Introduction

Biochemical textbooks define the mechanism of action of enzymes by the unifying view that enzymes accelerate chemical reactions by lowering the activation energy, i.e. the difference between the energy of the reacting species and the energy of the transition state. This lowering of the activation energy is supposed to be achieved through stabilization of the transition state, providing the explanation for the rate enhancement observed in enzymes compared with the corresponding reactions in solution [1-6]. Stabilization of the transition state as the actual mechanism underlying the enzyme-based rate enhancement is relevant, for example, in the case of serine proteases, where firm binding of the transition state lowers the energy barrier for the reaction [1,7]. However, in theory, it must be also feasible to lower the activation barrier of a reaction not by lowering the energy of the transition state but, instead, by increasing the energy contents, i.e. the reactivity, of the reactants. The present paper provides results that support this last concept for, in particular, aromatic hydroxyations in which the C(4a)-hydroperoxyflavin intermediate is involved.

Enzyme activities

Maximal conversion rates, $k_{\text{cat}}$, were taken from the literature. For phenol hydroxylase, data were recently reported by us for the enzyme purified from Trichosporon cutaneum [8]; for 4-hydroxybenzoate-3-hydroxylase, the $k_{\text{cat}}$ values were previously reported by Husain et al. [9].

Electronic calculations for the model substrates

For the fluorinated 4-hydroxybenzoates, electronic characteristics were calculated using the semiempirical PM3 Hamiltonian. Only the dianionic forms with a deprotonated carboxylic as well as deprotonated hydroxyl moiety were taken into account [10,11]. The mono-oxygenation is well accepted to occur through electrophilic attack by the C(4a)-hydroperoxyflavin cofactor on the reactive \( \pi \)-electrons of the substrates. The reactivity of these \( \pi \)-electrons to electrophilic attack increases when the energy of the corresponding orbital increases, i.e. becomes less negative [8,10]. Figure 1(a) presents a plot of $\ln k_{\text{cat}}$ for conversion of a series of fluorinated 4-hydroxybenzoates by 4-hydroxybenzoate-3-hydroxylase against the calculated energy of their reactive \( \pi \)-electrons. The correlation coefficient is \( r = 0.99 \).

The calculated chemical reactivity, i.e. the energy of the reactive \( \pi \) electrons, of a series of fluorinated phenolates (with a deprotonated hydroxyl moiety) varies from $-2.70$ (phenolate) to $-4.44$ eV (3-fluoro-4-nitrophenolate) [8]. These values suggest a much lower reactivity of the phenolates than of the dianionic 4-hydroxybenzoates towards the electrophilic C(4a)-hydroperoxyflavin. Extrapolation of the 4-hydroxybenzoate-3-hydroxylase QSAR (quantitative structure-activity relationship) of Figure 1(a) to the energy values of the reactive \( \pi \)-electrons of the phenolates predicts activities for phenol hydroxylase that would be several orders of magnitude lower than those for 4-hydroxybenzoate-3-hydroxylase. Nevertheless, in the presence of phenol hydroxylase, which uses also the C(4a)-hydroperoxyflavin cofactor, these phenolates are well converted with $k_{\text{cat}}$ values of the same order of magnitude as those reported for the conversion of the 4-hydroxybenzoates by 4-hydroxybenzoate hydroxylase [8,9].

Several working hypotheses can be put for-
Figure 1

Plot of ln $k_{cat}$ (in min$^{-1}$) for the conversion of a series of 4-hydroxybenzoates by 4-hydroxybenzoate-3-hydroxylase from *Pseudomonas fluorescens*

(a) The calculated energy ($E$) of the reactive \( \pi \) electrons of the substrates and (b) the relative heats of formation ($\Delta\Delta$HF) calculated for the reaction pathway proceeding through a cyclohexadienone intermediate as depicted in Figure 2.

Figure 2

Schematic presentation of the molecular structures of the molecules taken into consideration.

ward providing insight into the actual mechanisms that may underlie this seemingly strange chemical behaviour. Clearly, the two enzyme-substrate complexes must differ in such a way as to explain the differences observed, phenol hydroxylase being able to convert relatively less reactive substrates. For example, the actual distance between the cofactor and the reactive centre in the substrate might be smaller for phenol hydroxylase than for 4-hydroxybenzoate-3-hydroxylase. Furthermore, a different local dielectric constant in the active site of the two enzymes may contribute to the different reactivities in the two enzymes. The polarity of the surroundings could influence the status of the hydrogen bonds, with a more polar environment favouring complete proton transfer and disfavouring partial deprotonation owing to increased possibilities for solvation of localized charges in a polar environment [1-5]. One could propose either that the extent of protonation of the substrate varies in the two enzymes or that the extent of protonation of the C(4a)-hydroperoxylavin varies. The actual protonation state of both peroxyflavin and substrate will influence catalysis, because reactivity is increased when the hydroxyl moiety of the substrate is deprotonated and the peroxide moiety in the C(4a)-hydroperoxylavin is protonated [10-12]. A similar reactivity between the interacting electrophilic orbital of the C(4a)-hydroperoxylavin and the nucleophilic orbital of the reactive \( \pi \)-electrons of the substrate can be achieved for phenol hydroxylase and 4-hydroxybenzoate-3-hydroxylase when in the former deprotonation of the substrate and/or protonation of the C(4a)-hydroperoxylavin is more pronounced.

