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A computational investigation of the possible substrate binding sites in the hydroxylase of soluble methane monooxygenase

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Abstract

In this paper we report docked conformations for a diverse range of substrates within the hydroxylase component of soluble methane monooxygenase (sMMO). Based on energy minimisation calculations, three substrate binding sites have been elucidated. There is a unique site at which the lowest binding energy structures for methane, the in vivo enzyme substrate, acetylene (a potent suicide substrate), propene and pyridine are located. These four are designated group I substrates. The unique site is approximately 3 Å from the diiron site so that substrate oxidation can be easily achieved. The orientation of each of the group I molecules in the unique site reflects precisely the observed product formed in the oxidation reaction.

Substrates whose molecular volumes are greater than ≈ 71 Å^3 are not accommodated at the unique binding site. Rather, these group II molecules cluster at two further sites, termed A and B, both of which are approximately 14 Å from each of the iron atoms of the active site. The energy differences for binding of group II substrates at either site A or B are not great. Larger molecules bind preferentially at B, but size is not the only discriminatory factor between sites A and B. As the group II molecules are known sMMO substrates, a conformational change must occur which opens paths between sites A and B and the unique site to permit oxidation of these substrates by the high valent iron-oxo species. The required conformational change may be initiated by the regulatory protein B binding to the hydroxylase.

1. Introduction

The docking of small molecules into the active sites of much larger and highly complex enzymes has always posed difficulties, mainly because the potential energy hypersurface of such an interaction is highly detailed and is scattered with many local minima. If the active site of the host system is known, then the number of conformational possibilities is substantially reduced but *not* isolated. In the case of soluble methane monooxygenase, sMMO [1,2], the substrate binding site is *not known* and only recently has a crystal structure been reported [3–5]. Soluble methane monooxygenase is unique in that it is the only known enzyme which catalyses the reaction between methane and molecular oxygen, forming methanol as the sole product, at ambient temperature and pressure [6]. This is of interest because of its potential as a biological replace-

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ment for the more common synthesis gas process for methanol production [7] which requires much more harsh reaction conditions. The enzyme also catalyses the oxidations of a variety of other substrates including higher alkanes, alkenes, aromatics and alicyclic and heterocyclic compounds [6,8–10].

Soluble methane monooxygenase comprises three components; an hydroxylase, a reductase and a regulatory component, protein B. The hydroxylase has an $\alpha_2\beta_2\gamma_2$ structure and each of the α-subunits contains a bridged diiron site [3–5], similar to those in a growing list of non-heme iron proteins of vastly different functionality [11]. It is at or near the hydroxo-bridged [12–14] dinuclear iron centre that O_2 and substrate are activated. Electrons are transferred from NADH to the hydroxylase by the FAD and Fe_2S_2 groups of the reductase, which is a single polypeptide. Component B is also a single polypeptide containing no metal ions or cofactors and is believed to somehow regulate electron transfer to the diiron site of the hydroxylase [15,16]. The mechanism proposed for sMMO catalysed oxidations of methane and similar molecules is believed to involve H-atom abstraction from the substrate by a high-valent iron-oxo intermediate producing a substrate radical, which then rapidly reacts with iron bound –OH to form product [10,17–19]. Other substrate oxidations catalysed by sMMO may well proceed by different mechanistic pathways [11,18,20,21].

In this paper, we report conformations for a number of known sMMO substrates in newly identified binding sites which have been located by a semi-automated method for docking highly flexible guest molecules (substrates) within a rigid host (sMMO hydroxylase) structure. This methodology has been used previously in a successful application to the conformational variation and clustering effects of methane molecules in different zeolite structures and this work represents the transferral of the technique to a biological system. Computer assisted modelling has been used recently to predict the locations of substrate binding sites in lipases [22] and those compounds which might be alternate substrates for cytochrome $P450_{cam}$ [23]. The particular combination of Monte Carlo and molecular dynamics techniques used in this study has also correctly predicted which alternative substrates (other than camphor) will bind in the active sites of $P450_{cam}$ mutants [24]. This kind of technique is particularly useful in enzymes like sMMO where a crystal structure with the methane in place is unlikely to be obtained.

