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pH artifacts in reverse micellar enzymology: A warning

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Abstract - Kinetic and structural investigations of enzymes in reverse micelles can be affected by pH artifacts in different ways. Due to a lack in buffering capacity of the buffer generally present in reverse micellar systems, acidic substrates as well as acidic products formed during the course of the reaction can lead to changes in the apparent pH of the water pool. The intensity of these local pH changes depends on the actual w_o value (w_o=[H_2O]/[surfactant]), and can lead to misinterpretation of experimental kinetic data, as shown in detail for α-chymotrypsin. For example, bell-shaped curves of the enzymatic activity vs. w_o can be the artifactual result of these pH effects. A particular problem arises in the case of those lipase catalyzed reactions where fatty acids are either used as substrate or produced during the course of the reaction.

INTRODUCTION

During the last ten years a large number of studies have been carried out on the catalytic activity of enzymes in reverse micelles [e.g. 1-3]. Several aspects remain to be clarified, e.g. the bell-shaped profile of the activity vs. w_o; the "superactivity" observed for some enzymes; and the more general problem of the structure of water and how this affects the enzyme behavior.

Another problem has to do with the definition and determination of the concentration of solubilized guest molecules, including the concentration of H_3O^+, i.e. the (apparent) pH of the water pool [1]. Despite these conceptual problems, it has been shown that pH-dependent reactions in reverse micelles formed by the anionic AOT (sodium bis(2-ethylhexyl) sulfosuccinate) can be affected by acidic impurities either already present in the surfactant sample [4,5] or formed during long time storage of AOT solutions [4,6].

We believe that questions related to possible changes in the apparent water pool pH as caused by the substrate or by the products formed during the reactions has not received due attention. In the following, we will show that such pH changes can lead to artifacts and misinterpretations. We will do so by utilizing three different examples based on lipase, lipoxygenase and α-chymotrypsin.

PHENOL RED AS INDICATOR OF LOCAL pH CHANGES IN REVERSE MICELLES; THE CASE OF LIPASE AND LIPOXYGENASE

The first case is suggested by our recent work with lipases in reverse micelles. Lipase catalyzes the hydrolysis of fatty acid esters, and one of the products is an acid, which, being hosted partly by the water pool, may lower the apparent pH. This effect can be quantified on the basis of the method which has been developed to determine the content of free fatty acids in vegetable oils with the help of AOT-isooctane reverse micelles [7]. The principle of this determination is rather simple: the water pool

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Abbreviations: AOT, sodium bis(2-ethylhexyl) sulfosuccinate; w_o= [H_2O]/[AOT], ratio of molar concentrations of water to AOT; $[S]_{ov}$, $[S]_{wp}$, molar concentration of S as expressed with respect to the total volume or with respect to the water volume of the reverse micellar solution; Ac-Phe-NH-Np, acetyl-L-phenylalanine p-nitroanilide; Gr-Phe-NH-Np, glutaryl-L-Phe p-nitroanilide; Suc-Phe-NH-Np, succinyl-L-Phe p-nitroanilide.

contains the basic form of the indicator phenol red, and when free acid is formed in the micelle because of the hydrolysis of the fatty acid esters, there is a change in the color of the indicator from pink (λ = 560 nm) to yellow (λ = 430 nm) [8]. In this hydrolysis reaction, the apparent pH decreases from an initially alkaline value of approximately 9 (the pH of the added buffer solution, pH_{st}) to an acidic value around 5, the region of the pK_a of the released fatty acids: this very significant pH change (despite the presence of some buffer in the water pool, e.g. 0.1 M tris/HCl locally) is due to the lipase catalyzed reaction. Due to such pH changes it is therefore expected that the lipase reaction velocity changes with reaction time not only because the substrate concentration is decreasing, but also because the local pH - and therefore the catalytic activity of the enzyme - is decreasing with time. The reason for these pH effects in reverse micelles seems to be obvious: the relatively low amount of buffer ions in the system (1 mM overall in the case described above, w_o = 11.1) is by no means enough to buffer all the fatty acids either present in the oil substrates or produced during the course of the reaction. (Note, that buffer ions will also be used to neutralize acid impurities possibly present in the AOT surfactant, see [4,5]).

Basic kinetic investigations of lipases in reverse micelles can therefore significantly be affected by a drop in the apparent pH of the water pools caused by substrates containing 'free', non-esterified fatty acids and/or by fatty acid products formed during the reaction. Such pH effects have to be taken into account. It is also obvious that the study of the pH dependency of a lipase in reverse micelles with high concentrations of impure triglycerides has to be analyzed with great care. And in particular, pH-dependency studies for lipase catalyzed synthesis reactions with fatty acid educts require special attention [9,10]. In this case, one has to keep in mind that a rather high fatty acid concentration is used in order to shift the reaction equilibrium towards synthesis.

