Inhibition of 4-hydroxyphenylpyruvate dioxygenase by 2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione*  

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Received: 27 April, 2009; revised: 06 June, 2009; accepted: 14 July, 2009  
available on-line: 21 July, 2009

Triketone herbicides are inhibitors of 4-hydroxyphenylpyruvate dioxygenase (HPPD), a key enzyme of the tyrosine transformation pathway, common for plants and animals. One of these herbicides, 2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione (NTBC), is so selective and efficient that it can be applied as a medicine in a hereditary metabolic disease — tyrosinemia type I. In this paper the available information concerning the molecular mechanism of HPPD inhibition by NTBC, originating from experimental investigations as well as theoretical modeling, has been collected. It is supplemented by results of additional theoretical DFT and/or MP2 calculations of the energetic effects of individual elementary molecular transformations. All these data are discussed and a consistent picture of HPPD inhibition by NTBC is proposed.

Keywords: HPPD, NTBC, tyrosinemia type I

INTRODUCTION

The family of α-ketoacid-dependent non-heme iron(II) dioxygenases is a broad class of enzymes indispensable in various biotransformations essential for living organisms (Prescott & Lloyd, 2000; Hausinger, 2004). A member of this class, 4-hydroxyphenylpyruvate dioxygenase (HPPD), catalyzes the conversion of 4-hydroxyphenylpyruvate (HPP) to homogentisate (Fig. 1). This transformation is the second step of tyrosine catabolism, common for plants and animals (Lee, 1997; Lock, 1998). In plants homogentisate is a substrate necessary for the production of tocopherols and plastoquinones. It was discovered that in nature some plants use the specific inhibition of HPPD to compete with other species. A number of myrtaceous plants and lichens produce natural triketone herbicides, which causes bleeding of current plants and prevents their growth (Lee, 1997). These observations have become the starting point for inventing effective synthetic herbicides which are presently widely used for protecting various crops. One of the triketone herbicides, 2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione (NTBC, Fig. 2), which has appeared to be specific and extremely efficient, has found application as a life-saving medicine against a rare but fatal hereditary disease, tyrosinemia type I (Lock, 1998; Grompe, 2001; Russo, 2001). The underlying cause of this disease is a genetic error leading to the malfunction or deficit of fumarylacetoacetate hydrolase, another enzyme catalyzing one of the last steps on the tyrosine catabolism pathway (Fig. 1). As a result of this defect, the affected organism produces a pathognomonic metabolite, succinylacetone, which is highly toxic and causes stepwise degradation of liver, kidneys
and nervous system. The therapeutic action of NTBC relies upon blocking the tyrosine catabolism path at an earlier step and preventing formation of succinylacetone. A side effect of the permanent NTBC application can be accumulation of tyrosine in the organism, which is less dangerous and can be controlled by observing an appropriate diet (Tanguay et al., 1996; Grompe, 2001; Russo et al., 2001).

In search of the optimal herbicide a huge number of triketones have been synthesized and tested. These studies have led to some observations concerning the relationship between the structure and herbicidal activity of the compound. It was discovered that replacement of the isovaleroyl group in leptospermon (Fig. 2), a natural herbicide, by a substituted benzoyl group caused the desired effect. The herbicidal activity was strengthened, especially when the aromatic ring was substituted at the ortho position by an electron-withdrawing substituent. On the other hand, a bulky substituent at the para position diminished the activity of the potential herbicide (Lee et al., 1997; 1998; Mitchell et al., 2001). In the light of these findings one may notice that NTBC fulfills all the demands for a highly effective HPPD inhibitor. The above rules are, however, purely empirical and should find a rationalization invoking arguments coming from the molecular mechanism of HPPD functioning and its inhibition.

Investigations of these biochemical processes have been undertaken in numerous studies and most of the questions seem to have already been answered (Ellis et al., 1995; 1996; Serre et al., 1999; Kavana & Moran, 2003; Brownlee et al., 2004; Borowski et al., 2004; Neidig et al., 2004; 2005; Bassan et al., 2006), though some details concerning HPPD inhibition remain unclear. In this work we report some new results of theoretical calculations concerning molecular models of the species involved in the discussed biotransformations. These results shed light on some points in the proposed transformation mechanism and enable a consistent explanation of the available data.