2. Results

The docking procedure described in the methods section was applied to a number of substrate molecules differing in size, shape and functionality, but each has been experimentally observed to undergo an oxidation reaction catalysed by soluble methane monooxygenase [6,8–10]. The potential energy parameterisation employed in this study is the CVFF potential energy forcefield as applied in the suite of programs distributed by Biosym Technology [25]. The binding energy, BE, (defined as $E_{absorbed} - E_{isolated}$) for each docked substrate can be calculated and the average binding energy for each can be obtained upon consideration of the 20 next lower minima. The corresponding standard deviation can be thought of as giving a qualitative measure of how difficult it was to the locate the minimum and also the spread of other local minima on the complex potential energy surface. The overall binding energy results are reported in Table 1. In all cases there is preferential absorption of the hydrocarbon from the isolated state into the hydroxylase, although the range of absorption energies is broad. This range in energies reflects a number of factors in the substrates including varying numbers of atoms, atom types and bond orders. Strictly, a direct quantitative comparison of binding energies can only be made between molecules with the same number of atoms and the same atom types, i.e.

Table 1
The calculated values for the lowest energy minima, the average binding energies and the associated standard deviations for the lowest 20 minima

Substrate	Minimum binding energy kcal mol^{-1}	Average binding energy kcal mol^{-1}	Standard deviation
methane	−12.65	−7.66	1.30
acetylene	−30.93	−11.13	4.93
propene	−28.16	−13.82	3.75
pyridine	−40.72	−20.33	5.33
benzene	−22.61	−19.55	2.46
2-methylpropane	−13.04	−10.62	1.26
phenol	−27.89	−24.32	2.17
cyclohexene	−19.71	−16.97	1.34
toluene	−24.55	−21.53	2.35
pentane	−15.01	−12.55	1.34
vinylpyridine	−25.17	−21.68	1.43
1-methylcyclohexene	−21.46	−17.29	2.00
3-methylcyclohexene	−18.83	−16.70	0.66
2,3-dimethylpentane	−18.48	−14.92	1.51
cis-dimethylcyclohexane	−18.49	−14.76	1.59
trans-dimethylcyclohexane	−19.01	−14.59	1.86
biphenyl	−33.90	−30.03	2.57
adamantane	−14.86	−14.51	0.23
β-pinene	−20.03	−17.66	1.34

isomers. However, it is possible to get a broad qualitative comparison among all the substrate molecules by normalisation of the binding energies via the molecular volume of the substrate. Table 2 reports the binding energies for the energetically most favourable minima located

Table 2
Values for the lowest and average binding energies normalised with respect to the molecular volume (also given) of each substrate

Substrate	Molecular volume $Å^3$	Normalised minimum binding energy kcal mol^{-1} $Å^{-3}$	Normalised average binding energy kcal mol^{-1} $Å^{-3}$
methane	26.5	−0.48	−0.29
acetylene	29.1	−1.06	−0.38
propene	51.6	−0.55	−0.27
pyridine	70.5	−0.58	−0.29
benzene	74.5	−0.30	−0.26
2-methylpropane	75.1	−0.17	−0.14
phenol	81.3	−0.34	−0.30
cyclohexene	89.6	−0.22	−0.19
toluene	90.6	−0.27	−0.24
pentane	91.3	−0.16	−0.14
vinylpyridine	95.6	−0.26	−0.23
1-methylcyclohexene	105.6	−0.20	−0.16
3-methylcyclohexene	105.8	−0.18	−0.16
2,3-dimethylpentane	123.8	−0.15	−0.12
cis-dimethylcyclohexane	129.5	−0.14	−0.11
trans-dimethylcyclohexane	129.5	−0.15	−0.11
biphenyl	138.4	−0.25	−0.22
adamantane	141.0	−0.11	−0.10
β-pinene	144.8	−0.14	−0.12

Table 3
Comparison of the lowest and next lowest binding energies (BE) for group I substrates and the binding energies for group II substrates in sites A and B

Substrate group I	Minimum BE kcal mol^{-1}	Next lowest BE kcal mol^{-1}
methane	−12.65	−8.15
acetylene	−30.93	−12.93
propene	−28.16	−16.06
pyridine	−40.75	−22.65
group II	site A BE kcal mol^{-1}	site B BE kcal mol^{-1}
benzene	−22.61	−20.21
2-methylpropane	−11.88	−13.04
phenol	−27.89	−26.76
cyclohexene	−19.71	−17.18
toluene	−24.55	−22.11
pentane	−15.01	−14.16
vinylpyridine	−25.17	−22.51
1-methylcyclohexene	−21.46	−17.79
3-methylcyclohexene	−18.83	−17.53
2,3-dimethylpentane	−18.48	−16.25
cis-dimethylcyclohexane	−15.25	−18.49
trans-dimethylcyclohexane	−19.01	−17.82
biphenyl	−30.29	−33.90
adamantane	NA	−14.86
β-pinene	−16.75	−20.03

and the average binding energies which were obtained from normalisation with each molecular volume.

