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# Chemical probes into the active centre of a heme thiolate monoxygenase†

P K BHATTACHARYYA*, T B SAMANTA, A H J ULLAH and I C GUNSALUS

Department of Biochemistry, University of Illinois, Urbana, Illinois 61801, USA
*Department of Organic Chemistry, Indian Institute of Science, Bangalore 560012, India

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**Abstract.** Linalool-8-monoxygenase, a typical bacterial P-450 heme thiolase, shows a high degree of substrate specificity towards linalool. The active site of the pure enzyme has been probed with a large number of substrate analogues with systematic alterations or conformational variations in the linalool molecule. The comparison of three parameters, the mo → $m^{os}$ conversion of the enzyme as a result of substrate binding monitored at 392 nm, the $K_D$ of the analogues giving information about energies of association and the relative turnover as substrate have given information about the space-filling characteristics of the substrates in the enzyme cleft, the number of contacts the molecules make with the respective domains of the enzyme and the distance of the site undergoing hydroxylation from the oxygen site, respectively. The data permit the conclusion that linalool makes contact with the enzyme by hydrogen bonding with the hydroxyl group as well through hydrophobic association with all the eight carbons carrying hydrogen in the molecules.

**Keywords.** Enzymes; oxygenases; P-450 hydroxylases; structure of monooxygenase; substrate analogues; enzyme kinetics; intermolecular associations.

Linalool-8-monoxygenase has been purified and resolved into three components, the NADH-linked flavoprotein reductase, iron-sulphur electron transfer protein and cytochrome P450, the terminal oxidase (Ullah *et al* 1983).

The reaction *in vitro* with the purified components shows a high selectivity for substrate domains. In fact, the enzyme does not metabolize closely-related substrates such as geraniol, nerol or citronellol while linalool is converted into 8-hydroxy-linalool and linalool-8-carboxaldehyde under identical conditions (figure 1). The low $K_D$ (3.5 molar) for linalool indicates that linalool makes a precise fit around the active site of the enzyme. The contacts for certain exposed parts of the molecule indicate separate domains in the cleft of protein surrounding the oxygenating site.

It was evident from preliminary studies that the free hydroxyl at position 3 of linalool was an absolute requirement for a substrate as linalyl acetate (1a) did not show any binding or activity (figure 1). 6-Methyl-hex-5-en-2-one had no activity whereas the corresponding alcohol (8) was accepted as substrate (table 1).

As a simplication, the problem of designing molecules was visualized in creating a tetrahedral carbon with a hydroxyl group (figure 2) where $R^1$, $R^2$ or $R^3$ can be varied

* To whom all correspondence should be addressed.
† This paper originally meant for publication in the special issue of the Proc. Chem. Sci. to commemorate the Golden Jubilee of the Academy, could unfortunately not be included, as its submission got delayed.

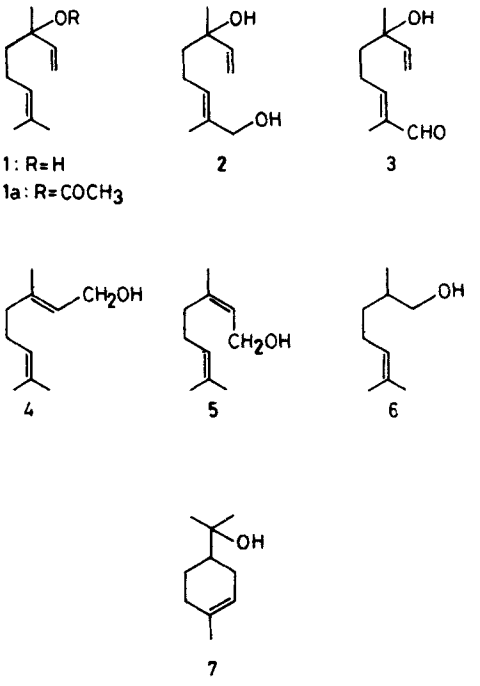



**Figure 1.**

keeping the other two groups constant and measuring the binding parameters and enzymatic rates.

*Catalytic rate measurements* were essentially carried out at 25°C with purified proteins of linalool 8-monooxygenase system. Of the three components, two were kept at saturating level, linalool redoxin-reductase (0·5 $\mu$M) and linalool redoxin (25 $\mu$M); the remaining component, linalool P-450, was kept at a limiting amount (0·025 $\mu$M). The enzyme assay solution (1·115 ml) contained $\beta$-NADH (300 $\mu$M) in 50 mM potassium phosphate, pH 7·0. The linalool 8-monooxygenase activity was stimulated with the addition of 50 $\mu$l linalool (200 mM) or other synthetic analogues (5 mM). Enzymatic activity was computed from the linear decrease in absorbance at 340 nm per minute under conditions demonstrating a first order rate dependence on the enzyme assayed. All the measurements were carried out in a Cary 219 spectrophotometer. The turnover number of linalool P-450 was estimated for various substrates by using the following formula:

$$\text{Turnover number} = \frac{\text{units of enzyme activity (n Kat) per second}}{\text{nmol of linalool P-450}}$$