In the case of lipoxygenase [11-13], the activity against linoleic acid as substrate is expected to be affected in a similar way by the fatty acid substrate. The higher the substrate concentration and the lower the surfactant concentration or the lower w_o (i. e. the lower the total amount of buffer), the more important the pH effect.

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACIDIC SUBSTRATES

Apart from lipases and lipoxygenases, other enzymes as well may show an unexpected activity behavior in reverse micelles which can be related to "pH artifacts". One example is α-chymotrypsin, if measured against acidic substrates at high substrate concentration, e.g. glutaryl-L-phenylalanine p-nitranilide (Gr-Phe-NH-Np) or succinyl-L-phenylalanine p-nitranilide (Suc-Phe-NH-Np).

Fig. 1 shows the effect of increasing the overall substrate concentration of Gr-Phe-NH-Np, Suc-Phe-NH-Np and Ac-Phe-NH-Np (acetyl-L-phenylalanine p-nitroanilide) - no enzyme added - on the absorption spectrum of 1.5 μM phenol red in 50 mM AOT/isooctane at w_o = 8 (prepared with 0.1 M tris/HCl, pH 8.0; and with the help of dimethyl sulfoxide in which the substrates have been dissolved before solubilization in reverse micelles). An increase in substrate concentration up to the solubility limit leads to a dramatic decrease in the apparent water pool pH in the case of the substrates with an acidic protecting group (Gr-Phe-NH-Np and Suc-Phe-NH-Np), while the pH remains constant for Ac-Phe-NH-Np.

What are now the consequences of this observation, if the activity of α-chymotrypsin against these three substrates is measured? Let us look at Fig. 2. We have measured at different fixed initial substrate concentrations ([S]) with a fixed overall enzyme concentration the initial velocity (v_{in}) of hydrolysis of the three substrates mentioned above. The results are reported in Fig. 2, together with the values obtained in water. Briefly, v_{in} vs. [S] curves in reverse micelles can be bell-shaped, depending on the substrate and the conditions used. In the case of Gr-Phe-NH-Np and Suc-Phe-NH-Np (Fig. 2A,B), v_{in} first increases with increasing substrate concentration and then - above a certain value - decreases. The decrease in v_{in} occurs at lower overall substrate concentration, the lower the buffer concentration used (data not shown) and the lower w_o, i.e. the lower the total amount of buffer solution in the system. If, however, v_{in} is plotted against water pool concentration, $[S]_{wp}$, the drop in v_{in} occurs for Gr-Phe-NH-Np as well as for Suc-Phe-NH-Np at all w_o values at the same $[S]_{wp}$ of 30-40 mM (data not shown). No such decrease has been observed with Ac-Phe-NH-Np in reverse micelles, or for all three substrates in aqueous solution.

These observed substrate effects have important consequences in those cases where the w_o dependency of the initial velocity is studied at fixed substrate concentrations. In particular, the nature of the v_{in} vs. w_o curve may depend on the substrate concentration used, as well as on the buffer salt, on the buffer concentration and on the pH of the buffer used. In order to illustrate this, consider in Fig. 2A, the case of Gr-Phe-NH-Np. If v_{in} vs. w_o is plotted for $[Gr\text{-}Phe\text{-}NH\text{-}Np]_{o,ov}$ = 0.2 mM, v_{in} decreases as w_o increases (Fig. 3). In contrast, if v_{in} measured at $[Gr\text{-}Phe\text{-}NH\text{-}NP]_{o,ov}$ = 0.4 mM or 0.65 mM are considered, the v_{in} vs. w_o curve is bell shaped with a maximum around w_o = 14 or 20, respectively (Fig. 3). As a consequence of these "pH artifacts", v_{in} vs. w_o profiles can be bell-shaped, while k_{cat} (or v_{max}) vs. w_o curves for the same enzyme substrate system are hyperbolic [14,15]. This new finding also explains to some extent the discrepancies between earlier literature reports on the kinetic behavior of α-chymotrypsin in AOT reverse micelles against the same substrate [14,16,17]. Barbaric and Luisi [16]

