PERKIN 2

Solid-state proton/sodium buffers: "chemical pH stats" for biocatalysts in organic solvents

Johann Partridge,* Peter J. Halling and Barry D. Moore

Department of Pure and Applied Chemistry, University of Strathclyde, Thomas Graham Building, 295 Cathedral Street, Glasgow, UK G1 1XL. Fax: (+44) 141 553 4124. E-mail: j.partridge@strath.ac.uk

Received (in Cambridge, UK) 1st November 1999, Accepted 24th December 1999

The useful application of enzymes in organic synthesis requires reliable and straightforward methods for maximising efficiency and selectivity. Here we describe how to control the protonation state of enzymes using a new class of solid-state buffers, applied for the first time in polar organic solvents. Remarkably these insoluble buffers are able to rapidly exchange H^+ and Na^+ ions with both the protein and reaction mixture as demonstrated here using propanol rinsed enzyme preparations (PREPs) of subtilisin Carlsberg and chymotrypsin. The buffers tested were generally mixtures of a zwitterionic biological buffer and its Na^+ salt with each buffer pair setting a characteristic fixed ratio of H^+ activity to Na^+ activity (a_{H+}/a_{Na+}) within the system. Dependent upon the solid buffer pair selected a wide range of different enzymatic activities could be observed. The variation in rate showed a fairly good but not exact correlation with the aqueous pK_a of the buffers, indicating that crystal lattice energies have less affect on acid–base strength than might be expected. The solid-state buffers were able to prevent detrimental changes to enzyme activity caused by the presence or build up of acids or bases in the organic reaction mixture (often undetected). They could also be used advantageously to tune the enzyme protonation state in solvent if a previous aqueous preparation step needs to be carried out at a pH not optimal for catalysis. Such buffering systems are expected to find wide-spread use as 'chemical pH stats' for reactions in non-aqueous media.

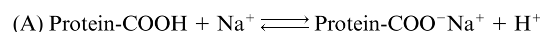
Introduction

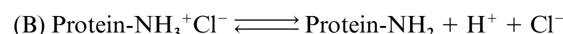
The use of biocatalysed reactions in organic solvents or other low water systems such as supercritical fluids or solid-to-solid conversions is becoming increasingly important.[1] Despite their widespread use, however, often insufficient attention is paid to how to maximise efficiency and selectivity of the biocatalyst. Candidate enzymes are often screened for a particular reaction simply by assaying the relative yield after a fixed time period under a fixed set of conditions. This is understandable since currently there is only limited fundamental guidance available on the choice of conditions for enzymes in non-aqueous media. Although there is some understanding of the effects of factors like solvent choice and water level, simple methods for tuning all relevant conditions would be valuable. These would allow more rigorous comparative studies to be undertaken and provide new possibilities in the screening of biocatalysts in organic media.

One important topic is acid–base effects. Early work on lyophilised powders demonstrated that catalytic activity in organic solvents depended on the pH of the aqueous solution from which the enzyme was dried ("pH memory").[2] This phenomenon is thought to arise because the protonation state of the protein is retained following the drying process. However, if the enzyme is exposed to acidic or basic species within the organic reaction mixture (reactants, products or impurities), the protonation state can change leading to large differences in catalytic activity. Thus, the phenomenon of pH memory generally can not be relied upon; at best it will fix the *initial* protonation state of the enzyme. To gain better control of such systems, we have been interested in the development of new methods for tuning and fixing enzyme protonation states in organic solvents.

Blackwood *et al.*[3] first introduced organic soluble acid–base buffers, and showed that the activity of immobilised enzymes could be controlled by the ratio of buffer species like triphenylacetic acid and its sodium salt. The concept of using organic soluble conjugate acid–base pairs was later extended to cross-linked enzyme crystals[4] and also applied in non-polar solvents using toluene soluble dendritic buffers.[5]

The equilibria involved in the action of buffers in low-water media are significantly different from those in water since the availability of counterions close to groups undergoing proton exchange also becomes important.[6] As a result, two different classes of buffer must be considered: those which accept or donate a proton–anion pair (*e.g.* $RNH_2{\cdot}HCl/RNH_2$); and those which exchange a proton for a cation (*e.g.* $RCO_2H/RCO_2^-Na^+$). In aqueous solution where the protein counterions are completely dissociated, these two buffer classes behave identically. In an organic solvent different behaviour may be obtained depending on which buffer class is used and how it alters the nature and extent of the resultant protein bound counter-ions (Scheme 1).†


(A) Protein-COOH + Na^+ ⇌ Protein-COO^-Na^+ + H^+


(B) Protein-$NH_3^+Cl^-$ ⇌ Protein-NH_2 + H^+ + Cl^-

Scheme 1 Equilibria involved in the action of buffers in low-water organic solvents. (A) Exchange of hydrogen ions and cations with acidic groups of the protein, *e.g.* carboxyl groups, require simultaneous exchange of H^+ with a cation such as Na^+. (B) Transfer of both a hydrogen ion and an anion onto basic groups of the protein, *e.g.* amino groups, bind or release H^+ and an anion, such as Cl^-, together.

Organic soluble buffers suffer from some limitations. The most important is that the ionic form of the buffer tends to have low solubility in the organic solvent, especially when this is of low polarity. Consequently the range of suitable nonpolar acid–

† If a solution is saturated in NaCl both systems will behave similarly.