*Determination of the dissociation constant ($K_D$) of linalool P-450 for linalool and other synthetic substrates.* All the measurements were carried out in a Cary 219 spectrophotometer at 25°C. The buffer in which binding of P-450 and the substrates were studied contained 100 mM KCl in 50 mM potassium phosphate, pH 7·0. The KCl was included to ensure complete conversion of the low spin form of P-450 to the high spin form (Gunsalus and Sligar 1978). The substrates were made to either 0·5 mM or 5 mM

**Table 1.** Structure and activity of synthetic substrates for linalool-8-oxygenase.

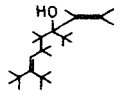
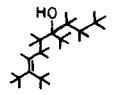
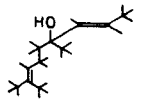
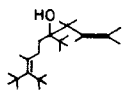
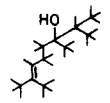
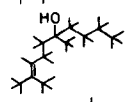
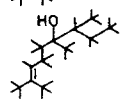
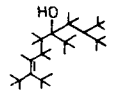
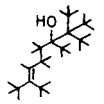
| No. | Structure | Rel. absorption maxima at 392 nm linalool = 100 | $K_D$ ($\mu$ mole) | Relative turnover (linalool = 100) | $AG_{ASSOC}$ $K_{CAL/MOLE}$ |
|---|---|---|---|---|---|
| | A probes into domains 1,2,70 and 1′ | | | | |
| 1 |  | 100 | 3.5 | 100 | −7.44 |
| 8 |  | 27.7 | 140 | 4.25 | −5.25 |
| 9 |  | 76.5 | 138 | 30 | −5.26 |
| 10 |  | 87.7 | 18.8 | 75 | −6.45 |
| 11 |  | 113.6 | 9.0 | 89 | −6.88 |
| 12 |  | 107.7 | 8.4 | 38 | −6.92 |
| 13 |  | 107 | 9.3 | 34.4 | −6.86 |
| 14 |  | 103.8 | 13.7 | 19 | −6.63 |
| 15 |  | 104.7 | 27.5 | 69 | −6.22 |
| 16 |  | 107.7 | 30.2 | 8.8 | −6.16 |
| 17 |  | 84.6 | 85.0 | 13.9 | −5.15 |
| 18 |  | 90.1 | 1.35 | 7.8 | −8.01 |
| 19 |  | 66.7 | 63.5 | 11.9 | −5.72 |

**Table 1.** (*Continued*)

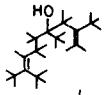
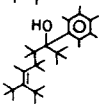
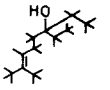
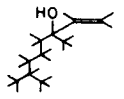
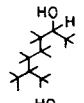
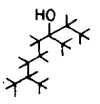
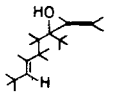
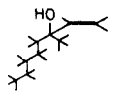
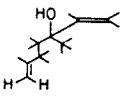
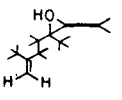
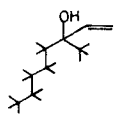
| No. | Structure | Rel. absorption maxima at 392 nm linalool = 100 | $K_D$ ($\mu$ mole) | Relative turnover (linalool = 100) | $AG_{ASSOC}$ $K_{CAL/MOLE}$ |
|---|---|---|---|---|---|
| 20 |  | 98.5 | 29 | 3.4 | −6.18 |
| 21 |  | 112 | 31.6 | 4.5 | −6.14 |
| 22 |  | 103.7 | 11.1 | 87.7 | −6.75 |
| | Probes into domains 6, 8 and 9 | | | | |
| 23 |  | 71.0 | 56.0 | 82 | −5.80 |
| 24 |  | 36.0 | 235.0 | 26 | −4.95 |
| 25 |  | 70.4 | 80.0 | 68 | −5.59 |
| 26 |  | 80.3 | 58.0 | 75.6 | −5.78 |
| 27 |  | 80.1 | 60.0 | 69.4 | −5.76 |
| 28 |  | 48.5 | 156.0 | 64.7 | −5.19 |
| 29 |  | 7.8 | 380.0 | 6.4 | −4.66 |
| 30 |  | 20.3 | 85.0 | 33.9 | −5.55 |
| 31 |  | 8.5 | 775 | 3.7 | −4.24 |