A unique binding site for CH_4, in which one of the methane H atoms is 2.5 Å from Fe1 and 2.71 Å from Fe2 is described by the docking procedure. Fe1 is defined as the active site iron which is ligated by non-bridging glu114 and his147 and Fe2 is that which is ligated by non-bridging glu209, glu243 and his246. This is the minimum energy structure for methane in the sMMO hydroxylase and its binding energy is 4.5 kcal mol^{-1} lower than that for the next lowest energy docked structure. The binding pocket surrounding methane is quite hydrophobic and is opposite the OH bridge which is present in the oxidised hydroxylase. Acetylene, propene and pyridine also have their lowest energy structures at this site. The minimum binding energies for these substrates are 18.0, 12.1 and 18.1 kcal mol^{-1} lower, respectively, than those of the next lowest energy structures. The residues which form the unique methane binding pocket are from helices B, C, D, E and F of the α-subunit. Of these, only helix D does not provide a ligand to either Fe1 or Fe2.

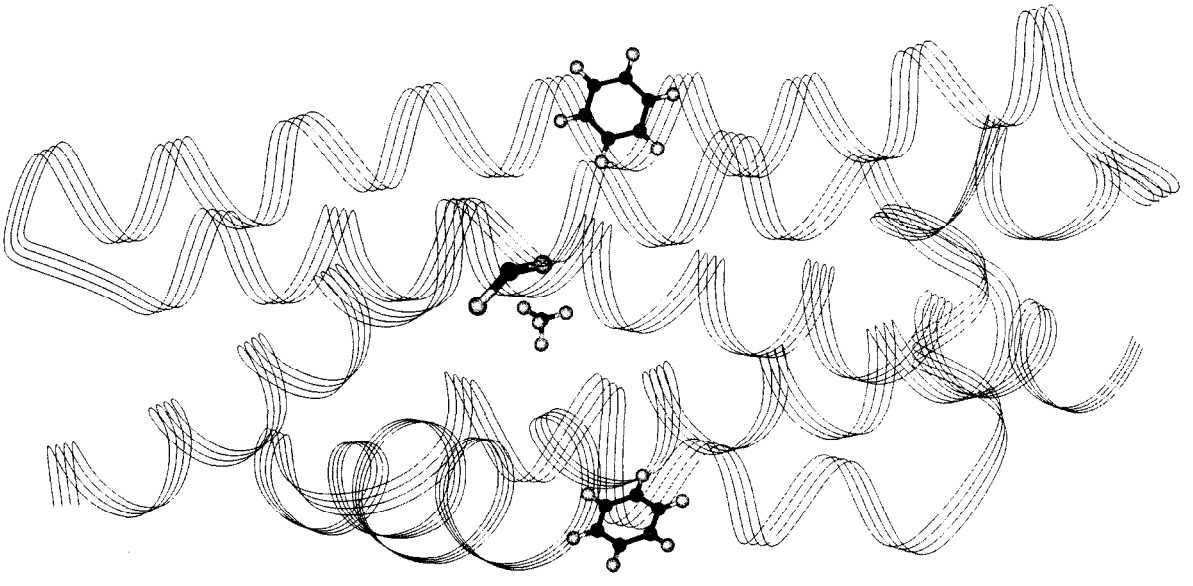

Fig. 1. Sites A (bottom) and B (top), with a group II substrate represented by benzene, shown in relation to the active site and the helices which encompass it. The distances of closest approach to Fe1 (left) or Fe2 (right) are indicated.