This journal is © The Royal Society of Chemistry 2000

base compounds is limited and the buffering capacity of most commercially available compounds tends to be too low to be useful. Furthermore, in synthetic applications there would be a need to separate dissolved buffers from reactants and products on completion of the reaction. To overcome these problems, we have recently begun the development of new methodology for use in non-aqueous enzymology: solid-state acid–base buffers.[7] In this type of system, two insoluble crystalline solids are employed that can be interconverted by exchange of a proton and a counterion. As shown in Scheme 1, proteins can theoretically exchange protons and counterions with the solid buffer pairs and reach a fixed equilibrium protonation state.

In initial studies, we showed that suitable solid-state buffer pairs for control of equilibrium (B) included the zwitterionic amino acid lysine and its monohydrochloride.[7] Both forms are insoluble in hexane and toluene, the non-polar organic solvents used. Hence, together the solids generate a fixed concentration (or more precisely activity) of HCl in the solution, whatever their relative amounts. When an enzyme is introduced into the solvent it exchanges HCl with the solid-state buffers so that the same constant HCl activity is attained throughout the system. Surprisingly, we found this equilibration process to be remarkably fast. Solid buffer pairs can therefore effectively function as "chemical pH stats" and should give precise control of an enzyme protonation state throughout the reaction, even where acidic and basic species are being produced or consumed.

In this paper we describe the action of a new class solid-state buffers for control of equilibrium (A), complementary to those described before. They are able to exchange a proton for a sodium ion, and will hold constant the ratio of H^+ activity to Na^+ activity (a_{H^+}/a_{Na^+}). While this parameter may appear a little abstract, it can be seen from Scheme 1 that this is precisely the ratio that effects the ionisation state of carboxylate groups on a protein. We show here that remarkably this type of solid-state buffer can also rapidly alter the protonation state of an enzyme in organic media. These new experiments also use much more polar solvents than in the previous study and a novel highly active form of biocatalyst, propanol rinsed enzyme preparations (PREPs). Previous work with PREPs showed two important reasons for wishing better control of acid–base conditions.[8] Firstly, if the enzyme of interest is a protease, using the pH for optimal activity during the aqueous immobilisation process can lead to significant loss of activity due to autolysis. Secondly, during enzyme catalysed transesterification, small amounts of acidic by-products, arising from competing hydrolysis, were found to cause a significant decline in enzymatic activity as the reaction proceeded.

Results and discussion

We selected a range of compounds for test as solid-state H^+/Na^+ buffers. The selected buffers had aqueous pK_a values covering the pH range over which this enzyme is active in aqueous solution. In the main, acid and sodium salt forms of commercially available biological buffers were employed: AMPSO (pK_a 9.0), CAPSO (pK_a 9.6), HEPPSO (pK_a 7.8), MOPS (pK_a 7.2), PIPES (pK_a 6.8), TAPS (pK_a 8.4) and TES (pK_a 7.4). In addition, HEPES (pK_a 7.5) and its potassium salt and KH_2PO_4/K_2HPO_4 (pK_a 7.2) were used. As in the study of Zacharis *et al.*,[7] zwitterionic salts were chosen in order to insure buffer solubility in organic solvent was minimal.‡

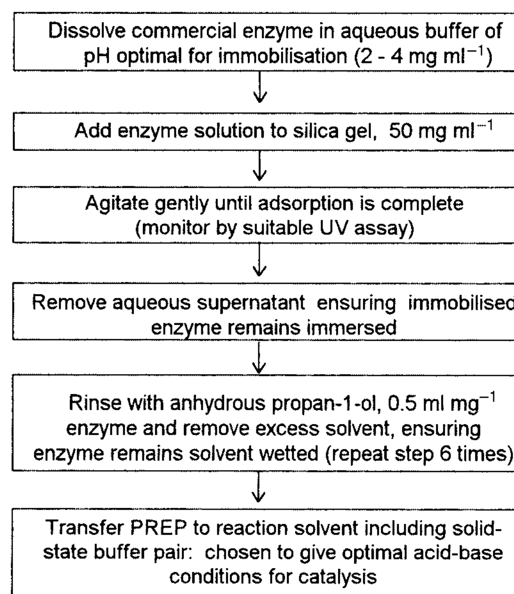


Fig. 1 Production of propanol rinsed enzyme preparations (PREPS) for use in organic solvent. For further experimental details see Experimental section.

The buffers were tested in a model transesterification reaction in acetonitrile at a_w of 0.22 (1% water by volume).§ The enzymes were used in the very active PREP form. One attractive feature of PREPs is that they can be prepared very reproducibly; they therefore provide an excellent system for fundamental studies. In light of our present findings, Fig. 1 illustrates the recommended procedure for producing PREPs for use in organic media.

Effect of solid-state buffers on immobilised subtilisin activity in organic solvents

In our previous work with subtilisin PREPs, the enzyme was immobilised in aqueous solution at a pH close to the optimum for catalytic efficiency in aqueous solutions. However, we were interested to see whether solid-state acid–base buffers could be used to change the protonation state of PREPs which had been immobilised at a pH lower than the optimum for catalysis. A major advantage of immobilising at low pH, particularly for proteases such as subtilisin and α-chymotrypsin, is the fact that autolysis should be substantially reduced and a greater amount of active enzyme bound to the support.

