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Solvation of the active site of cytochrome P450-cam

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SUMMARY

Energetically favorable water binding sites in the substrate pocket of cytochrome P450-cam have been predicted by a molecular mechanics method. Binding sites corresponding to all the experimentally observed water sites in this region of the enzyme were located. The calculations also indicate the presence of two further water binding sites. One of these is located in a hydrophobic region of the protein where a water molecule would not bind tightly to the substrate-free enzyme. However, in the substrate-bound enzyme, a water molecule in this region could donate a hydrogen bond of optimum geometry to the carbonyl oxygen atom of the camphor substrate and could therefore contribute to the correct positioning of the camphor substrate for 5-exo-hydroxylation. These calculations also suggest that a steric analogue of camphor, containing an alkyl group which could prevent a water molecule from binding in this region, might inhibit cytochrome P450-cam by forming a more stable enzyme–ligand complex than camphor itself.

INTRODUCTION

Cytochrome P450-cam catalyzes the hydroxylation of camphor to 5-exo-hydroxycamphor using molecular oxygen. Its structure has been observed by X-ray crystallography with [1,2] and without [3] the camphor substrate bound, and also in the presence of a number of inhibitors [4] and alternative substrates [5]. In the absence of other ligands, six waters are seen in the camphor pocket [3], one of which acts as a ligand to the heme Fe atom (see Fig. 1f).

The solvation of the active site was studied by the GRID method [6,7]. This is a method of determining energetically favorable ligand binding sites on molecules of known structure. The interaction energies of a probe water molecule and a probe methyl group with cytochrome P450-cam were calculated at positions of these probes on an array of points in the region of the active site of the enzyme. These energies were contoured and displayed using computer graphics (with pro-

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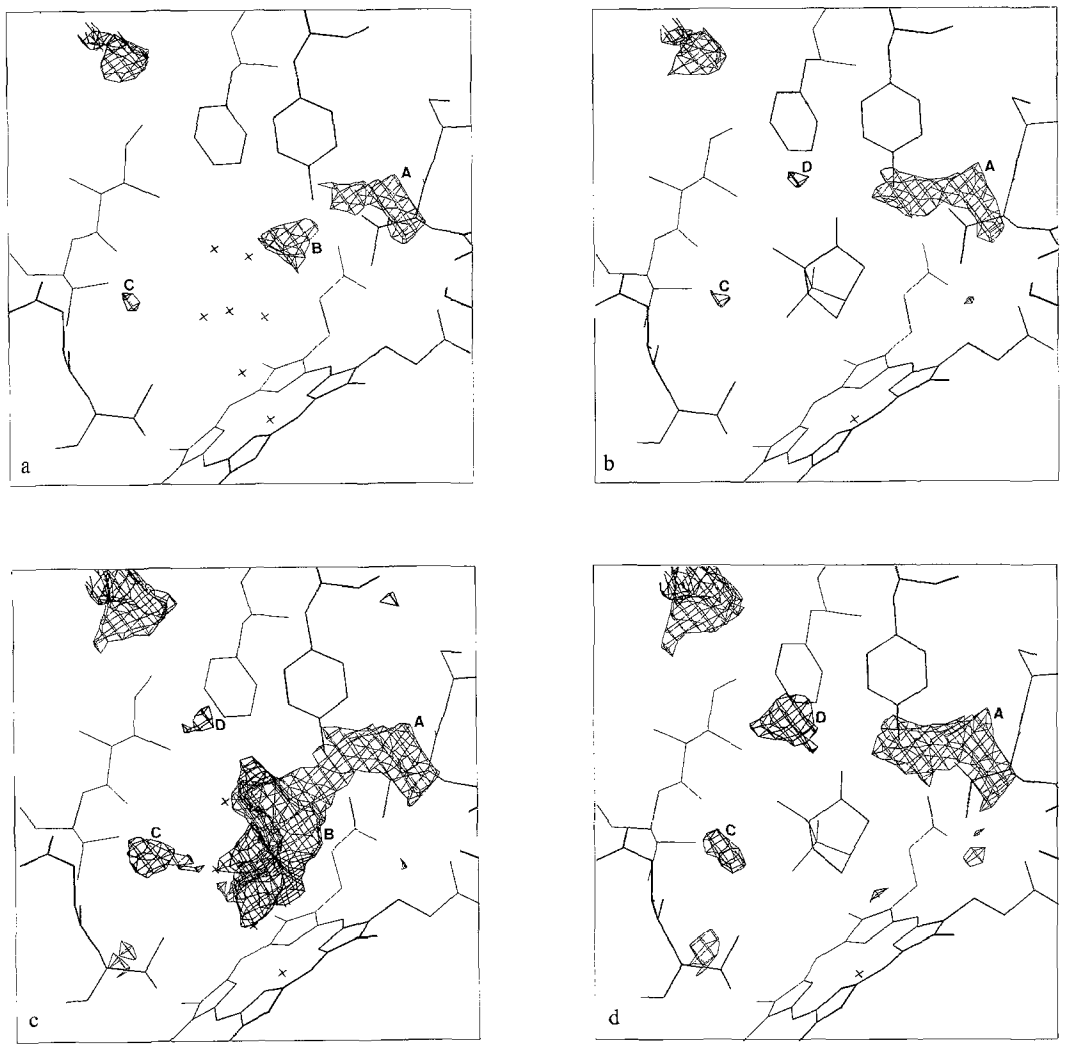