None of the other substrates listed in Tables 1 and 2, with molecular volumes greater than ≈ 71 Å^3, exhibits a minimum energy structure at the unique CH_4 site, nor were any of their next 20 minima found at this site. Instead the minimum binding energies for these substrates fell into two groups which describe the additional binding sites A and B (Table 3). From here on, for simplicity, the substrates which bind at the unique site (methane, acetylene, propene and pyridine) will be referred to as group I and those at sites A and B as group II. The binding energy differences for placement of each group II substrate in either site A or B are significantly less than the differences observed between the lowest and second minima for each group I substrate (Table 3). Examination of Table 3 shows that for most of the group II substrates the lowest energy structure is at site A, although for pentane and phenol the differences between structures located in sites A and B are only 0.85 and 1.13 kcal mol^{-1} respectively, and the minimum energy structure for 2-methylpropane is at site B. Larger group II substrates are not easily accommodated in site A and in the case of adamantane, none of the structures represented by the first 20 minima was found to bind there. Sites A and B are approximately equidistant (≈ 14 Å) from the diiron center, as shown in Fig. 1, with benzene used as an example of a group II substrate. The residues which surround site A are from helices A, C, E and F of the α-subunit and helices 1 and A of the β-subunit while those around site B come from helices B, E, F, G and H of the α-subunit and helix 1 of the β-subunit.

3. Discussion

An advantage of the method used in this study is that a large number of interactions of each flexible substrate molecule with the hydroxylase can be searched in a semi-automated manner and thus, a multitude of docked structures are considered. The docking calculations allowed all conformations for each substrate to sample the entire hydroxylase in a volume of approximately 17000 Å^3 and not simply small regions near the active site. The resulting structures discussed below are only a small sample (the 20 lowest minima at most) of the 100 docked conformations produced for each substrate and for group I molecules represent the lowest energy minima calculated by the method. For most of the group II substrates there is more than one significant structure whose binding energies may vary marginally.

3.1. The unique methane binding site

Methane, the in vivo substrate for methane monooxygenase, is found to bind in a pocket surrounded by residues leu110, glu111, val112, gly113, tyr115, ala117, ile118, gln140, phe188, phe192, leu204, gln205, gly208, thr213 and ile217 as shown in Fig. 2. The majority of the residues are hydrophobic (60%) or neutral (33%) and there is only one charged amino acid (other than Fe ligands), glu111, within 7 Å of the center of the unique site. The CH_4 binding site is located essentially opposite the OH diiron bridging group and histidines 147 (Fe1 ligand) and 246 (Fe2 ligand) (Fig. 4). In the 1.7 Å Fe(III)Fe(III) hydroxylase X-ray crystal structure the acetate ion is no longer a bridging ligand but participates in hydrogen bonds with thr213 and a bridging water molecule [5]. It appears to be clamped in place by phe188 and phe192 and it is proposed that methane is located in the same position during the catalytic cycle. This is precisely the location described by the lowest binding energy structure for CH_4 (and the other group I substrates) determined by the docking method used in this study. Three of the four methane H-atoms are less than 3 Å from either Fe1 or Fe2 (defined above) and are positioned for facile abstraction by an activated iron bound oxygen species

Only three other known sMMO substrates, acetylene, propene and pyridine, also have their lowest energy structures located at the unique,

group I, site. Acetylene is a powerful mechanism based inhibitor of sMMO and as such would be expected to bind tightly to the hydroxylase. Each of the carbon atoms in HC≡CH in less than 3 Å from both Fe1 and Fe2. Its immediate oxidation product is probably ketene which may react with a residue at or near the active site resulting in complete, irreversible inactivation of the enzyme. Propene is a very good sMMO substrate, whose specific activity is comparable to that of methane, and catalysis of its epoxidation is routinely used as an assay for the enzyme. In its lowest energy structure the propene $-CH_3$ group points away from the diiron center and each of the carbon atoms which participates in the olefinic bond is 2.5 Å, or less, from Fe1 or Fe2. The propene molecule is nicely positioned for attack at the double bond which is consistent with formation of the epoxide. We have never observed production of allyl alcohol, the other possible product of propene oxidation. Pyridine is the fourth of the group I substrates and could represent the size limit for substrate location in the unique site. Its molecular volume is 70.5 $Å^3$ and all of the group II substrates are larger than this (Table 2).