To this end, subtilisin was adsorbed onto the silica support from aqueous sodium pyrophosphate buffer at pH 5.7. As an initial experiment we followed a typical reaction with this PREP in the absence of solid-state buffer pairs. The activity in acetonitrile at a_w of 0.22 was found to be 15.8 nmol mg^{-1} min^{-1}. In a similar set of experiments, a selected buffer pair was added after 25 min, and the effect on product formation monitored.

‡ It is useful to choose zwitterionic compounds as solid-state buffers because both acidic and basic forms will be charged, so that both are expected to be essentially insoluble in non-polar media. However, there is also a less obvious advantage. Zwitterionic compounds with an ionisable weak basic group can effectively act as acidic (proton/cation) buffers, and *vice versa*. This makes a greater choice of acid–base strengths available in both classes of buffer. For example, consider a molecule with an amine group. This weakly basic group would lead one to expect the compound to act as a buffer binding H^+ and Cl^-. This would indeed be true if the molecule contains no other charged groups, or an additional positive charge. However, consider a zwitterionic amine, with for example a sulfonate group as well. The two solid forms related by protonation of the amine will be: i) a zwitterion with zero net charge; and ii) a salt with an appropriate cation (*e.g.* Na^+) to balance the sulfonate charge. Thus the ionisation of the amine group now buffers the exchange of Na^+ and H^+. Similarly, a carboxylic acid group may lead to a H^+ plus Cl^- buffer. If the molecule also contains a quaternary ammonium group, for example, then the two solid forms will be: i) the zwitterion; and ii) the salt with only the ammonium group charged and a negative counterion (*e.g.* Cl^-).

§ Although the maximum catalytic efficiency of this enzyme is attained at a_w of 0.44, we decided to carry out the buffer reactions at lower a_w to ensure buffer insolubility in the polar solvent. In addition, as pointed out by Harper *et al.*,[9] some of the salts may be capable of forming hydrates at sufficiently high a_w values. Keeping water levels as low as possible should help avoid this complication.

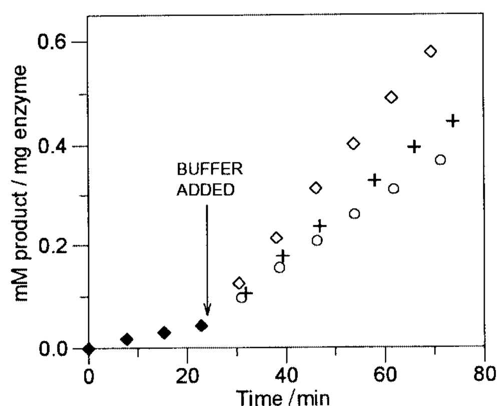


Fig. 2 Addition of solid-state acid–base buffer pairs to subtilisin Carlsberg PREP catalysed transesterification reaction. Enzyme was immobilised from aqueous sodium pyrophosphate buffer (pH 5.7), dried by washing with propan-1-ol and suspended directly in acetonitrile at a_w of 0.22. The rate of product formation was followed on addition of substrates (◆). After 25 min, a solid-state buffer pair was added to the reaction mixture and the subsequent rate of transesterification measured. Buffers used were TAPS/Na-TAPS (◇), TES/Na-TES (+) and HEPES/K-HEPES (○). Each form of the buffer pair were present at a concentration of 10 mg ml^{-1}.

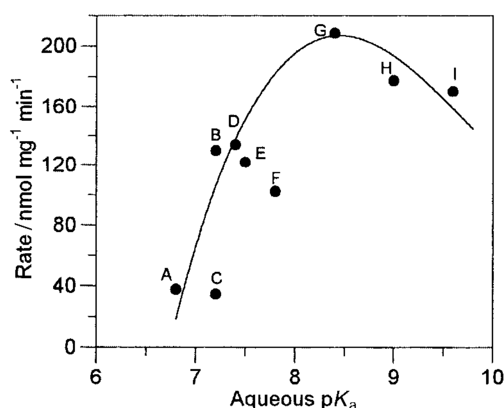


Fig. 3 Subtilisin Carlsberg PREP catalysed transesterification rate as a function of solid state buffer aqueous pK_a. Enzyme was immobilised from sodium pyrophosphate buffer (pH 5.7) and dried by rapid solvent rinsing. PREP was then used directly to catalyse reactions in acetonitrile at a_w of 0.22 in the presence of different buffer pairs. The buffers were PIPES/Na-PIPES (A), KH_2PO_4/K_2HPO_4 (B), MOPS/Na-MOPS (C), TES/Na-TES (D), HEPES/K-HEPES (E), HEPPSO/Na-HEPPSO (F), TAPS/Na-TAPS (G), AMPSO/Na-AMPSO (H) and CAPSO/Na-CAPSO (I).

The results obtained are shown in Fig. 2. With each pair tested the catalytic rate was observed to increase instantaneously!