Fig. 1. a and b: GRID energy contours at −6 kcal/mol for a water probe in the active site of cytochrome P450-cam are shown in the substrate-free enzyme (a) and in the substrate-bound enzyme (b). Regions A, B, C and D are distinct water binding regions predicted by program GRID. c and d: GRID energy contours at −3 kcal/mol for a water probe are shown in the substrate-free enzyme (c) and in the substrate-bound enzyme (d).

Lennard–Jones, electrostatic and hydrogen bond terms was used. The probes were modelled as extended atoms with the geometry of the hydrogen bonds formed considered explicitly.

GRID energy contour maps for water and methyl probes in the region of the active site of cytochrome P450-cam were calculated for substrate-free and substrate-bound structures; these are displayed in Fig. 1. Only small conformational changes between the substrate-bound and the substrate-free enzyme structures have been observed [3]. Therefore, the calculated GRID energy maps for a water probe in the substrate-bound and the substrate-free enzyme structures (Figs. 1a–d) may be compared to each other, and also to the GRID energy map for a methyl probe in

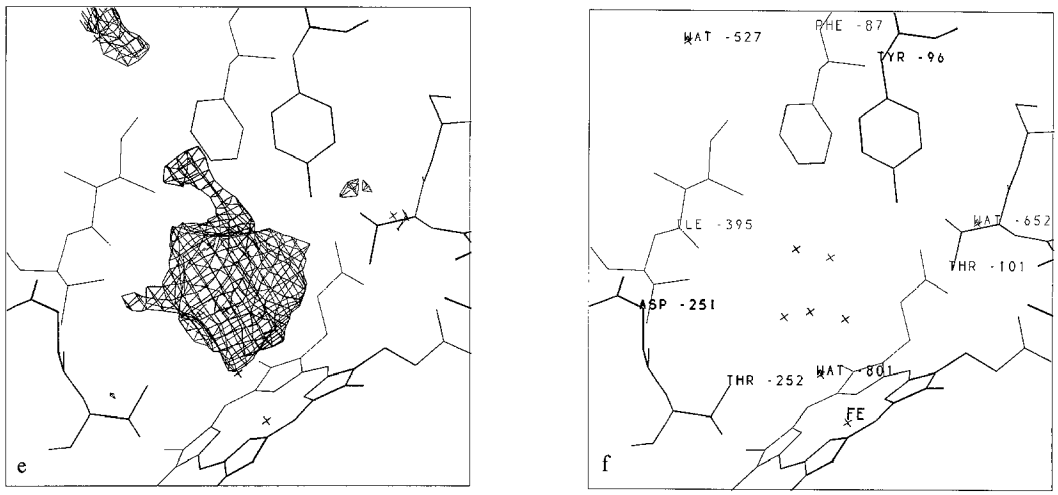

Fig. 1. e: GRID energy contours at 0.5 kcal/mol for a methyl probe in the substrate-free enzyme. These contours show the shape of the substrate pocket. f: The six crystallographically determined water molecules (W801-806) in the camphor pocket of the substrate-free enzyme are shown. W801 is the aqua-ligand to the heme Fe atom. Also shown is water W652, which lies within water binding Region A predicted by program GRID. See text.