**Table 1.** (*Continued*)

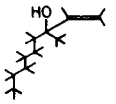
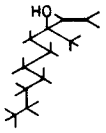
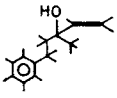
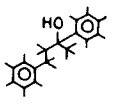
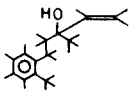
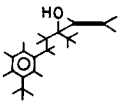
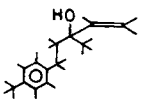
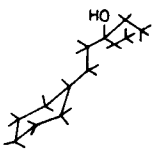
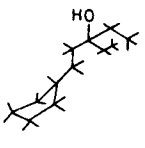
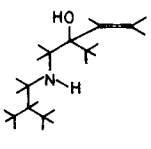
| No. | Structure | Rel. absorption maxima at 392 nm linalool = 100 | $K_D$ ($\mu$ mole) | Relative turnover (linalool = 100) | $AG_{ASSOC}$ $K_{CAL/MOLE}$ |
|---|---|---|---|---|---|
| 32 |  | 47.2 | 150 | 45 | -5.22 |
| 33 |  | 12.0 | 95 | 7.8 | -5.49 |
| 34 |  | 84.0 | 1.4 | 145.3 | -7.98 |
| 35 |  | 35.2 | 0.64 | 12 | -8.45 |
| 36 |  | 29.6 | 1.3 | 76 | -8.03 |
| 37 |  | 48.0 | 0.9 | 53 | -8.25 |
| 38 |  | 39.7 | 18 | 11.1 | -6.47 |
| 39 |  | 47.8 | 6.54 | 2.1 | -7.07 |
| 40 |  | 61.6 | 4.62 | 20.3 | -7.28 |
| Probes into domains 5 and 6 | | | | | |
| 41 |  | 1.9 | 7.5 | 0 | -6.99 |

**Table 1.** (*Continued*)

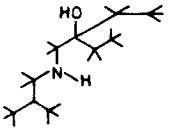
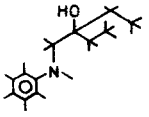
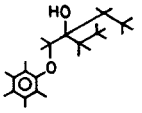
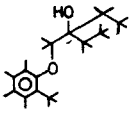
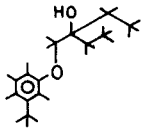
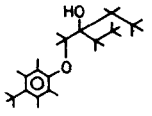
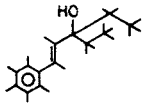
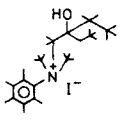
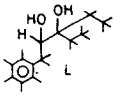
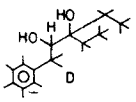
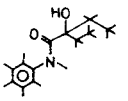
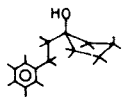
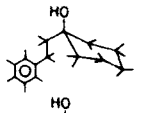
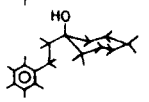
| No. | Structure | Rel. absorption maxima at 392 nm linalool = 100 | $K_D$ ($\mu$ mole) | Relative turnover (linalool = 100) | $AG_{ASSOC}$ $K_{CAL/MOLE}$ |
|---|---|---|---|---|---|
| 42 |  | 0 | 0 | 0 | — |
| 43 |  | 17.0 | 100 | 25.5 | −5.46 |
| 44 |  | 91.0 | 30.0 | 128 | −6.17 |
| 45 |  | 100 | 5.7 | 59 | −7.15 |
| 46 |  | 42.7 | 88 | 30 | −5.53 |
| 47 |  | 48 | 66 | 13.1 | −5.70 |
| 48 |  | 93.7 | 14.03 | 103 | −6.62 |
| 49 |  | 18.9 | 3.5 | 0 | −7.49 |
| 50 |  | 31.9 | 31.0 | 53 | −6.15 |
| 51 |  | 10.3 | 30.0 | 9.3 | −6.77 |

**Table 1.** (*Continued*)

| No. | Structure | Rel. absorption maxima at 392 nm linalool = 100 | $K_D$ ($\mu$ mole) | Relative turnover (linalool = 100) | $AG_{ASSOC}$ $K_{CAL/MOLE}$ |
|---|---|---|---|---|---|
| 52 |  | 1.6 | 16 | 1.1 | -6.54 |
| 53 |  | 42.7 | 218 | 0 | -4.99 |
| Probes into orientation of hydroxyl | | | | | |
| 54 |  | 61 | 3.3 | 100 | -7.48 |
| 55 |  | 44.7 | 5.8 | 35 | -7.14 |
| 56 |  | 49.7 | 12.3 | 8.4 | -6.70 |

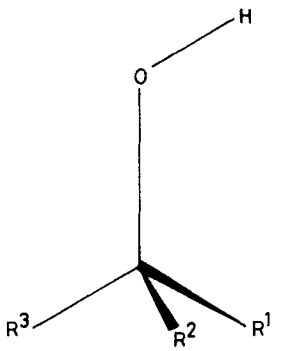



**Figure 2.**

stock concentration. The optical measurements were carried out in quartz cuvettes of 1 cm path length while a plastic spatula was employed to mix the P-450 and the substrate analogue efficiently. In a typical binding assay, approximately 4 nmol of oxidized substrate-free form of linalool P-450 were adjusted to 1 ml in substrate binding buffer and the absolute absorbance of the protein recorded at 392 nm and designated $A_0$. The solution in the cuvette was then titrated with an increasing amount

of substrate and the absorbance recorded at 392 nm for each addition of substrate and designated $A_i$. The final absorbance of linalool P-450 (at infinite concentration) when maximum absorbance at 392 nm was observed was designated $A_\alpha$.

The $K_D$ value of linalool P-450 for substrates was then computed from the formula:

$$K_D = \frac{A_\alpha - A_i}{A_i - A_0} \times \text{concentration of the free substrate.}$$

Most of the analogues were synthesized by a combination of the Hauser process for methyl ketones (Boatman and Hauser 1967) and Grignard reactions (figure 3). The synthesis of substrates and the identification of the reaction products will be published elsewhere.