We then went on to measure the initial transesterification rates for PREPs in acetonitrile with each of the chosen buffer pairs present from the start of the reaction. The catalytic activities are plotted in Fig. 3 against the known aqueous pK_a of the acid–base buffers. It can be seen that there are wide variations in the activity observed. Thus, remarkably even with both the biocatalyst and acid–base buffer in the solid-state, interchange of sodium ions and protons can occur moving the enzyme into different equilibrium protonation states.[¶,‖,**,††]

At first sight it would appear that the bell-shaped curve in Fig. 3 is the same as would be obtained for subtilisin in aqueous solution. However, on closer inspection it can be seen that there are a number of points which do not follow the smooth progression. For example, the point corresponding to the solid-state pair HEPPSO/Na-HEPPSO looks like it should be shifted further to the left: away from HEPES/K-HEPES and closer to that representing PIPES/Na-PIPES. This is not due to experimental error: the rate measurements for each buffer were carried out at least in triplicate and were accurate to better than 10%. Rather it is the result of trying to directly correlate aqueous pK_a of the buffers with catalytic activity when the parameter set by the solid-state buffers is in fact (a_{H^+}/a_{Na^+}). This parameter will be affected at the molecular level by the pK_a of the groups involved but also at a supramolecular level by the relative lattice energies of the acidic and basic forms. If these lattice energies differ significantly from each other, the a_{H^+}/a_{Na^+} value will be perturbed, *e.g.* if the sodium salt is more stable (higher lattice energy), the ratio will decrease as the sodium ions will be bound more tightly in the solid-state. In fact it can be seen that for most of the buffer pairs selected, the effect of lattice energy is limited, or else, more likely in view of their related molecular structures, there is a similar perturbation to the ratio across the series. Nevertheless, the deviations from the curve indicate our rate data would be better presented as a function of the H^+/Na^+ exchange potential for each of the solid-state buffer pairs used.

Recently, an organo-soluble indicator which measures H^+/Na^+ exchange potential has been successfully developed.[9] When catalytic rates for an immobilised form of subtilisin in toluene were plotted with respect to experimentally measured indicator response, a smooth progression of points was obtained. Interestingly in the study of Harper *et al.*,[9] the data point representing the solid-state buffer pair HEPPSO/Na-HEPPSO was also a noticeable outlyer of the fitted curve when plotted against aqueous pK_a. However, the indicator response to this buffer was found to be 0.076: confirming that it gave a H^+/Na^+ exchange potential closer to PIPES (indicator response of 0.044) than HEPES (indicator response of 0.78).

¶ We have confirmed that buffers are predominantly present in the solid state as opposed to being dissolved in ACN (1% v/v). Solubility in the solvent was always less than 18 mg l^{-1}. We were also interested to see whether contact with solid buffers could effect the acid–base conditions of the solvent and thus the protonation state of the enzyme and its subsequent catalytic activity. A series of reactions were carried out whereby a chosen solid-state buffer pair was added to acetonitrile, shaken and allowed to settle. Solvent was then filtered to remove all solid particles and used as the reaction medium. Buffers tested were: AMPSO, TAPS and TES. In all cases, **initial** rates were similar to those obtained when the solid-state pair were present throughout the reaction. However as the reactions progressed, product formation noticeably dropped when solid buffers were absent. Presumably in this case as small amounts of acid by-product build up, acid–base conditions can no longer be controlled, thus leading to reduced rates. In addition, when reactions were purposely spiked with acid, we found that catalytic rates were significantly lower in the absence of solid buffers as compared to those carried out in their presence. Our results therefore confirm that the acidity/basicity of the solvent, and thus the performance of the enzyme can be effected by contact with solid-state buffers. Nevertheless, control of acid–base conditions can NOT be achieved unless the solid-state buffer is present throughout the reaction time course.

‖ In all cases it was found in the absence of enzyme, no activity was observed with the buffers.

**It should be noted that some buffer salts have small amounts of water associated with them. One might therefore suggest that the effects demonstrated here are due to water rather than pH, or a combination of the two. However, the variation in water content of reaction mixtures with each of the buffer pairs was found to be insignificant (as tested by Karl Fischer). This is in part due to the fact that reactions were carried out in acetonitrile with 1% water v/v, so that small changes in system water content will not significantly alter a_w. Nevertheless, if these reactions were performed in a hydrophobic solvent such as hexane, it is likely that the low levels of water associated with the buffers would result in significant increases in system a_w. Therefore, more care would have to be taken to equilibrate the buffers to the required a_w level prior to use in hydrophobic media.

†† There is no need to allow the enzyme to equilibrate with the buffer pairs in acetonitrile (a_w = 0.22). Lag periods in initial rates for reactions in acetonitrile were not observed for the majority of the buffers used here. Noted exceptions were PIPES/Na-PIPES and KH_2PO_4/K_2HPO_4. However, even in these cases, after 10 min the initial rates were linear. Thus, it would appear that transfer of ions between enzyme and buffers is relatively quick in acetonitrile.

Table 1 Variation of catalytic rate in the presence of different solid-state buffers

Solid-state buffer[a]	Rate nmol mg^{-1} min^{-1}	
	α-Chymotrypsin in ACN[b]	Subtilisin in THF[c]
None	11.9	2.2
AMPSO/NaAMPSO	108	52.0
HEPES/KHEPES	59.0	33.3
MOPS/NaMOPS	35.9	12.1

[a] Each form of solid-state acid–base buffer pair was present at a concentration of 10 mg ml^{-1}. [b] α-Chymotrypsin PREP catalysed transesterification reaction between 10 mM *N*-acetyl-L-tyrosine ethyl ester with 1 M propan-1-ol in acetonitrile (1% H_2O by volume). [c] Subtilisin Carlsberg PREP catalysed transesterification reaction between 10 mM *N*-acetyl-L-tyrosine ethyl ester with 1 M propan-1-ol in THF (0.45% H_2O by volume).