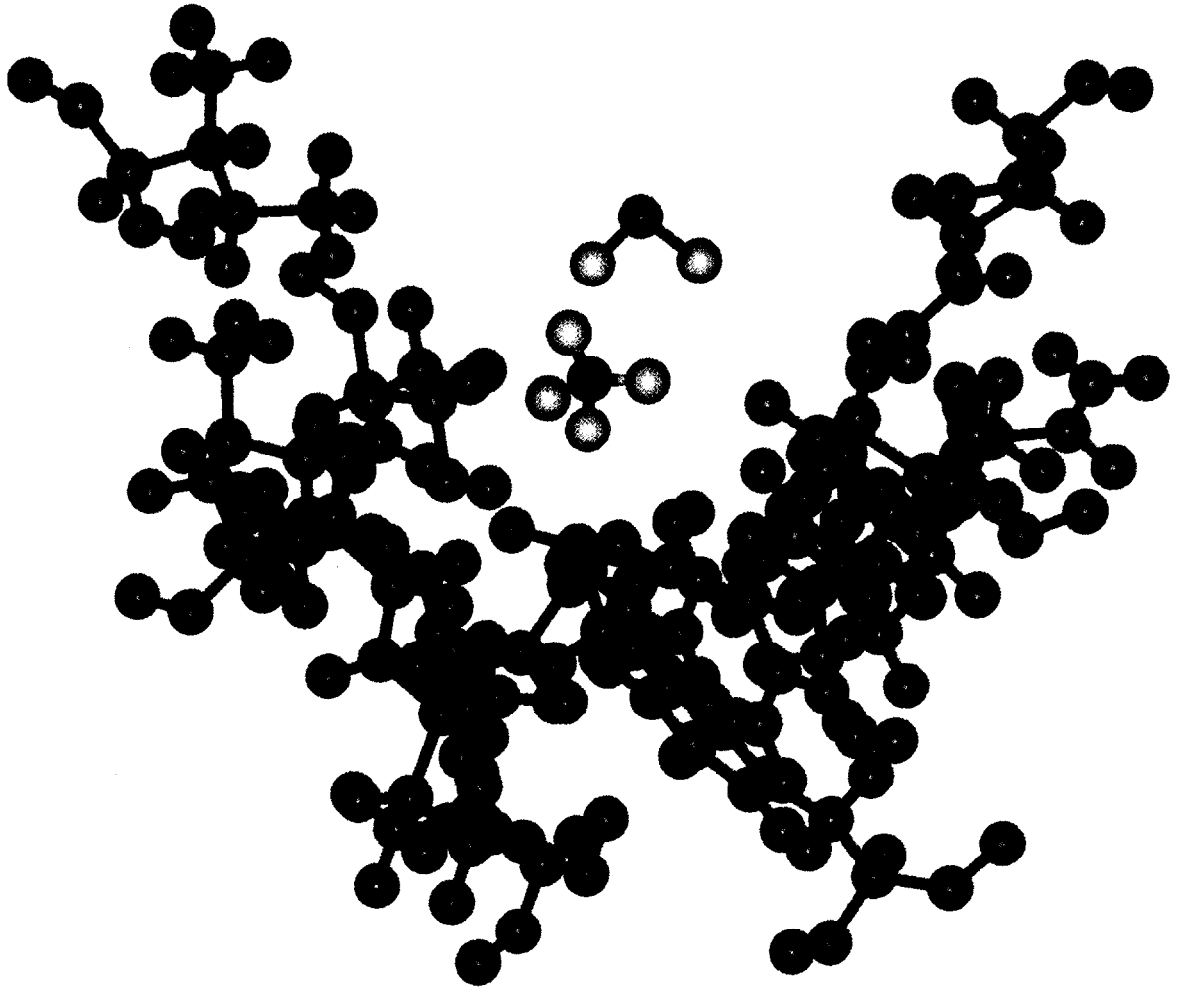

Fig. 2. The hydrophobic residues surrounding the unique site (with bound methane) are shown in blue. The Fe(III) ions are colored silver and the μ-OH group is red. Fe1 is to the right and Fe2 to the left.

Neither benzene (74.5 Å^3) nor 2-methylpropane (75.1 Å^3) is much larger than pyridine, but none of their first 90 structures is located at the unique site. The lowest energy pyridine structure is oriented such that attack at the ring nitrogen atom (2.44 Å from Fe1, 2.53 Å from Fe2) would be expected and the N-oxide is the only observed product. Although each of the group I substrates has at least one of the first 20 minima located at site A or B (or both), the energy differences between these and the lowest energy structures are significantly large (Table 3) so there is no ambiguity as to the location of the unique site.