*Methyl ketones*

$$R-X + CH_3COCH_2COCH_3 \xrightarrow[CH_3CH_2OH]{K_2CO_3} R-CH_2COCH_3$$

*Carbinols*

$$RCH_2COCH_3 + R^1MgX \rightarrow RCH_2\overset{\displaystyle OH}{\overset{|}{\underset{\displaystyle R^1}{\underset{|}{C}}}}CH_3$$

**Figure 3.** General schemes for synthesis.

*Discussion*

Linalool has ten carbons and a hydroxyl group. The two quarternary carbons 3 and 7 are too deeply embedded in the molecule to permit any contact with the inner surface of the enzyme. The methyl groups at positions 8, 9 and 10, the methylene groups at positions 1, 4 and 5, and the methine groups at position 2 and 6 are exposed enough to bind to vicinal domains (figure 4).

Figure 4 depicts an idealized representation of the approximate ground state conformation of the linalool molecule and the postulated domains on the inner surface of the enzyme to make specific contacts with different parts of the linalool molecule. In the conformation shown, the co-planarity of the C–O bond with the $R^3$ carbon skeleton, $C_3C_4C_5C_6C_7C_8$ and $C_9$ has been presumed. However, the efficiency of models in which the C–O has to lie in the same plane with the $R^3$ carbon skeleton (compounds 54, 55, 56) as substrates substantiate this assumption.

The principle used in this investigation is that any loss, substitution or conformational alteration of carbons at different positions of linalool would alter the binding parameters or the rates or both giving information about the existence of the specific domains on the enzyme for that particular moiety.

There are a few limitations in this approach to make a quantitative assessment of the energies of contact of different parts of the linalool molecule with their respective domains on the enzyme. The $K_D$ values have been determined from the absorption at 392 nm, a characteristic of all P-450 systems in conversion of $m^o$ to $m^{os}$ form depicting a transition of heme-iron from a low spin to a high spin state. A filling of space in the

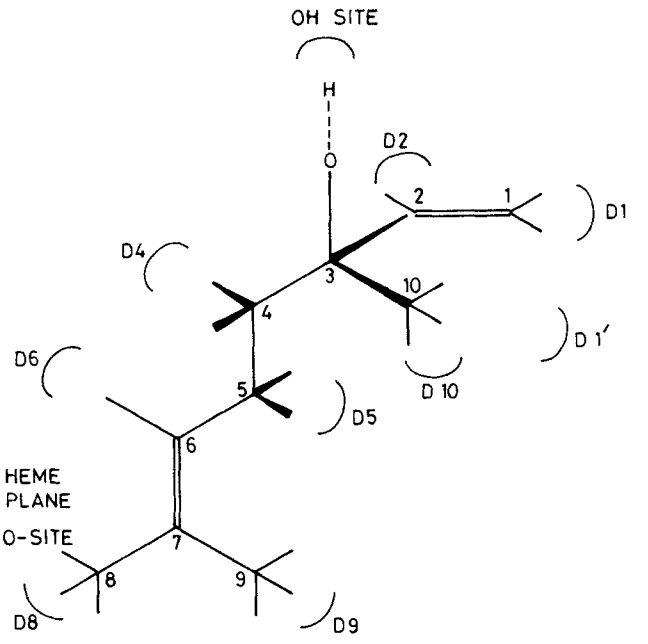



**Figure 4.** Alignment of linalool in the active site of linalool monoxygenase.

substrate cleft causes the iron to be displaced from the porphyrin plane. The $K_D$values represent the amount of substrate that brings about 50% of the maximum $m^{os}$ absorption obtained for the compound under test. It is obvious from table 1 that there is a wide range of variation in the 392 nm absorption maxima in the model compounds employed as compared to that of linalool (1.6% to 114%). There may be some doubt whether the $K_D$ values represent a true picture of equilibria for compounds with low conversion efficiencies.

The other hindrance in the quantitation of contact energies is due to cooperativity of the binding process of the substrate with the enzyme. As an illustration of this, the $\Delta G$ association of linalool with the enzyme with a $K_D$ of 3·5 $\mu$M works out to be − 7·44 kcal/mol. The usual energies expected for a hydrogen bond is of the order of − 4·0–4·5 kcal/mol. The contact of methyl group is about 0·3 to 0·4 kcal/mol, that of a methylene 0·2 to 0·3 kcal/mol, and of a methine is around 0·1 kcal.

The calculated $\Delta G$ association of the entire linalool molecule with maximum contact would therefore be between − 5·7 and − 6·8 kcal/mol. The excess energy of − 0·64 to − 1·74 kcal/mol must be due to a tighter hydrogen bonding and perhaps tighter contact of the $CH_2$ groups with their respective domains. As a further illustration of the cooperativity, the loss of methylene group at position 1 in linalool (table 1, compound 10) results in a rise in $K_D$ from 3·5 $\mu$M to 18·8 $\mu$M, an energy loss of − 1 kcal/mol which is obviously too high for hydrophobic contact for a methylene group. Compound 10 also shows a reduction (87·7%) in conversion efficiency in the 392 nm $m^{os}$ maximum indicating that the cleft space has not been efficiently filled.

