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Modelling the enantioselectivity of subtilisin in water and organic solvents: insights from molecular dynamics and quantum mechanical/molecular mechanical studies†

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Through molecular dynamics and quantum mechanical/molecular mechanical calculations we found that differential charge distributions due to the enzyme and to the different solvents can determine the reactivity of subtilisin in different media.

The possibility to use enzymes in organic media[1,2] has widened the range of applicability of proteins to almost all synthetic organic reactions. In these media, the absence of a continuous aqueous layer around the enzyme makes it possible for it to interact directly with the non-aqueous solvent, which results in modifications of the properties of the enzyme in terms of stability, activity and specificity/selectivity:[2] thus, enzymes like hydrolases and proteases can catalyse esterification and transesterification readily and with high product yields.[1] Serine proteases like subtilisin have also been the subject of many computational studies involving either the investigation of their structure–activity relationships[3–5] or of their catalytic mechanism using different theoretical approaches.[6–8] In a previous paper[9] we have examined the origin of enantioselectivity of the serine protease subtilisin in DMF through the use of molecular dynamics (MD) and free energy perturbation (FEP) simulations. As a model reaction we studied the resolution of a racemic mixture of *sec*-phenethyl alcohol by transesterification reaction with the acylating agent vinyl acetate in organic solvents (Scheme 1, ESI data†). The transition state (in which enantioselectivity is determined) leading to the ester formation in organic solvents is the same as the one leading to ester hydrolysis in water, and is represented by the tetrahedral intermediates (Figs. 1 and 2). A critical aspect of our previous study was the determination of the charge distribution on the two (*R* and *S*) tetrahedral intermediates and on the residues of the catalytic triad through the use of a combined quantum mechanical/molecular mechanical (QM/MM) electrostatic potential (ESP) fitting methodology. Our approach could reproduce the experimental $\Delta\Delta G^*_{(S-R)}$ value very well thanks to the application of a flexible charge model for the two diastereomeric intermediates. The experimental $\Delta\Delta G^*_{(S-R)}$ resulted 0.4 kcal mol^{-1}, while the calculated value was *ca.* 1 kcal mol^{-1}.[9]

We now extend the QM/MM treatment to other two solvents: water and hexane. The program ROAR 1.0[10] was used to carry out all the calculations, with all-atom AMBER parameters for the protein,[11] OPLS parameters for the organic solvents[12] and TIP3P parameters for water.[13] The QM region for QM/MM ESP calculations comprised the substrate and the residues involved in catalysis (Ser 221, Asp 32, His 64, Asn 155). The PM3[14,15] Hamiltonian was used for the minimisation stage, while MNDO[14,15] was used for the ESP stage. We used the MNDO Hamiltonian for ESP fitting because MNDO has been shown to give ESP fitted charges that are well correlated to HF/6-31G* ESP derived charges, while PM3 does not.[14,15] The systems (*R* and *S* complex in the three different environments) were MD equilibrated for 300 ps, and the structures for QM/MM calculations were saved every 15 ps over the last 180 ps of MD.

Two enantiomers in an achiral environment have the same physico-chemical properties, and hence, the same charge distribution on the corresponding atoms. The same enantiomeric substances bound or complexed to the enzyme, however, experience a chiral environment, which gives rise to two diastereoisomeric complexes. Lipkovitz *et al.*[16] pointed out that chiral auxiliaries can induce a desymmetrization of the frontier orbitals at the reaction site, making them chiral as well. This desymmetrization will be reflected in the electron (*i.e.* charge) distributions. In the tetrahedral complexes we have studied, the two substrates are perturbed by the chiral environment determined by the enzyme and thus by differential electrostatic fields, giving rise to differential charge distributions on analogous atoms of the substrate in the complex.

The concept of 'electrostatic stereodifferentiation',[9] together with steric factors, can be important in the determination of the energy difference between the two transition states leading to selectivity. The availability of QM/MM electrostatic potential fitting methods allows us to carefully take this aspect into consideration, by explicitly considering polarization and charge transfer effects due to the solvents (water, DMF, hexane).

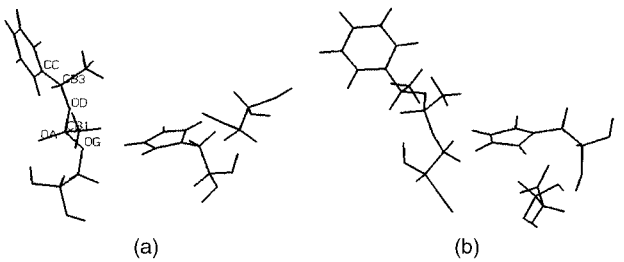

Fig. 1 Representation of the equilibrated *S* (a) and *R* (b) tetrahedral intermediates in organic solvents.

† Electronic supplementary information (ESI) available: average charge values for the reactive intermediate of Scheme 1. See http://www.rsc.org/suppdata/cc/a9/a909680i/

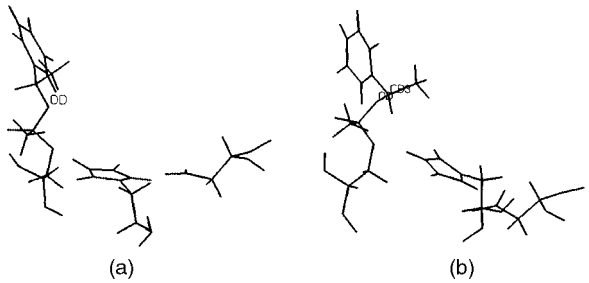

Fig. 2 Representation of the equilibrated *S* (a) and *R* (b) tetrahedral intermediates in water.