**COMPUTATIONAL DETAILS**

Theoretical calculations of molecular structures and their energies were performed for molecular models of the molecules participating in the investigated biotransformations. Such an approach, though dictated by computational limitations, has been proven to be useful and effective (Bassan et al., 2006; Siegbahn & Borowski, 2006). In calculations concerning the complexation of ferrous ion in the HPPD active site the enzyme was modeled using 4-methylimidazoles in place of histidine ligands and acetate instead of glutamate. Simultaneously, the natural enzyme substrate, HPP, as well as the investigated inhibitor, NTBC, were simplified by substituting their aromatic rings with a hydrogen atom and a –CN group, respectively. All calculations were performed using Gaussian 03 quantum chemistry program (Frisch et al., 2003). The molecular geometries of all the investigated species involving Fe(II) were optimized starting from the appropriately truncated X-ray structure of the HPPD–Fe(II)(–H_2O)–NTBC complex (Brownlee et al., 2004), using the DFT method, the standard Becke-Lee-Yang-Parr B3LYP hybrid functional, Los Alamos DZ pseudo potential for the Fe atom, and the 6-31G basis set for other atoms. In

![Figure 1. Tyrosine catabolism in plants and animals. HPP, 4-hydroxyphenylpyruvate; HPPD, 4-hydroxyphenylpyruvate dioxygenase; HG, homogentisate; FAH, fumaroylacetacetate hydrolase.](image1)

![Figure 2. Examples of natural and synthetic herbicides. a, Leptospermon; b, tetramethyl-NTBC; c, NTBC; d, sulcotrine.](image2)
the calculations of the molecular energy for optimal structures the basis set was increased to 6-31G(d)
for the first-row atoms and to 6-311G(2d) for the Fe atom. Keeping in mind that the calculations were
performed for the model compounds rather than the original molecules participating in the discussed biotransformation, the calculated energies were not corrected for zero-point vibrations and the effect of the bulk solvent. In our opinion the reliability of such corrections would be limited and — which is even more important — for total energy differences, being the only point of interest in this work, such corrections are practically irrelevant.

Reactions 3 and 5, i.e. transformations of the E–S and E–I complexes including an additional water
table molecule (6C species) into the appropriate water-free complexes (5C species) (Figs. 4 and 6), are
dissociation type reactions: AB → A + B. The energetic effects of these transformations were calculated taking into account the BSSE correction (counterpoise calculation) (Boys & Bernardi, 1970) and relaxation energies of the appropriate “monomers” A and B to their optimal molecular geometries. Reactions 2 and 4 are of the type: AB+C → AC+B, and their energetic effects could be calculated by subtracting the dissociation energies of complexes AB and AC. During the computation of the binding energy of HPP in the HPPD–Fe(II)(–H2O)–HPP complex the calculation of the geometry relaxation energies for monomer A originating from AB and monomer A originating from AC could actually be avoided. Instead, only the difference between the appropriate single-point energies of the common fragment A had to be taken into account.

Since the standard DFT methods have been shown to be inadequate in describing π–π stacking interaction (Ye et al., 2004), the energy of the E–I complex stabilization by this interaction was estimated using the counterpoise Møller-Plesset MP2 calculation with the cc-pVdz basis set for H
and F atoms and the aug-cc-pVdz set for other atoms. Also in this case the structures of interest had to be modeled by simpler objects. Thus, the aromatic rings of phenylalanine residues were represented by two benzene molecules and NTBC — by 2-nitro-4-trifluoromethylbenzaldehyde with the aldehyde group oriented perpendicularly to the aromatic ring (Fig. 7). The parts of the sandwich were arranged according to the situation found in the crystal (Brownlee et al., 2004). In the counterpoise calculation 2-nitro-4-trifluoromethylbenzaldehyde was treated as “monomer1” and the two benzenes as “monomer2”.