The third limitation is the possibility of minor conformational changes of the substrate analogs in the enzyme-bound form. The rotational barrier of a C–C bond in a

methylene chain may not be completely overcome by the release of $\Delta G$ association but small torsional displacements are possible. The data presented show that in some cases, such displacements do occur (compounds 30 and 51).

Because of the above limitations, no attempts have been made for a linear free energy correlation, although such correlations are possible in a limited number of compounds with increasing chain length or branching (figure 2).

*Domains* 1,2 *and* 10 *and* 1′. The necessity for discussing these domains together and postulating the hypothetical domain 1 (figure 4) is due to the fact that both D and L linalool are accepted by the enzyme with almost equal proficiency and the 10-methyl group and the 1,2 vinyl group are spatially interchangeable. Unfortunately, the divinyl compound which is predicted to be a better substrate than linalool, obtained as a minor compound in the Grignard reaction proved difficult to purify. Further, no attempts have been made to resolve the synthetic analogues into their optical antipodes. The diethyl ($R' = R^2 = Et$) compound (22) which was expected to make full contact with domains 2 and 10 and partial ones with domains 1 and 1′ had a $K_D$ of 11·1 $\mu$M ($\Delta G^{Assn.}$ 0·69 kcal/mol) lower than that of linalool.

The free energy difference in association of compound 23 and compound 25 with the enzyme is of the order of −0·21 kcal/mole. This more realistically represents the possible contact energy of methylene 1 with domain 1 or 1′ of the enzyme than that observed from the data on linalool and compound 10 ($\Delta G = -1{\cdot}0$ kcal/mol). Both 23 and 25 show the same conversion efficiency to $m^{os}$ at 392 nm and show comparable enzyme rates 82 and 68 % of that of linalool respectively. It is not possible, however, to estimate realistically the energy of contact of the methyl group 10 and the methine at 2 with domains 10 and 2, as the difference in $\Delta G^{Assn.}$ between compounds 25 and 24 is −0·64 kcal/mol and that between 10 and 9 is −1·19 kcal/mol. Since the $m^{os}$ 392 nm conversion efficiencies are not comparable for these pairs, it is likely that the loss of one contact at position 2 lead to weakening of contacts at other points also including the displacement of the hydroxylation target from domain 8. The total loss of $R^1$ and $R^2$ groups decrease the $\Delta G^{Assn.}$ only nominally, a difference of −0·01 kcal/mol between compounds 9 and 8, but a big alteration in the $m^{os}$ 390 nm conversion from 76·5 % to 27·7 % and the fall in enzymatic rates 3·0 to 4·25 indicate an alteration of the binding modes of the compounds.

The data however demonstrate the existence of domains 1, 2, 10 and 1′ in the enzyme.

It was not possible to assess the contribution of the 1 and 2 vinylic double bond of linalool in the association process because of the displacement of the target methyl group from the hydroxylation site due to overcrowding at sites 1 and 1′ in the models used. A comparison of data for compounds 12, 13 and 14 would show that in their space-filling characteristics as reflected in higher $m^{os}$ 392 nm conversion all three were better than linalool. The association energies were similar, −6·92, −6·86 and −6·63 kcal/mol respectively, but the enzymatic rates were nearly a third of that of linalool for compounds 12 and 13 and a fifth for compound 14 indicating a displacement of the 8-methyl group from the oxygen site.

The three parameters chosen for study give different information about the binding process. The absorption map at 392 nm relates the space filling characteristics of substrates, the $\Delta G$ associations give the magnitude of contact for the overall structures with the enzyme and the turnover number gives the displacement of the target site from the active oxygen.

The following order was observed for the substrates studied:

For 392 nm absorption,

$R^1$ = methyl, $R^2$ = ethyl > $R^1$ = methyl, $R^2$ = phenyl > $R^1$ = methyl, $R^2$ = *n*-propyl > $R^1$ = methyl, $R^2$ = *n*-butyl > $R^1$ = methyl, $R^2$ = propenyl >

$R^1$ = methyl, $R^2$ = *i*-propyl > $R^1$ = ethyl, $R^2$ = ethyl > $R^1$ = methyl, $R^2$ = vinyl > $R^1$ = methyl, $R^2$ = *i*-butenyl > $R^1$ = methyl, $R^2$ = *i*-butyl >

$R^1$ = methyl, $R^2$ = *i*-butyl > $R^1$ = methyl, $R^2$ = H > $R^1$ = methyl, $R^2$ = methyl > $R^1$ = H, $R^2$ = H.