Testing the generality of the solid-state buffers

Given the results with subtilisin, we were keen to determine the generality of our findings with respect to different enzymes and other reaction solvents. As mentioned, one advantage of immobilising at low pH is the limitation of autolysis during adsorption. This is a potentially greater problem with α-chymotrypsin than subtilisin and so we carried out a similar set of experiments with this enzyme. The enzyme was immobilised successfully at low pH under similar conditions to those employed for subtilisin (see Experimental section). The model transesterification reaction catalysed by chymotrypsin PREP in acetonitrile was then followed in the presence and absence of several solid-state acid–base buffer pairs. The results in Table 1 confirm that the ionisation state of this PREP can also be readily adjusted in solvent too. Since the two enzymes exhibit completely different secondary and tertiary structures, the solid-state buffering process would appear to be widely applicable. To further confirm this, we moved on to study the effect of changing reaction solvent. Experiments were performed using subtilisin PREP in THF at a_w of 0.22. Again, addition of solid-state buffer pairs to the reaction mixture was found to effect enzymatic activity (Table 1). However, in THF, we observed lag periods in the initial rates of up to 30 min. In this case, initial catalytic rates were measured from the eventual linear section of the progress curves. Evidently, although transfer of ions between enzyme and buffer pairs is slower in THF than in acetonitrile, equilibrium is still reached fast enough for the method to be useful.‡‡ Together these results show solid-state buffers are likely to be applicable for a wide range of biocatalyst systems.

Buffering enzymes prepared from different aqueous pH

At this point we were interested to investigate how solid-state acid–base buffer pairs would affect the activity of subtilisin PREP immobilised in aqueous sodium phosphate buffer at its optimum pH of 7.8. This preparation displayed a high activity of 154 nmol mg^{-1} min^{-1} in acetonitrile at a_w of 0.22. Since the initial protonation state of the enzyme in organic media is expected to be dictated by the pH of the aqueous buffer from which it was dried, we anticipated that the addition of solid-state buffers would not increase the initial catalytic rate and was more likely to decrease it. This was indeed the case and a plot of catalytic performance in acetonitrile for this PREP is shown against aqueous buffer pK_a in Fig. 4. The trend afforded is similar to that of enzyme immobilised at pH 5.7 (Fig. 3): TAPS

‡‡ Where lag periods are observed, it is possible to shake the enzyme in the organic phase with buffers present for a period of time prior to initiating the reaction. This should allow sufficient time for transfer of ions and eliminate the lag phase.

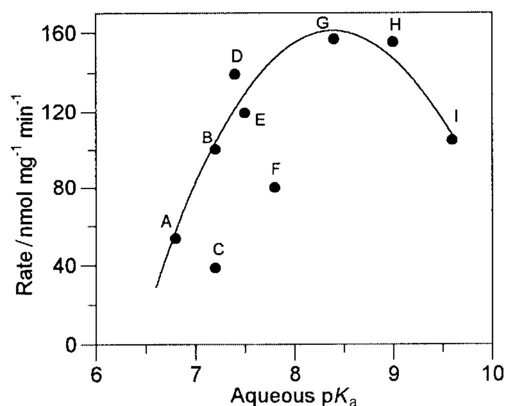


Fig. 4 Subtilisin Carlsberg PREP catalysed transesterification rate as a function of solid state buffer aqueous pK_a. Enzyme was immobilised from sodium phosphate buffer (pH 7.8) and dried by rapid solvent rinsing. PREP was then used directly to catalyse reactions in acetonitrile at a_w of 0.22 in the presence of different buffer pairs. The buffers were PIPES/Na-PIPES (A), KH_2PO_4/K_2HPO_4 (B), MOPS/Na-MOPS (C), TES/Na-TES (D), HEPES/K-HEPES (E), HEPPSO/Na-HEPPSO (F), TAPS/Na-TAPS (G), AMPSO/Na-AMPSO (H) and CAPSO/Na-CAPSO (I).

produces the maximum rates, whilst MOPS gives the lowest. However, it is interesting to note that the actual magnitude of the rates differ depending on the pH used to immobilise the enzyme. In most cases, enzyme immobilised at pH 7.8 gave lower rates than that which had been immobilised at 5.7: with the solid-state buffer pair CAPSO/Na-CAPSO, the observed rate was almost 40% less for the enzyme adsorbed from aqueous pH of 7.8. In part, the observed differences in rates can be attributed to the fact that the buffers chosen in this study can only control the parameter (a_{H^+}/a_{Na^+}). In order to fully describe acid–base effects for biocatalysts in low water organic media, we need to consider their behaviour as a function of both (a_{H^+}/a_{Na^+}) and ($a_{H^+}\cdot a_{anion}$). Since we have not controlled the latter parameter, the presence of different anions in the two aqueous buffers could quite likely give rise to the variations observed here.