The actual way in which phenol hydroxylase and 4-hydroxybenzoate-3-hydroxylase accelerate the chemical reaction compared with the reaction in solution may result from activating the substrate as well as the cofactor through changing their protonation state in a way that could never be achieved in solution at one pH, but which activates the two reactants for the aromatic hydroxylation. This would imply that, for this type of reaction, the enzyme does not act merely by stabilization of the transition state but rather by increasing the reactivity of the interacting species. This observation was further confirmed by additional calculations using 4-hydroxybenzoate-3-hydroxylase as the model system.

**Calculated relative heats of formation**

Using 4-hydroxybenzoate-3-hydroxylase as the model enzyme, relative heats of formation ($\Delta\Delta$HF) for the conversion of the dianionic hydroxybenzoate derivatives to their supposed hydroxycyclohexadienone-type reaction intermediate was calculated. Figure 2 schematically presents the molecular structures of the molecules taken into consideration. The hydroxycyclo-
Proposed reaction pathway intermediates for the conversion of 4-hydroxybenzoate and its fluorinated analogues

Note that for hydroxylation at a fluorinated C-3 position defluorination proceeds through initial formation of a quinone-like intermediate that is chemically reduced to give the hydroxylated product. This pathway was proven to occur in previous studies [9]. Under physiological conditions the rate-limiting step is assumed to be in the first reaction steps of this scheme leading from the dianionic substrate to the cyclohexadienone intermediate and is probably the actual attack of the C(4a)-hydroperoxyflavin on the aromatic π-electrons [10].

The results of Table 1 also indicate that the increase in ΔΔHF with increasing number of fluorine substituents originates from a stabilizing effect of the fluorine substituents on the dianionic reactive form of the substrate that is larger than the stabilizing effect of the fluorine substituents on the supposed hydroxycyclohexadienone reaction intermediate. Therefore, these results support the view that for this aromatic hydroxylation by 4-hydroxybenzoate-3-hydroxylase the activation energy of the reaction decreases going from tetrafluoro-4-hydroxybenzoate to 4-hydroxybenzoate not because of an increased relative stabilization of the transition state, but because of a decreased relative stabilization, i.e. a relative activation, of the dianionic reactive form of the substrate. This observation supports the concept that lowering of the activation barrier can be achieved not only by stabilization of the transition state, but by destabilization, and thus activation, of the reacting substrate.

Summary

Flavin-dependent proteins can be involved in a wide variety of biochemical reactions. Among these reactions are electron transfer, oxidase activity, dehydrogenase activity and mono- and dioxygenations. Furthermore, the oxygenation
Table I
Relative heats of formation (ΔHf) for the various 4-hydroxybenzoate derivatives and for their supposed hydroxycyclohexadienone reaction intermediates, and calculated heats of formation for the formation of the reaction intermediate (ΔΔHf = ΔHfhydroxycyclohexadienone − ΔHf4-hydroxybenzoate).

\[ k_{cat} \] values were taken from the literature [8]. All values are presented on a relative scale, the lowest values of the series set at 0 kcal/mol. Hydroxylation is at C-3.

<table>
<thead>
<tr>
<th>4-hydroxybenzoate</th>
<th>( \Delta H_{f, 4-hydroxybenzoate} ) (kcal/mol)</th>
<th>( \Delta H_{f, hydroxycyclohexadienone} ) (kcal/mol)</th>
<th>( \Delta \Delta H_f ) (kcal/mol)</th>
<th>( k_{cat} ) (min⁻¹)</th>
</tr>
</thead>
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<td>0</td>
<td>0</td>
<td>3300</td>
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<td>-45.3</td>
<td>6.0</td>
<td>2100</td>
</tr>
<tr>
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<td>-44.3</td>
<td>4.1</td>
<td>1050</td>
</tr>
<tr>
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<td>-95.0</td>
<td>6.8</td>
<td>550</td>
</tr>
<tr>
<td>Tetrafluoro-</td>
<td>-196.6</td>
<td>-180.2</td>
<td>16.4</td>
<td>84</td>
</tr>
</tbody>
</table>

Steps may proceed by either electrophilic or nucleophilic reaction mechanisms. The present paper focuses on aromatic hydroxylation by a nucleophilic C(4a)-hydroperoxyflavin cofactor. The reactivity of this intermediate is highly dependent on its actual protonation state as well as on the (de)protonation of the substrate to be converted. Electrophilic attacks by the peroxylavin intermediate on the substrate are favoured by a protonated C(4a)-hydroperoxyflavin and a (partially) deprotonated hydroxyl moiety in an aromatic substrate. Thus, the actual mechanism of enzyme catalysis is basically that the enzyme provides possibilities for the coexistence of (de)protonation states of cofactor and substrate that would normally not be possible in one solution at one pH value.

Another concept presented in this paper, originating from results obtained in molecular orbital studies on flavin-dependent mono-oxygenation reaction by phenol hydroxylase and 4-hydroxybenzoate-3-hydroxylase, questions the actual unifying concept found in some biochemical textbooks that proposes that enzymes accelerate chemical reactions by stabilization of the transition state only. The results of the present study demonstrate that increasing the reactivity of the substrate and/or cofactor may be another important mechanism to decrease the activation barrier of a chemical reaction.

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