For G association,

$R^1$ = methyl, $R^2$ = *i*-butyl > $R^1$ = methyl, $R^2$ = vinyl > $R^1$ = methyl, $R^2$ = *n*-propyl > $R^1$ = methyl, $R^2$ = ethyl > $R^1$ = methyl, $R^2$ = propenyl >

$R^1$ = ethyl, $R^2$ = ethyl > $R^1$ = methyl, $R^2$ = allyl > $R^1$ = methyl, $R^2$ = methyl > $R^1$ = methyl, $R^2$ = *i*-propyl > $R^1$ = methyl, $R^2$ = *n*-butyl >

$R^1$ = methyl, $R^2$ = phenyl > $R^1$ = methyl, $R^2$ = *t*-butyl > $R^1$ = methyl, $R^2$ = *i*-butyl > $R^1$ = methyl, $R^2$ = H > $R^1$ = H, $R^2$ = H.

For enzymatic rates,

$R^1$ = methyl, $R^2$ = vinyl > $R^1$ = methyl, $R^2$ = ethyl > $R^1$ = ethyl, $R^2$ = ethyl > $R^1$ = methyl, $R^2$ = methyl > $R^1$ = methyl, $R^2$ = isopropyl >

$R^1$ = methyl, $R^2$ = *n*-propyl > $R^1$ = methyl, $R^2$ = propenyl > $R^1$ = methyl, $R^2$ = H > $R^1$ = methyl, $R^2$ = allyl > $R^1$ = methyl, $R^2$ = *i*-butyl >

$R^1$ = methyl, $R^2$ = *t*-butyl > $R^1$ = methyl, $R^2$ = *n*-butyl > $R^1$ = methyl, $R^2$ = *i*-butyl > $R^1$ = methyl, $R^2$ = phenyl > $R^1$ = H, $R^2$ = H >

$R^1$ = methyl
$R^2$ = butenyl

The data collected are likely to be useful in the refinement of the tertiary structure of the active site once it is established.

*Domains* 6, 8 *and* 9. Saturation of the 6,7 double bond of linalool changes the conformation of the isobutylenic side chain of linalool in three ways (figure 5): (1) lengthening of the 6–7 bond; (2) the orientation of C–H at position 6 out of plane and (3) the displacement of the 9-methyl group out of the skeletal plane (figure 5). The 6, 7 and 8 carbons would still lie in the same plane as the carbon skeleton of $R_3$ of linalool in spite of slight displacement of the 8-methyl from the active oxygen site, compounds 23, 25 and 26 are still good substrates (82 %, 68 % and 75·6 %). The loss in association free energies due to the saturation of 5,6 bond can be assessed from 4 pairs of compounds 1 and 23 (−1·64 kcal/mol) 9 and 24 (−0·31 kcal/mol), and 10 and 25 (−0·86 kcal/mol) and 11 and 26 (−1·1 kcal/mol). The $m^{os}$ 392 nm absorptions unfortunately, show variations. The average value of the contact energy difference is of the order of −0·98 kcal/mol. Apart from the three changes mentioned, this value also encompasses the displacement of the other parts of the molecule as well as the weakening of the hydrogen bond.

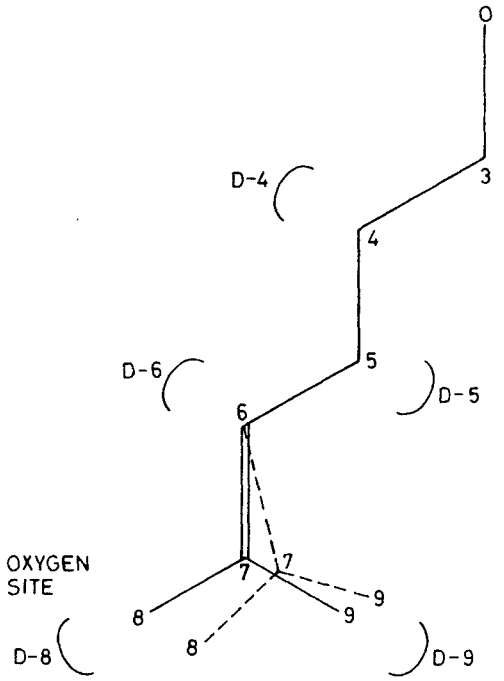



**Figure 5.** Relative orientations of the sidechains of linalool (—) and 6,7 dihydrolinalool (– – –) in domains 4, 5, 6, 8 and 9 of the enzyme.

The difference in $\Delta G^{Assn.}$ between linalool (1) and 9-nor linalool (27) (−1·68 kcal/mol) is obviously too high for the contact of 1 methyl group with domain 9. The 20% loss in $m^{os}$ 392 nm conversion and fall of 30% in turnover number indicate that other displacements are involved. In the 6,7 saturated compounds the difference of $\Delta G^{Assn.}$ of compounds 23 and 28 (−0·61 kcal/mol) also gives an indication of the overall weakening of binding of the entire molecule (in compound 24, the 9-methyl group would be away from domain 9) and the loss of this contact would not be reflected in the figure of −0·61 kcal/mol.

The reduction of the 9-nor linalool (27) causes the net loss of contact energy by −0·57 kcal/mol. Since the efficiencies as substrate of both compounds are comparable, the terminal methyl groups approach near the active oxygen site. The energy difference apparently reflects the contact of the CH group with domain 6 and the overall relaxation of the contacts at other loci.