Buffering acidic hydrolysis by-products

For pH 7.8 immobilised enzyme, addition of solid-state acid–base buffer pairs lowers the initial rate of catalysis. However, the advantage of having buffer present is that the ionisation state of the protein will be maintained throughout the reaction, regardless of whether acidic/basic products are produced. This is illustrated well in the reaction progress curves in Fig. 5. The *initial* rates of transesterification are very similar with and without the presence of the selected buffer pairs. However, as the reaction proceeds, the progress curve with buffer remains nearly linear while without it there is definite curvature. The difference can be attributed to the build up of small but detectable amounts of acidic by-product which change acid–base conditions, protonate active site residues and hence slow the enzyme catalytic rate. The advantage of using these buffers in synthetic reactions becomes more evident when we consider the propyl ester yields for these reactions after 24 hours (Table 2). With the buffers present, the yields are as high as 76%; without buffer, propyl ester formation is limited to 46%.

Buffering acidic or basic species present within a reaction mixture

Since in the absence of buffer pairs, small amounts of the acid by-product evidently lower the catalytic rate as the reaction progresses, we went on to investigate the effect of purposely adding acid or base to reaction mixtures.

We firstly studied the effect of spiking reactions catalysed by subtilisin PREP (immobilised from pH 7.8) with acetic acid. Reaction progress was followed in the presence and absence

Table 2 Product yields in the presence and absence of solid-state acid–base buffers[a]

Solid-state buffer[b]	*N*-Acetyl-L-tyrosine propyl ester[c] (%)	*N*-Acetyl-L-tyrosine[c] (%)
None	46	0.7
TAPS/Na-TAPS	72	2.5
AMPSO/Na-AMPSO	76	2.5
TES/Na-TES	60	1.3

[a] For the subtilisin Carlsberg PREP catalysed transesterification reaction between 10 mM *N*-acetyl-L-tyrosine ethyl ester with 1 M propan-1-ol in acetonitrile (1% H_2O by volume). [b] Each form of solid-state acid–base buffer pair was present at a concentration of 10 mg ml^{-1}. [c] Yields of propyl ester and acid after 24 hours determined by HPLC.

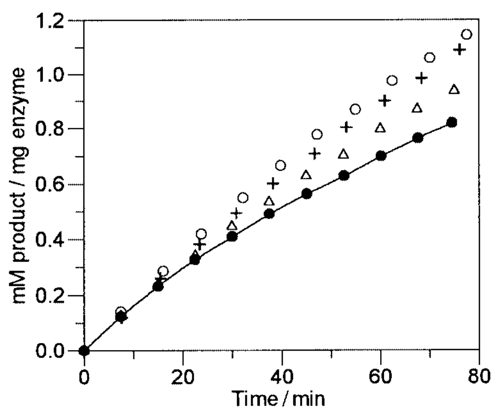


Fig. 5 Reaction progress for subtilisin Carlsberg PREP catalysed transesterification in the presence and absence of solid state buffers. Subtilisin Carlsberg was immobilised from aqueous buffer at pH 7.8. The aqueous enzyme suspension was then dried by washing with propan-1-ol and suspended directly in acetonitrile at a_w of 0.22. On addition of substrates, the rate of product formation was followed in the absence of solid state buffers (●) and in the presence of TAPS/Na-TAPS (○), AMPSO/Na-AMPSO (+), and TES/Na-TES (△).

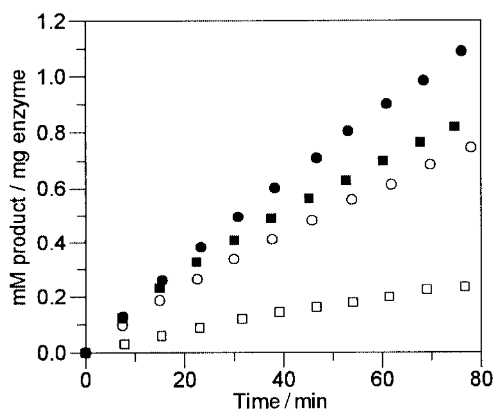


Fig. 6 Progress of PREP catalysed transesterification reaction spiked with acid in the presence and absence of solid state AMPSO/Na-AMPSO. Subtilisin Carlsberg was immobilised from aqueous buffer at pH 7.8. The aqueous enzyme suspension was then dried by washing with propan-1-ol and suspended directly in acetonitrile at a_w of 0.22 containing 4 mM acetic acid. On addition of substrates, the rate of product formation was followed in the presence AMPSO/Na-AMPSO (○) and in the absence of the buffer (□). Progress of reactions without acid in the presence of AMPSO (●) and in its absence (■) are shown for reference.

of solid-state buffers (AMPSO, TAPS and TES). The results obtained in acetonitrile at a_w of 0.22 with AMPSO/Na-AMPSO are shown in Fig. 6. In the absence of buffer species, acetic acid causes a dramatic loss in catalytic rate (rate falls by more than 70% compared to the control). In the presence of AMPSO, the acid also causes a decrease in rate. However, in this case the decrease is not so high (*i.e.* rate falls by 10% compared to the control). Similar results were obtained for TAPS/Na-TAPS and TES/Na-TES. Since these buffers will tend to control exchange of Na^+ with protons, setting a_{H^+}/a_{Na^+}, it is perhaps not surprising that they do not fully eliminate the effects of organic soluble acetic acid on the catalytic rate.