3.2. Sites A and B

Inspection of Table 1 shows that for group II molecules the averages of the 20 next lower minima are not greatly different (< 5 kcal mol^{-1}) from those of the minimum binding energies for these substrates. For all group II substrates a number of the 20 minima are the same, but the structures represented by these energies are not randomly scattered throughout the hydroxylase. Instead they cluster at two locations which have been designated sites A and B. Because sites A and B are both approximately 14 Å from the diiron cluster (Fig. 1),

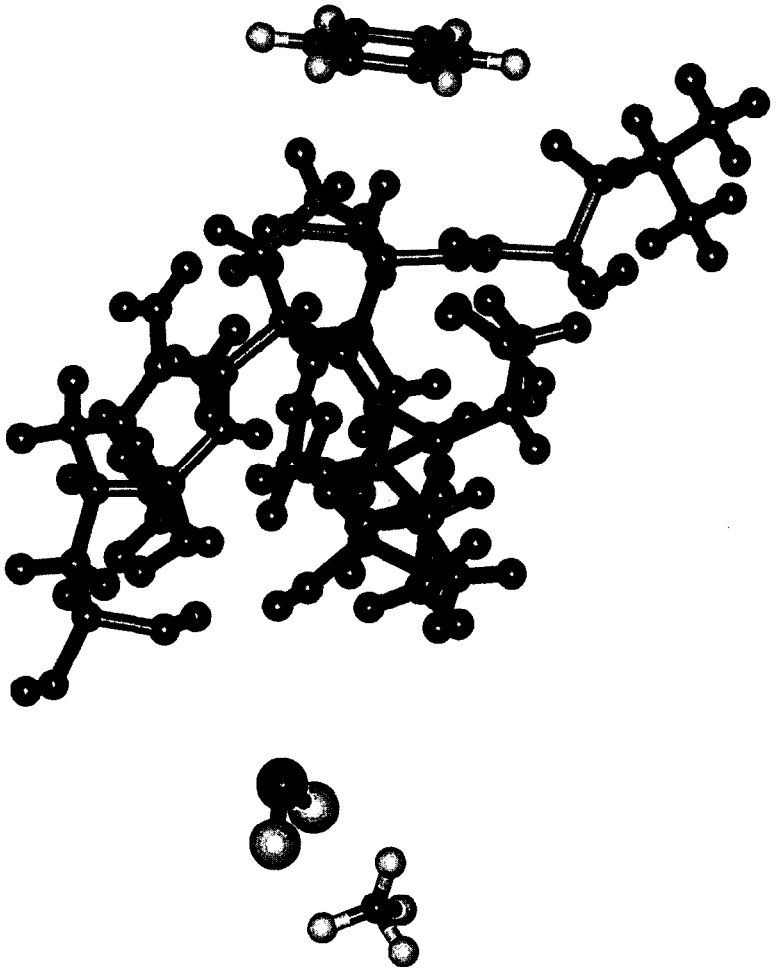

Fig. 3. View showing the residues which block access to the active site (right) for group II substrates, represented by benzene (left), bound at site A.

neither can be an actual binding site at which substrate oxidation occurs. Sites A and B may be 'holding areas' where the group II molecules locate prior to a conformational change (perhaps induced by protein B binding) which allows access to the unique binding site. Protein B is known to alter several of the properties of the diiron site in the hydroxylase, affect product distributions and accelerate the rates of formation of catalytic cycle intermediates [16,19,26–33]. All of these observations could be the result of a conformational change initiated by the binding of protein B to the hydroxylase. The migration of group II substrates from sites A and B to the unique site could also take place following such a conformational change. There is a canyon formed by the intersection of the α and β subunits of the two hydroxylase protomers where protein B has been postulated to bind [3–5]. Helices E and F of the α subunit are exposed on the walls of the canyon and would be most affected by binding of component B. Site A lies between helices A and F and site B is surrounded by helices E, F and H. Therefore, any conformational change would be communicated to group II substrates located at these remote sites. An equally satisfactory alternative which could explain the migration of group II substrates to the active site is the general overall mobility of the protein side chains which block access. Such flexibility is well characterised in a number of enzymes.

In the *Methylosinus trichosporium OB3b* sMMO system different substrate concentration dependencies were observed for some of the rate constants associated with the intermediates in the catalytic cycle [19,33,34]. It was suggested that substrates like nitrobenzene might bind at a site away from the diiron centre and in so doing somehow affect the rate of formation of the intermediate termed Q. Intermediate Q is the species which reacts with bound substrate forming the enzyme–product complex [19,32–35]. Nitrobenzene (91.6 $Å^3$) would be a group II substrate and would therefore be located at either site A or B. Neither methane nor furan (56.7 $Å^3$) increased k_f for intermediate Q which is consistent with their being group I substrates already bound at the unique site. Assuming that oxidation of all substrates occurs when they are bound at the unique site, a direct path from site A is obstructed by residues arg146, glu150, leu237, ser238, ile239, glu240, thr241 and asp242 (Fig. 3). Access to the unique site for