The loss of both the methyl groups of linalool (compound 29) causes an overall reduction of association energy by −2·78 kcal/mol. From the 9-nor compound (27) to (29) there is a loss of −1·1 kcal/mol of association energy. The 392 nm absorption of compound 29 is only 7·8% of linalool. It is somewhat surprising that compound 29 and the saturated compound 31 are still accepted as substrates although feebly (6·4% and 3·7%).

The higher association energy of −0·89 kcal/mol—the higher 392 nm absorption (20%) and the good enzymatic rate (34%) observed with compound 30 after introduction of a methyl group at position 6 in compound 29 present an enigma; the

molecule 30 has to undergo some conformational distortion in the enzyme bound form to present a terminus, possibly the methylene end near the oxygenation site.

The difference in $\Delta G$ association of compound 31 and 29 ($-0{\cdot}42$ kcal/mol) again includes the contribution of the methine CH at position 6 with domain 6. It may be noted that 392 nm absorption of compounds 29 and 31 is comparable. The data also indicate but do not prove that there may be some degree of interaction with the 5,6 double bond with some vicinal $\pi$-electron systems on the enzyme. It is difficult to explain the high losses in association energies through reduction of the 5,6 double bond from only a steric consideration.

Lengthening of the side chain in compound 28 by one more methylene (compound 32) reduced only the enzymatic rates (65 to 45) without affecting the other parameters. Increase of one more methylene as in compound 33 adversely affected both the rate and the 392 nm absorption.

The most striking results are obtained when an aromatic ring is substituted for the isobutylenic side chain in linalool. It is obvious that some of the aromatic compounds are better substrates for the enzyme than linalool itself as far as the binding energies and enzymatic rates are concerned (table 1).

An alignment of the aromatic analogues with the linalool molecule (figure 6) would show that 3 consecutive positions in the aromatic ring 1',2' and 3' occupy approximately the same positions as carbon atoms 6,7 and 8 of linalool with 3 aromatic carbons, 4',5' and 6' protruding outside, possibly underneath the porphyrin ring system. The excess of $\Delta G$ association (compound 34-compound 1) is $-0{\cdot}54$ kcal/mol. It is difficult to visualize contact points for the 3 aromatic methine groups at the heme site. It is more probable that the excess energy represents some stacking interactions with the porphyrin system or some vicinal aromatic residues on the enzyme. The $\Delta G^{Assn.}$ of

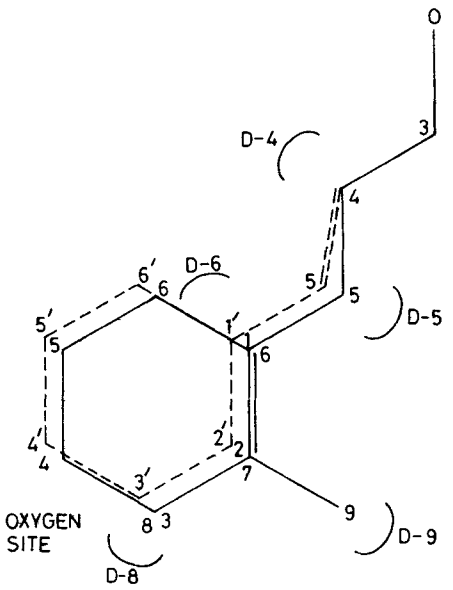



**Figure 6.** Relative orientations of the sidechains of linalool (═), 5-phenyl (—) and $\Delta^4$ 5-phenyl analogs (– – –) in domains 4, 5, 6, 8 and 9 on the enzyme.

compounds 35 and 21 differ by −0·59 kcal/mol. Neither of these compounds is a good substrate indicating the displacement of the 5-phenyl group in compound 35 far into the porphyrin zone resulting in a higher stacking interaction and displacement from the active site.

In the conformation represented in figure 6 coplanarity of the phenyl ring with the 3-carbon skeleton has been presumed although the rotational barrier of the C–Ph bond is far lower than that in a C–C bond in the polymethylene chain. A confirmation of the co-planarity of the main skeletal chain of the substrate can be obtained from the data on compound 48 which is as good a substrate as linalool (103 %) although the phenyl ring is somewhat displaced from the domain 8. Compound 48 has to be planar because of extended conjugation.

It will be evident from figure 6 that in the orientation shown, there should be no contact between compounds 35 and 48 with domain 9. Introduction of a methyl group in the ortho position should make a contact feasible at this location. The *o*-methyl compound 36 has about −0·69 kcal/mol higher $\Delta G^{Assn.}$ over compound 35. However, the loss in the 392 nm absorption (from 67·0 to 24·5 %) and the fall in the enzymatic rates (from 145·0 to 76) indicate that in the process of establishing contact with the aromatic C-methyl with domain 9, the phenyl ring is displaced from the active oxygen site with some sacrifice in the stacking interactions. The existence of domain 9 is also confirmed by the higher $\Delta G^{Assn.}$ of compound 45 containing an *o*-Me group over compounds 44, 46 and 47. Slight enzymatic activity is observed with the amide 52. It is curious to observe that out of all compounds binding with the enzyme, compound 52 shows the poorest $m^{os}$ 392 nm absorption (1·6 %).