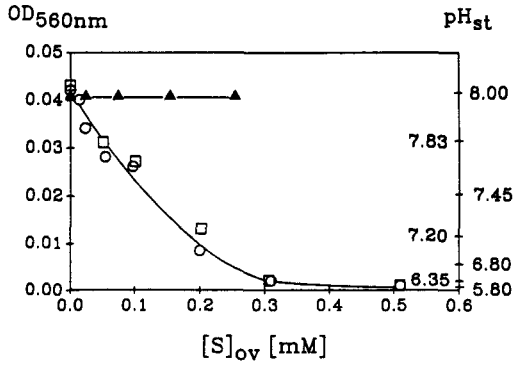

Fig. 1:
Effect of the overall substrate concentration on the absorption at 560 nm of phenol red in 50 mM AOT/isooctane reverse micelles at w_o=8. [Phenol red]$_{ov}$ = 1.5 μM, 0.1 M tris/HCl, pH 8.0. ○ : Gr-Phe-NH-Np; □: Suc-Phe-NH-Np; ▲ : Ac-Phe-NH-Np. The pH indicated on the right hand side (pH_{st}) corresponds to the pH value of phenol red containing 0.1 M buffer solutions with which 50 mM AOT-reverse micelles (w_o=8) have been prepared in the absence of any substrate, and which gave the corresponding optical density at 560 nm indicated on the left hand side. [Phenol red]$_{ov}$ = 1.5 μM; path length: 1 cm. Concerning the behavior of phenol red in AOT reverse micelles, see [25].

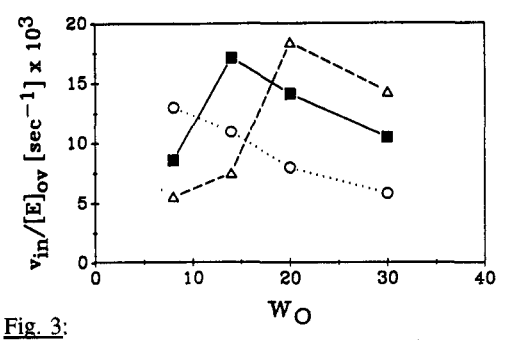

Fig. 3:
α-Chymotrypsin catalyzed hydrolysis of Gr-Phe-NH-Np in 50 mM AOT/isooctane reverse micelles at 25 °C. Initial velocity (v_{in}) as a function of w_o as determined with three different overall substrate concentrations, 0.2 mM (○), 0.4 mM (■), and 0.65 mM (△). For experimental details, see [15].

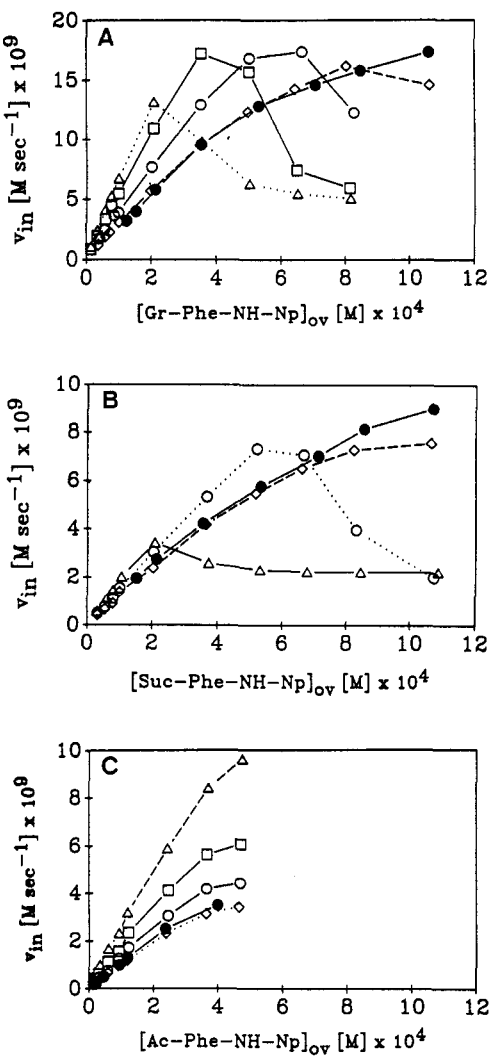

Fig. 2:
α-Chymotrypsin catalyzed hydrolysis of Gr-Phe-NH-Np (A), Suc-Phe-NH-Np (B), and Ac-Phe-NH-Np (C) in 50 mM tris/HCl, pH 8.0 (●) and in 50 mM AOT/isooctane reverse micelles at 25 °C. W_o dependence of initial velocities as a function of the overall substrate concentration, w_o=8 (△), 14 (□), 20 (○), and 30 (◇), prepared with 0.1 M tris/HCl, pH 8.0; [α-chymotrypsin]$_{ov}$ = 1 μM, [dimethyl sulfoxide]$_{ov}$ = 28 mM.

and more recently Rao et al. [17] showed initial velocity data in their papers, while Fletcher et al. [14] determined k_{cat}. As discussed earlier, the AOT purity (i.e. the presence of acidic impurities) may also affect the kinetic behavior of enzymes in AOT reverse micelles [4,5]. (In a broader sense, an acidic substrate can be considered as an acidic impurity!)