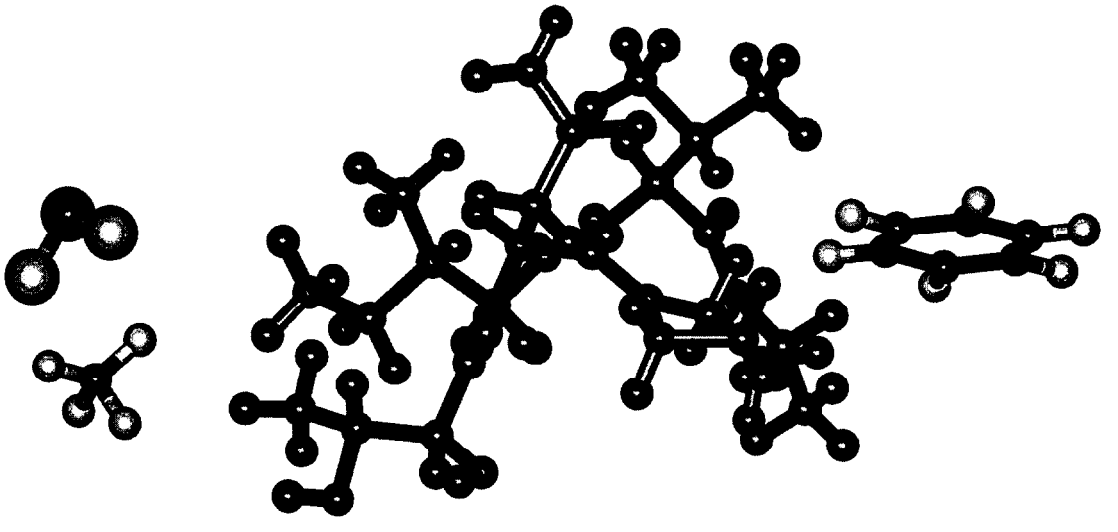

Fig. 4. View showing the residues blocking access to the active site (right) for group II substrates, represented by benzene (left), bound at site B.

molecules at site B is blocked by residues, thr213, asn214, pro215, ile217, val218 and ala219 (Fig. 4). In a recent EXAFS study, it was concluded that 1-bromo-1-propene does not form a π complex with either Fe1 or Fe2 because no backscattering from the bromine atom was observed [31]. The molecular volume of 1-bromo-1-propene is 100.9 Å^3 and therefore it would be predicted to bind at either site A or B. The EXAFS results were for dithionite reduced hydroxylase to which protein B and the brominated substrate had been added. It is possible that substrate binds only after reduction to the Fe(II)Fe(II) state, but there is evidence which indicates that binding occurs with the fully oxidised Fe(III)Fe(III) hydroxylase [36]. If protein B alone was required for a conformational change allowing access from sites A and B, the brominated olefin would be expected to have moved to the unique binding site where backscattering from Br should have been observed. Possibly, the sMMO reductase is also necessary for initiation of the required conformational change(s) or perhaps chemical reduction of the diiron center does not produce a suitable conformation for the repositioning of substrates from sites A and B [31,32].

As was observed for the unique site, most of the residues which make up sites A and B are either hydrophobic (A, 62%; B, 72%) or neutral (A, 11%; B, 6%). These are residues within an 11 Å radius from the centre of each site. Generally, the minimum binding energy structure for the smaller group II molecules locate at site A in preference to B (Tables 2 and 3). Molecules whose volumes are greater than $\approx$ 130 Å^3 appear to favor site B and only adamantane, one of the largest of the group II substrates, has no representative structures at site A. However, size cannot be the only criterion for differentiation between sites A and B. The minimum binding energy structure for 2-methylpropane (75.1 Å^3) is found at site B and the *cis* and *trans* isomers of dimethylcyclohexane, whose molecular volumes are the same, are located at B and A respectively.

Sites A and B may be analagous to the regions within hemoglobin and myoglobin where ligands (O_2, NO, CO) are known to reside (albeit on a very different time scale) for a time between being photolytically dissociated from Fe and release into bulk solvent [37–39]. In addition, there is known to be a hydrophobic patch in carbonic anhydrase where CO_2 binds before moving on to bind to the Zn(II) in the active site [40,41]. The hydrophobic patch, which is approximately 6 Å from the Zn^{2+}, and a second intermediate CO_2 binding site ($\approx$ 10 Å from Zn^{2+}) have been studied using molecular dynamics calculations [42].