Compounds 39 and 40 with cyclohexyl and cyclopentyl rings in the *iso*butylenic region bound to the enzyme with the cyclopentyl compound exhibiting the best substrate characteristics (61·6 % 392 nm absorption; $\Delta G^{Assn.}$ −7·28 kcal/mol and 20·3 % turnover). The binding of the cyclohexyl analogue 39 was good but the turnover was poor.

*Domains* 4 *and* 5. The existence of the domains was indicated by the data on compound 48 which has a much reduced $\Delta G$ association as compared to compound 39. Figures 6 and 7 would indicate a loss of contact of methylene groups at positions 4 and 5 with corresponding domains on the enzyme by the introduction of the 4,5-double bond. Unfortunately the phenyl ring is also displaced in compound 48 from the domains 6 and 8. Introduction of an NH group at position 5 (figure 7) is not expected to change the overall conformation of the substrate. However, all compounds with nitrogen at domains 5 and 6 showed unexpected behaviour possibly due to some polar interactions with some vicinal charged group on the enzyme. Of all the analogues employed, compound 43 showed activity (25·5 %). The quaternary dimethyl ammonium derivative of 43 (49) showed stronger binding but no activity. The amide 52 had also a trace of activity but the aliphatic N-*iso*butyl compound (42) was totally inactive. Substitution of the methylene at position 5 with oxygen eliminated the association with domain 5 but also caused displacement of the oxygenation site due to changes in bond angles. The $\Delta G^{Assn.}$ difference between compounds 34 and 44 (−2·18 kcal/mol) is far in excess of what one expects from the contact of a single methylene group ignoring the difference of substitution at positions $R^1$ and $R^2$. All the phenolic ethers (44–97) were however fair to extremely good substrates for the enzyme.

The existence of the domain 4 was established from the data on the diols 54 and 55.

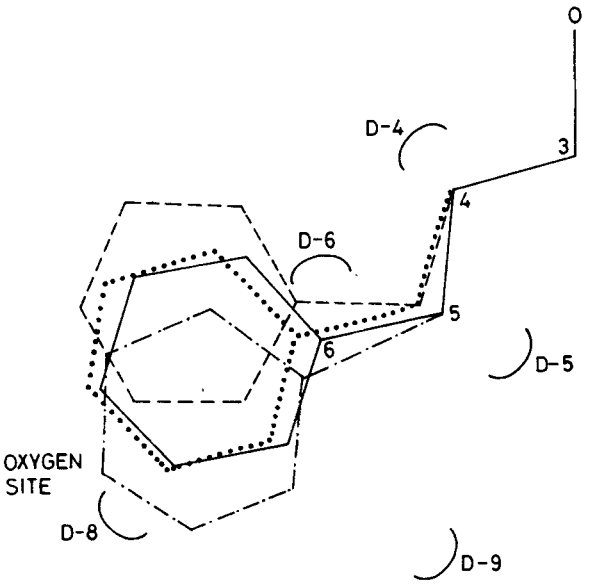



**Figure 7.** Relative orientations of the $R^3$ side chains $-CH_2CH_2C_6H_5$ (—), $-\overset{O}{\overset{\|}{C}}CH_2C_6H_5$ (– – –), $-\overset{O}{\overset{\|}{C}}NHC_6H_5$ (– – –), $-CH_2OC_6H_5$ (– –), and $-\overset{O}{\overset{\|}{C}}OC_6H_5$ (· · ·), in domains 4, 5, 6, 8 and 9 of the enzyme.

Because of the vicinal 3-hydroxyl group the preparation of stable substrates with heteroatoms at position 4 was not possible. The secondary alcohols 50 and 51 could be obtained in optically active forms from L and D phenylalanine. They had almost identical $\Delta G^{Assn.}$ but showed differences in 392 nm absorption (31·9% and 10·5%) and enzymatic activity (53% and 9·3%). The data definitely indicate that the D compound suffers a substantial conformation distortion in the enzyme bound form.

Data on compound 53 with a hydroxymethylene substitution in compound 8 did not add any new information about domain 4. However, the $\Delta G^{Assn.}$ of compound 53 was −0·26 lower than that of compound 8.

*The orientation of the hydroxyl group and the nature of the OH binding site of the enzyme.* The excellent substrate characteristics of compound 54 with regard to $\Delta G^{Assn.}$ (−7·48 kcal/mol) compared to 34 and the excellent turnover (100%) definitely establish the co-planarity of the hydroxyl group with the carbon skeleton of linalool and the aromatic substrate. The cyclohexane analogue of 54 (55) also shows efficient binding and reasonable turnover inspite of steric crowding at domains 1, 2, 10 and 1′.

While the sequencing of the amino acids on the linalool-8-hydroxylase and the identification of products from some of the analogues are in progress, the acceptability of a large number of synthetic substrate models by the enzyme open up a broad vista in probing the structure of the active site. The knowledge of the domains permits designing of photolabile affinity groups for different domains with a certain degree of confidence particularly for domains 1 (1′), 4, 5 and 9.

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