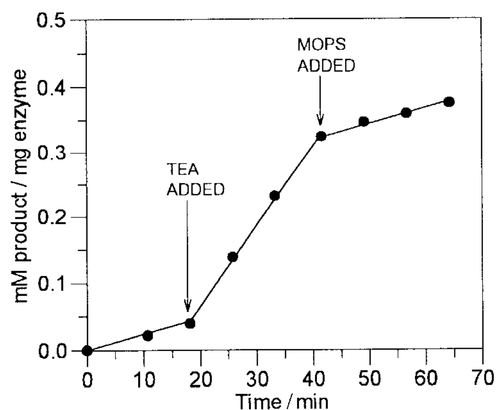


Fig. 7 Progress of PREP catalysed transesterification reaction on addition of triethylamine followed by MOPS/Na-MOPS. Subtilisin Carlsberg was immobilised from aqueous buffer at pH 5.7. The aqueous enzyme suspension was then dried by washing with propan-1-ol and suspended directly in acetonitrile at a_w of 0.22. On addition of the substrates, the reaction was monitored. After 20 min, the reaction was spiked with 4 mM triethylamine. Subsequently, 10 mg ml^{-1} each of MOPS/Na-MOPS were added.

We also looked at the effect of adding triethylamine to a reaction catalysed by PREP immobilised from sodium pyrophosphate at pH 5.7. The progress of a reaction in acetonitrile is shown in Fig. 7. After 20 min, the reaction was spiked with 4 mM triethylamine, causing a dramatic increase in the generally low rate observed for this enzyme. At some later time, the solid-state buffer pair MOPS/Na-MOPS was added. As illustrated, the buffer immediately lowered the rate of catalysis—thus, demonstrating that the effects of the soluble base could also be effectively dealt with by the solid-state buffer pair.

Conclusion

Control over enzyme protonation state is a critical requirement for obtaining reliable biocatalysis in non-aqueous media. We have shown that pairs of crystalline solids, interconvertible by exchange of a sodium ion for a proton, can rapidly alter and set the protonation state of immobilised enzymes in organic solvents. The solid-state buffer pairs therefore function as 'chemical pH stats' fixing the sodium/proton activity ratio within the reaction mixture to precise values. By using different buffer pairs a wide range of activity ratios can be attained providing access to most enzyme protonation states. The buffers were shown to work well in the synthetically useful solvents acetonitrile and THF and with two unrelated enzymes. Their ease of use and general applicability suggest they will find widespread use in biocatalysis. In addition they may find interesting other synthetic applications where precise control of protonation state is required.

Experimental

Chemicals

Subtilisin Carlsberg Protease, Type VIII from *Bacillus licheniformis*, 13.5 units mg^{-1} solid (P5380) and α-chymotrypsin, Type II from Bovine Pancreas, 52 units mg^{-1} solid (C-4129) were purchased from Sigma Chemical Co. (Poole, U.K.). *N*-Acetyl-L-tyrosine ethyl ester (A-6751) and silica gel (S-0507) were also

obtained from Sigma. All anhydrous solvents were from Merck Ltd. (Poole, U.K.). These were stored over 3 Å molecular sieves and used without further purification. All inorganic salts were of analytical grade from Merck. Biological buffers: AMPSO (3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid), CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid), HEPPSO (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-hydroxypropanesulfonic acid]), MOPS (3-[*N*-morpholino]propanesulfonic acid), PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]), TAPS (*N*-tris[hydroxymethyl]-methyl-3-aminopropanesulfonic acid), TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) and their sodium or potassium salts were purchased from Sigma.

Preparation of supported enzymes

Subtilisin was dissolved (2 mg ml^{-1}) in 20 mM sodium phosphate buffer pH 7.8 or 20 mM sodium pyrophosphate pH 5.7 (this buffer was prepared from 20 mM sodium pyrophosphate and pH adjusted with H_3PO_4). 20 ml of enzyme solution was mixed with 1 g of untreated silica gel and the mixture was shaken for 1 h at 25 °C. Protein assay (Biorad) of the supernatant was then used to determine the amount of enzyme adsorbed onto the silica support. Enzyme loading was found to be 92.5% and 85% at pH values of 7.8 and 5.7 respectively. Chymotrypsin was immobilised at 4 mg ml^{-1} in 20 mM sodium pyrophosphate (pH 5.7). The aqueous enzyme solution was shaken in the presence of the silica support for 4 hours at 25 °C. After this time, the enzyme loading was determined as 80%.

In all cases, immobilised enzymes were stored in aqueous buffer at 4 °C until required for use in organic solvents. In the presence of 0.02% sodium azide, these aqueous enzyme suspensions can be stored for at least 3 weeks with negligible loss of acivity.[8] Catalytic activities of different batches of the same enzyme in aqueous solution were found to vary by no more than 10%, indicating high reproducibility.

Enzyme preparation for use in organic solvent (propanol rinsed enzyme preparations, PREPs)

An aliquot of immobilised enzyme (generally 1 ml) was removed from the stock suspension and allowed to settle out in an eppendorf tube. Excess aqueous buffer was then removed carefully so that silica–enzyme particles were not disturbed. Subsequently, the immobilised enzyme was dried by washing with anhydrous propan-1-ol (6 × 1 ml). After each wash, the enzyme was allowed to settle out and the solvent was then removed. In this work, enzyme preparations were used to catalyse reactions in organic media without further delay. In general, the solvent washing procedure and subsequent transfer to reaction solvent takes no more than 15 minutes. Note that it is important to use PREP immediately to achieve maximum catalytic rates. When solvent wet pellets are left to dry out for more than a few minutes, lower rates are noted.