gram FRODO [8] on an Evans & Sutherland Picture System 330). Contours at negative energy levels indicate favorable binding regions for the corresponding probe while those at positive energies show regions where repulsive interactions occur. An empirical energy potential consisting of the substrate-free enzyme structure (Fig. 1e). The latter, contoured at a small positive energy level, delineates the shape of the active site and is analogous to a solvent-accessible surface [9].

These maps show that four distinct water binding regions (labelled A, B, C and D) were predicted by program GRID in the vicinity of the substrate pocket of cytochrome P450-cam. Two of these (A and B) contain experimentally observed water molecules while the other two (C and D) suggest the presence of water molecules which have not been reported.

PREDICTED WATER BINDING REGIONS WHICH CONTAIN EXPERIMENTALLY OBSERVED WATER SITES

Region A

The prediction of water binding in Region A demonstrates the ability of program GRID to reliably determine water sites which correspond to those observed experimentally.

GRID energy contours at -6 kcal/mol in both structures (see Figs. 1a and b) show that binding of water is favored in Region A adjacent to the camphor pocket. This region is occupied by the experimentally observed water molecule W652 in both the substrate-free structure (see Fig. 1f) and the substrate-bound structure. Program GRID predicted that, at its experimentally observed position, this water molecule would make hydrogen bonds to atoms O Thr^{101} and OG Ser^{83} and

would bind to the protein with an energy of about −9 kcal/mol. As this particular water molecule was predicted to remain present on binding of the substrate, it may assist in stabilizing an adjacent sharp turn in the protein (residues 98–102). Water W652 may also interact with the camphor substrate via a chain of cooperative hydrogen bonds in which it donates a hydrogen bond to O Thr^{101}, which in turn donates a hydrogen bond to OH Tyr^{96}, which in turn donates a hydrogen bond to the oxygen atom of the camphor substrate. It may thus contribute to the formation of a strong hydrogen bond between the ligand and the protein.

Region B

The GRID program also shows that all the experimentally observed water sites from which water molecules are displaced on the binding of camphor are in energetically favorable positions. At all these water sites, the calculated binding energy is better than −2 kcal/mol. Three of these water sites are within the large region of contours seen at an energy level of −3 kcal/mol in Fig. 1c. Within this contoured region, a GRID energy minimum for a water probe was calculated at −5.4 kcal/mol. This energy minimum lies close to the experimentally determined iron-linked aqua-ligand W801, at a distance of 0.23 Å on the opposite side of this aqua-ligand from the heme ring. The experimentally observed aqua-ligand is 2.28 Å from the Fe atom and has a low temperature factor (16.5 $Å^2$) and full occupancy. It has been suggested [3,5] that a hydroxide ion might occupy this observed site and this would approach closer to the Fe atom than a neutral water molecule because it could make more favorable electrostatic interactions. Thus, it might be expected that the predicted water binding site would be further from the Fe atom than the experimentally observed aqua-ligand.

The other experimentally determined water sites (W802-806) in the camphor pocket were found in a large region where the probe was calculated to have a weak attractive binding energy. They were not predicted to be positioned in defined energy minima by program GRID. This is consistent with crystallographic observations [3] in which these five water sites were located within one large region of electron density and could not be individually resolved. On average, they were observed to have higher temperature factors and lower occupancies than the ordered surface waters suggesting that these water sites are not well defined and that the cavity contains a mobile water structure [3].

Displacement of these mobile waters on the binding of camphor would be favorable as it would result in a gain in entropy. In fact, the binding of camphor is thought to be largely entropically driven due to the desolvation of the camphor substrate and the release of the active site waters allowing camphor to bind in a region of low dielectric constant [3,10]. However, the GRID energy maps suggest that water may play a more complex role in the binding of camphor as shown by the next two binding regions discussed.