SOLUBILIZATION OF PROTEINS

A third example where pH effects may lead to unexpected results is the solubilization of proteins at high concentration. This may be required in the case of spectroscopic studies such as uv/vis absorption, circular dichroism or light scattering, or in the case of stopped flow kinetic experiments. If the pH of aqueous protein solutions is not controlled and adjusted before solubilization in reverse micelles, the apparent pH in reverse micelles may be significantly different from the pH of the buffer in which the proteins have been dissolved. Fig. 4 shows the case of α-chymotrypsin and lysozyme in 50 mM AOT/isooctane reverse micelles at w_o = 30, containing 1.5 μM phenol red (overall). The proteins were dissolved in 0.1 M tris/HCl, pH 8, without adjusting the pH before solubilization (open symbols) and with pH adjustment (closed symbols). For these two enzymes, the pH effect is more significant in the case of α-chymotrypsin, and the higher the overall enzyme concentration, the lower the apparent water pool pH. Similar pH effects may be relevant in solid-liquid protein extraction experiments with reverse micelles, where an experimental control of the water pool composition is particularly difficult.

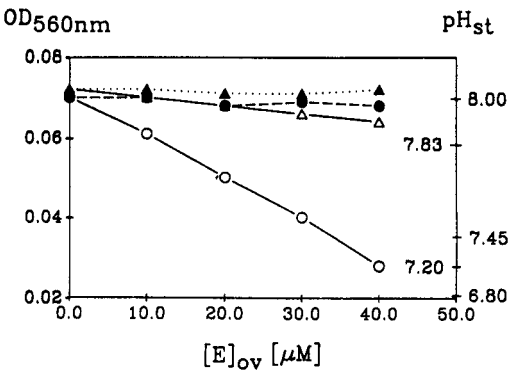

Fig. 4:
Effect of bovine pancreatic α-chymotrypsin (○ , ● ; from Sigma, Saint Louis, MI, USA) and hen egg lysozyme (△ , ▲ ; from Fluka, Switzerland) on the absorption of phenol red (1.5 μM overall) at 560 nm in 50 mM AOT/isooctane reverse micelles at w_o=30 (0.1 M tris/HCl, pH 8.0) without adjustment (open symbols) or with adjustment (closed symbols) of the pH of the aqueous protein solution before solubilization; path length: 1 cm. For the meaning of pH_{st}, see Fig. 1.

CONCLUDING REMARKS

In summary, we believe that at least some unexpected results in the literature on enzymes in reverse micelles can be explained by pH effects caused by high enzyme concentrations, by acidic (or basic) substrates, by the presence of acidic (or basic) impurities in the substrates, or by acidic products formed during the reaction. The latter problem is in particular difficult to solve in the case of lipase catalyzed hydrolysis reactions where high substrate concentrations are used and high reaction yields are desired. Fundamental studies should in these cases only be carried out with very sensitive lipase assays, where the initial release of only small amounts of free fatty acids can be detected [8]. An alternative would be the use of physiological irrelevant chromogenic fatty acid esters, such as p-nitrophenyl alkanoates [25]. In all the future work on fundamental studies of enzymes in reverse micelles, we recommend that the questions related to possible "pH artifacts" should be discussed and considered more carefully. It may help to understand better the action of certain enzymes in reverse micellar systems.

Generally, we recommend to specify in all the enzyme kinetic measurements in reverse micelles not only the origin and the purity of the surfactant [18], but also give enough experimental details. In particular, in the case of activity vs. w_o profiles, it is important to specify what is meant with activity (k_{cat}, v_{in} at a certain substrate concentration, or an assumed v_{max}). This specification is particularly important if one likes to explain theoretically the kinetic behavior of enzymes in reverse micellar systems [19-24]. In order to detect possible "pH artifacts" the use of cosolubilzed pH-indicators (such as phenol red) may be useful [25]. In order to avoid "pH artifacts" caused by the substrate itself, the

pH of the aqueous substrate solution should always be adjusted, before solubilization, to the desired pH with acid or base. In those cases where the substrate is dissolved with the help of a polar organic solvent (such as dimethyl sulfoxide), or where the influence of water-miscible polar solvents on the enzyme activity is studied, special attention should be taken to possible pH effects.

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