Typical reaction conditions for rate studies

The model reaction was the transesterification of 10 mM *N*-acetyl-L-tyrosine ethyl ester with 1 M propan-1-ol. A solution of the ester substrate in anhydrous propanol was added to a suspension of PREP in 10 ml of acetonitrile containing 1% water by volume (to give a controlled a_w of 0.22).[10] After brief mixing, the zero time sample was removed. The mixture was then incubated at 24 °C with constant reciprocal shaking (150 min^{-1}). Samples from the reaction mixture were taken at regular intervals and analysed by HPLC on a Gilson 715 equipped with an ODS2 reverse phase column (Hichrom). The mobile phase consisted of 40% acetonitrile mixed with an aqueous phase (pH2 with orthophosphoric acid). The retention times of the acid, ethyl ester and propyl ester were 2.1, 2.9 and 3.9 min respectively.

Experimental errors

In this study, all reactions were carried out at least in triplicate and errors in reported rates were always less than than 15%.

Addition of solid-state buffers to progressing reactions

Reactions with PREP immobilised from sodium pyrophosphate (pH 5.7) were carried out in acetonitrile ($a_w = 0.22$) as described above. In the absence of solid-state buffer pairs, reactions were monitored for 25 min. After this time, the chosen buffer pair was added to the reaction mixture (100 mg of each form) and product formation followed as usual. Buffers tested were TAPS/Na-TAPS, TES/Na-TES and HEPES/K-HEPES.

Reactions in the presence of solid state buffers

Reactions were performed in acetonitrile ($a_w = 0.22$) in a manner similar to that described above. The chosen solid buffer pair (10 mg of each form per ml of reaction mixture) was added to a suspension of PREP in solvent just prior to substrate addition. Buffer pairs tested were the free acid and sodium salt forms of AMPSO, CAPSO, HEPPSO, MOPS, PIPES, TAPS and TES. In addition, the free acid and potassium salt of HEPES and KH_2PO_4/K_2HPO_4 were examined. Experiments were carried out with PREP immobilised from aqueous buffer at pH 5.7 and 7.8.

Reactions in THF

These were carried out in the presence and absence of solid-state buffer pairs in a fashion similar to that discussed above. THF at a fixed a_w of 0.22 was prepared by adding H_2O to give 0.45% by volume.[10]

Spiking reaction systems with acid

Reactions in acetonitrile (1% water v/v, a_w of 0.22) were carried out in the presence or absence of solid state buffer pairs using enzyme immobilised from sodium phosphate (pH 7.8), as described above. Prior to substrate addition, the reactions were spiked with acetic acid (final concentration 4 mM). Buffers tested were AMPSO/Na-AMPSO, TAPS/Na-TAPS and TES/Na-TES.

Spiking reaction systems with base

A reaction in acetonitrile ($a_w = 0.22$) catalysed by PREP immobilised from sodium pyrophosphate (pH 5.7) was carried out as described above. After 20 min, the reaction mixture was spiked with triethylamine (4 mM final volume) and reaction progress followed for a further 25 min. After this time, 100 mg each of MOPS/Na-MOPS was added and the reaction rate followed as before.

Acknowledgements

The authors are grateful to the Biotechnology and Biological Sciences Research Council for financial support.

References

1 Y. L. Khmelnitsky and J. O. Rich, *Curr. Opin. Chem. Biol.*, 1999, **3**, 47; M. T. De Gomez-Puyou and A. Gomez-Puyou, *Crit. Rev. Biochem. Mol. Biol.*, 1998, **33**, 53 and A. M. Klibanov, *Trends Biotechnol.*, 1997, **15**, 97.
2 A. Zaks and A. M. Klibanov, *Proc. Natl. Acad. Sci. U.S.A.*, 1985, **82**, 3192 and A. Zaks and A. M. Klibanov, *J. Biol. Chem.*, 1988, **263**, 3194.

3 A. D. Blackwood, L. J. Curran, B. D. Moore and P. J. Halling, *Biochim. Biophys. Acta*, 1994, **1206**, 161.
4 K. Xu and A. M. Klibanov, *J. Am. Chem. Soc.*, 1996, **118**, 9815.
5 M. Dolman, P. J. Halling and B. D. Moore, *Biotechnol. Bioeng.*, 1997, **55**, 278.
6 A. D. Blackwood, B. D. Moore and P. J. Halling, *Biocatalysis*, 1994, **9**, 269 and E. Skrika-Alexopoulos and R. B. Freedman, *Biotechnol. Bioeng.*, 1993, **41**, 887.
7 E. Zacharis, B. D. Moore and P. J. Halling, *J. Am. Chem. Soc.*, 1997, **119**, 12396.
8 J. Partridge, P. J. Halling and B. D. Moore, *Chem. Commun.*, 1998, 841.
9 N. Harper, M. Dolman, B. D. Moore and P. J. Halling, *Chem. Eur. J.*, 2000, in the press.
10 G. Bell, A. E. M. Janssen and P. J. Halling, *Enzyme Microb. Technol.*, 1997, **20**, 471.

Paper a908673k