FURTHER WATER BINDING REGIONS PREDICTED BY PROGRAM GRID

Region C

GRID energy contours at −6 kcal/mol show a distinct water binding region (labelled C in Figs. 1a and b) near Val^{247} (not shown) and Asp^{251} in both substrate-free and substrate-bound structures although a water site has not been reported in this region.

From the methyl contours in Fig. 1e, it can be seen that Region C lies in a small crevice leading from the main volume of the active site, and so the position of Region C is well defined by van der Waals contacts. In addition, a water molecule in Region C could form hydrogen bonds to atoms O Val^{247} and OD1 Asp^{251}. This suggests that a water molecule might bind in Region C and that it would not be displaced by the camphor substrate but would instead fill space unoccupied by the substrate.

Region D

In the substrate-bound enzyme, a third binding region (labelled D in Fig. 1b) is indicated by the GRID energy contours at -6 kcal/mol. This binding site is not shown by contours at this energy in the substrate-free structure but it is seen at a less attractive energy contour level of -3 kcal/mol (see Fig. 1c). Region D is in a crevice of the substrate pocket as shown by the methyl probe contours (see Fig. 1e). Experimental observation of a water molecule has not been reported in this region.

Region D is surrounded by residues Phe^{87}, Phe^{98}, Val^{247} and Tyr^{96}, and there are no nearby hydrogen-bonding atoms. It is therefore hydrophobic in the absence of camphor and thus a water molecule may only interact weakly at this site in the substrate-free enzyme. However, on substrate binding, a water molecule at Region D might be stabilized by a hydrogen bond which it could donate to the camphor oxygen positioned about 2.9 Å away as shown in Fig. 1b. This hydrogen bond would be optimally oriented at the camphor oxygen at the trigonal angle to the C-O bond and would lie approximately in the plane of the oxygen lone pair orbitals. This hydrogen bond might therefore serve, in conjunction with that from OH Tyr^{96} to the camphor oxygen (which is also of optimum geometry), to orient the substrate correctly with the 5-carbon exposed to the molecular oxygen and the heme group so that the highly specific hydroxylation reaction can occur. A water molecule at Region D might thus play a significant role in positioning the bound substrate.

However, the presence of a water molecule in Region D when the substrate is bound would be entropically unfavorable. This suggests that modification of camphor by the addition of an alkyl moiety which could fill Region D and exclude water from it may result in a ligand with a greater binding affinity. Binding of such a ligand would permit the formation of enthalpically favorable van der Waals contacts with the hydrophobic protein residues surrounding Region D and ensure the entropically favored release of any water that may be present in Region D. Indeed, such a ligand might bind sufficiently strongly to act as an inhibitor of cytochrome P450-cam.

CONCLUSIONS

In this study of the binding of camphor to cytochrome P450-cam, it has been shown that water may play several different roles in ligand binding. It may stabilize the tertiary structure of the active site of the protein. It may hydrogen-bond directly to the ligand and thereby play a part in orienting the ligand correctly for a subsequent biological process. It may facilitate hydrogen bond formation between the protein and the ligand by participating in a cooperative hydrogen bond chain involving the enzyme and the ligand. It may fill space in the active site which is unoccupied by the bound ligand. It may make an entropic contribution to the free energy of ligand binding upon desolvation of the active site and the unbound ligand.

The above factors favor the protein–ligand interaction, but water may also destabilize the enzyme–ligand complex as it may be trapped in hydrophobic regions of the protein where its full hydrogen-bonding capacity cannot be satisfied, and because it may be constrained by the protein and the bound ligand so that its motion is restricted and is therefore entropically unfavorable. The balance of these favorable and unfavorable effects of water on ligand binding makes the overall action of water in protein–ligand interactions difficult to predict.

Nevertheless, these GRID calculations suggest that it may be possible to design enzyme inhibitors by modification of the substrate so as to remove cavities at the protein-substrate interface which could be occupied by water. To test this proposal, we plan to synthesize analogues of camphor and measure their binding to cytochrome P450-cam. If this approach is successful in the case of cytochrome P450-cam, it might lead to a strategy which is generally applicable to the rational design of inhibitors from substrates.

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