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The steric factors in the two organic solvents resulted very similar. Once the complex is equilibrated the phenyl group of the *S*-substrate fits very nicely into a hydrophobic pocket defined by residues 126, 127, 128 and Asn 155, in both DMF and hexane [Fig. 1(a)]. For the *R*-enantiomer [Fig. 1(b)] the phenyl ring is oriented towards the surrounding solvent, giving rise to favourable hydrophobic solvation interactions with the organic environment. The methyl group of the alcoholic moiety of the *R*-complex is now pointing into the hydrophobic pocket, and in total, these rearrangements contribute to disrupt the catalytically essential H-bonds (OD to HE2). We will show later on that this can have important consequences in terms of charge distribution.

In water, the phenyl ring tends to be removed from the unfavourable contact with the polar hydrogen bonding solvent, and resides in the hydrophobic pocket for both *R* and *S*-complexes. The methyl groups point towards the solvent. The difference in the two structures can be found in the conformation around the OD–CB3 bond. In the *S*-complex, the phenyl ring is in *anti*-conformation with respect to the rest of the bulky tetrahedral intermediate, while, for the *R*-complex, it assumes a more hindered and unfavourable *gauche*-conformation. The energetically unfavourable conformation of the *R*-complex will be a factor in depressing the reactivity of this enantiomer. The diastereoisomeric arrangement that determines the steric differences will be reflected in the charge distributions on the atoms which are directly involved in the formation (organic solvents) or disruption (water) of the tetrahedral intermediate (Table 1, ESI data†). Moreover, not only do we notice differences between enantiomers, but also on the same atoms of the same enantiomer in different solvents. In particular this is true for the stereogenic center CB3 and the atoms forming its environment. In fact, CB3 shows a charge value for the *S*-complex in water which is lower than in the two organic solvents. The same consideration applies also to CB3 in the *R*-complex, even if the charge differential among the solvents is less pronounced in this case. The case of CC (phenyl atom directly bound to the stereocenter) is also representative of this solvent effect for the *S*-complex: the charge on CC has a value of 0.11 in water and is negative for both hexane and DMF. For the *R*-complex the charges for this atom are very similar. Small but noticeable differences due to solvent effect can be noticed in HB3, as well. We believe that all these effects involving the differentiation of charges on analogous atoms of the same enantiomer in different solvents is mainly due to the differential polarising characteristics of the solvents.

When the same solvent environment is considered, the most dramatic differences between analogous atoms of the two enantiomers can still be noticed on the stereogenic (CB3) center and in the atoms around this center. This factor can be ascribed to the stereodifferentiating environment determined by the enzyme. In particular, we notice that the alcoholic OD has a higher charge concentration (*i.e.* more negative charge) in all solvents for the *S*-complex. On the other hand, a higher concentration of positive charge is localized on the tetrahedral carbon (CB1) in the *S*-complex in all solvents. This makes the CB1–OD bond (the one formed in organic solvents and cleaved in water) more highly polarised in the transition state leading to the *S*-product. A higher concentration of negative charge on the nucleophilic atom, coupled with a higher positive charge on the electrophilic carbon (CB1), will favour the approach between the alcoholic moiety and the acyl enzyme in the absence of other nucleophiles. Moreover, the higher negative charge concentration on OD will favour the formation of a stable hydrogen bond between OD and the acidic HE2 hydrogen on His 64 for the *S*-complex (the average distance between OD @Ser 221–HE2 @His 64 in this case is 2.6 ± 0.6). On the other hand, in the *R*-complex, the catalytically essential hydrogen bond is disrupted because of both steric and charge factors: OD is, in fact, less negative than in the *S*-complex case. These observations suggest that, in the transesterification reaction the *R*-alcohol cannot readily donate its proton to the catalytic residue His 64, which is essential for catalysis.[17] For hydrolysis this very factor will favour the cleavage of the reactive bond, by concentrating more negative charge on the more electronegative carbon (OD), in the tetrahedral intermediate for *S*-complex. A smaller polarization is found for the CB1–OD bond in the *R*-complex in all solvents. This will slow down the corresponding reaction leading to the preferential synthesis or hydrolysis of the compounds with *S* absolute configuration. By considering the charge distribution, we can garner deeper insights into how the electrostatically stereodifferentiating environment imposed by the enzyme can influence reactivity and selectivity. Our results are in agreement with, and help us rationalize, the experimental observation of reactivity in several environments.[18]

Summarising, we showed that steric and electrostatic factors play an important role in determining asymmetric induction and selectivity for enzymes in different environments. The application of QM/MM simulation techniques can be of valuable help in identifying the residues near the active site and of the binding pocket which are important for catalysis. The steric differentiation between two bound enantiomers can thus be magnified by mutating non-bulky residues of this region to sterically demanding ones. Maximising the charge differential between the two tetrahedral intermediates through mutations of non-polar to polar residues, which are involved in recognition, can also improve selectivity. Finally, focussing on the solvation patterns of the two simulated bound intermediates can help us find solvents which could better solvate the exposed parts of one of the two enantiomers.

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