4. Methods

Our approach is based on the 'docking' of absorbed molecules into other larger macrostructures by a combination of the Monte Carlo (MC) [43] and molecular dynamics (MD) [44] techniques. The method was developed originally for the investigation of the sorption of butene isomers into zeolite type structures [45]. In this study, the absorbed molecules are substrates and the macrostructure is the sMMO hydroxylase. The procedure is as follows: in the first stage, an MD trajectory simulation is performed at a high temperature (1500 K in this simulation) thus generating a collection of possible conformations of the substrate molecule. For small molecules like methane and acetylene a set of conformations is clearly not required, but such a collection becomes increasingly important for larger more flexible molecules. In addition, a more stringent conformational search procedure may be required for large molecules in the second stage. A Monte Carlo algorithm is used to insert a randomly chosen substrate conformation into a random position within the host structure (i.e. the hydroxylase). Only those conformations whose energies fall below a user specified energy threshold are accepted for subsequent minimisation. The MC algorithm is vital if the binding site is unknown, as the entire

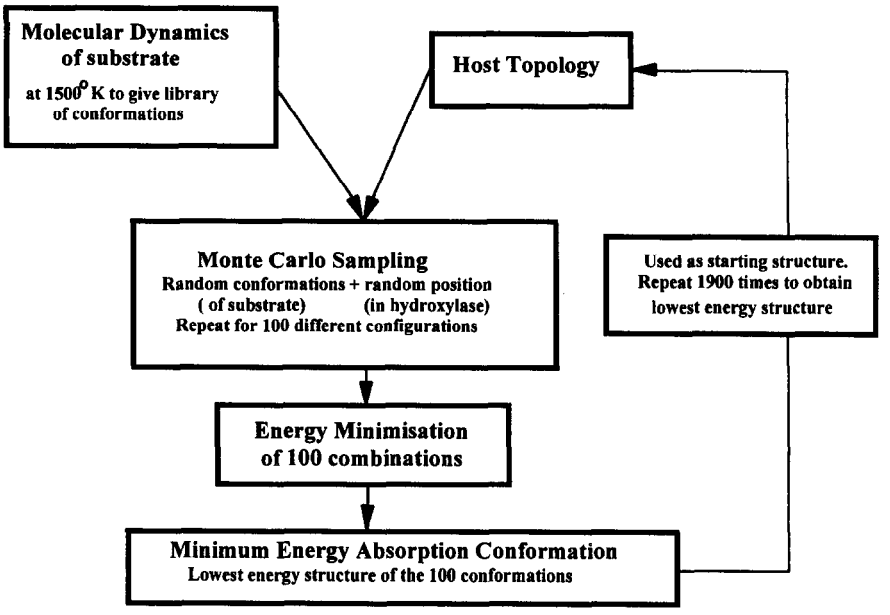

Fig. 5. Flow diagram representation of the 'Docker' methodology.

tertiary structure of the hydroxylase has to be probed. The resulting docked structures are minimised with a molecular mechanics forcefield, holding the hydroxylase rigid. The approximation of a rigid host is valid in such a preliminary binding site investigation in which a broad outline of the substrate location(s) is demonstrated. The procedure can be extended to include host (enzyme) relaxation [46], but at greatly increased computational cost. The present simulations used the potentials embodied within the constant valence forcefield (CVFF), as incorporated in the DISCOVER MD code, which is distributed by BIOSYM Technologies [25]. In the calculations, the non-bonding and coulombic interactions were summed over all atom pairs within the short range cutoff of 50.0 Å and the dielectric constant was set to be distance dependent. The non-bonded potentials for iron were included in the forcefield and were obtained from Rappe et al. [47]. The total energy minimum structure can be extracted from the sampled conformations, while the average energy of the lowest 20 conformations yields a measure of the affinity of each substrate for binding to the hydroxylase. The lowest energy structure gives an excellent indication of a possible substrate conformation at the newly identified binding site. This methodology is outlined in the flow diagram shown in Fig. 5. The calculations were performed on a Silicon Graphics Iris workstation, with the average complete run taking approximately 24 h of cpu time. The host coordinates [3] used in this study are those for the sMMO hydroxylase isolated from *Methylococcus capsulatus* (Bath) and were taken from the Brookhaven Protein Databank (file = 1mmo). Molecular volumes were calculated in SYBYL.

Acknowledgements

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