-state buffers: Subtilisin Carlsberg catalysed the transesterification of Ac-Tyr-OEt (10 mM) with propan-1-ol (1M) in toluene at a_w 0.53. The enzyme was covalently immobilised[4] to PolyHipe SU 500, a porous support with a polydimethylacrylamide surface layer, rinsed in deionised water, then stored over molecular sieves. The catalyst, solid buffer and solvent (toluene containing 1M propan-1-ol) were separately preequilibrated at a_w 0.53 in the presence of a saturated solution of magnesium nitrate. The buffers were equilibrated directly from the bottle. HEPES and its Na^+ salt were not used in this experiment because they could not be brought to equilibrium at a_w 0.53 in a reasonable time.

Ac-Tyr-OEt straight from the bottle was added to the equilibrated solid buffer (100 mg of each form) along with the catalyst (10 mg) and the solvent (5 mL). The reaction vials were shaken at 600 min^{-1} at 20 °C. Aliquots (50 μL) were taken periodically and the solvent removed with nitrogen. The samples were redissolved in acetonitrile/water (1:1 *v*/*v*), filtered and analysed by HPLC with a C_{18} reverse-phase column (Hi-Chrom, UK) and UV detection at 280 nm. The mobile phase composition was 55 % water (adjusted to pH 2 by addition of orthophosphoric acid) and 45 % acetonitrile. The percentage conversion was calculated from the ratio of the area of the propyl ester peak to the sum of areas of the ethyl and propyl esters and Ac-Tyr-OH. A control reaction was also run in which no buffer was added.

Detection of changes in a_{H^+}/a_{Na^+} during the enzyme reaction: The reaction was the transesterification of Ac-Tyr-OEt with propan-1-ol in toluene at a_w 0.53 and 0.85, catalysed by subtilisin Carlsberg immobilised to a polyHipe SU 500 support (see above). The dry catalyst was washed in sodium phosphate (20 mM) at pH 9.9 (2.5 mg catalyst per mL buffer) by shaking at 600 min^{-1} for 10 min. The buffer was filtered off and the wet beads dried in a vacuum desiccator.

The sodium salt of the indicator was prepared by shaking the protonated indicator (23.5 μM) with glycine and its Na^+ salt (0.37 mmol of each form), followed by filtration (0.2 μm, PTFE). The catalyst and organic phase (toluene containing 1M propan-1-ol and 23.5 μM sodium salt of indicator) were then separately preequilibrated at a fixed a_w (at a_w 0.53 and 0.85 with the appropriate saturated salt solution). When equilibrated, the catalyst (10 mg) was added to 5 mL of organic phase (in the absence of Ac-Tyr-OEt) and shaken at 200 min^{-1}. The spectra were obtained periodically until equilibrium was reached. Solid Ac-Tyr-OEt was then added (to 13 mM) straight from the bottle. (Note that the volume may have reduced due to sampling.) The spectra were recorded during the course of the reaction. The reaction progress was followed by sampling (50 μL) the reaction mixture periodically. A control reaction was also run in which the indicator was not present, but all other conditions were identical. See above for details of analysis.

For the reaction in the presence of CAPSO/Na^+ salt, the above method was followed except that the catalyst was not prewashed in phosphate buffer. The solvent, catalyst and buffer were separately preequilibrated at a_w 0.53. The indicator was then equilibrated with both the catalyst and buffer suspended in the solvent, then the substrate was added.

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