RESULTS AND DISCUSSION

Structure of the enzyme active site

4-Hydroxyphenylpyruvate dioxygenases occur in various organisms, from photosynthetic bacteria
to plants and mammals. These enzymes are active as homodimers, homotrimers or homotetramers. The appropriate monomers exhibit remarkable structural variability, however, in all hitherto characterized cases the enzyme active site remains the same (Prescott & Lloyd, 2000; Hausinger, 2004). After some initial controversies it has eventually been evidenced that the enzyme activity results from the presence of an Fe(II) rather than an Fe(III) ion in its structure (Kavan & Moran, 2003; Neidig et al., 2004; Bassan et al., 2006). In the first report on the crystal structure of HPPD it was found that the enzyme from Pseudomonas fluorescens was active as a homotrimer and that each polypeptide chain bound one ferrous ion (Serre et al., 1999). Similarly as in other α-ketoacid dependent dioxygenases the ferrous ion was liganded with two histidines and a glutamate located far from each other in the polypeptide chain (Fig. 3). In the investigated crystal the coordination sphere of the metal ion had a distorted tetrahedral symmetry with the fourth site occupied by an acetate anion, probably originating from the buffer used during crystallization. Later crystallographic investigations have shown that in HPPD separated from other organisms the metal ion is hexacoordinate (6C species): three facial coordinations are occupied by the His-His-Glu triad and the remaining ones by three water molecules, one of them being weakly bonded (Fritze et al., 2004; Yang et al., 2004). Independent experimental evidence coming from NIR, CD and MCD spectroscopy has shown that, besides the 6C centers, pentacoordinate ferrous centers (5C) are present in this enzyme, at least at liquid helium temperatures (Neidig et al., 2004). These data strongly suggest that

Figure 3. Structure of the active site of 4-hydroxyphenylpyruvate dioxygenase (HPPD).
Model of 5C complex — optimized molecular geometry for S = 2 species, and model of 6C complex — molecular geometry according to the crystal structure (Fritze et al., 2004)
also in the natural biological environment the resting enzyme active centers exist as a mixture of 5C and 6C species in a dynamic equilibrium:

\[
[[HPPD–Fe(II)–3·H_2O]^+ \rightleftharpoons [HPPD–Fe(II)–2·H_2O]^+ + H_2O]
\] (1)

The results of our theoretical calculations performed for the enzyme model are in agreement with the above findings. The molecular structures of the [HPPD–Fe(II)–2·H_2O]^+ complex, as well as of 5C type E–S and E–I complexes, were optimized assuming various multiplicities of their state functions. The total energies calculated for the species of various spin multiplicities are collected in Table 1. A comparison of these values leads to a conclusion that in the ground state the resting enzyme active site should be a quintet species (\(S = 2\)), similarly as other Fe(II) complexes involved in the enzymatic transformation of HPP into homogentisate (Bassan et al., 2004; 2006; Krebs et al., 2007). It is interesting that when the molecular structure optimization starts from the arrangement of the ligands observed in the crystal structure, the complex containing three water molecules appears to be energetically unstable — during unrestricted geometry optimization one water molecule is eventually pushed out of the iron coordination sphere. The 5C form of the enzyme active site formed this way takes part in the discussed enzymatic transformations.

**Enzyme–substrate complex**

The first step of the reaction sequence leading eventually to the conversion of HPP into homogentisate is the formation of a complex of the substrate with the enzyme. This complex, obtained in anaerobic conditions, has recently been investigated by CD and MCD spectroscopy at liquid helium temperatures (Neidig et al., 2004; 2006a; 2006b). It was proven that HPP was bidentately bonded and that the complex occurred in two 5C and 6C forms (Fig. 4). Moreover, as it was mentioned, in the ground state the 5C E–S complex formed this way takes part in the discussed enzymatic transformations.

\[
[HPPD–Fe(II)–2·H_2O]^+ + [HPP] → HPPD–Fe(II)(–H_2O)–HPP + H_2O
\] (2)

The exact calculation of the free energy changes during the above reaction would be very difficult due to the overall impact of the medium. Nevertheless, all the data show that the energy gain in this reaction has to be huge (\(\Delta E_2 = 400.0 \text{ kJ/mol}\)). On the other hand, the removal of the second water molecule from the 6C type E–S complex and its transformation into the 5C complex (Eqn. 3) demands, according to our calculations, investing some energy (\(\Delta E_3 = 36.4 \text{ kJ/mol}\)).

\[
HPPD–Fe(II)(–H_2O)–HPP \rightleftharpoons HPPD–Fe(II)–HPP + H_2O
\]

The experimental data cited above suggest that these two complexes are in dynamic equilibrium, apparently with the high prevalence of the 6C one as judged from the total energy difference. On the other hand, only the 5C species can coordinate the dioxygen molecule and irreversibly rearrange to a complex of 4-hydroxyphenylperacetate and \(\text{CO}_2\) (Fig. 5), which readily releases \(\text{CO}_2\). The remainder of the substrate is transformed through several steps into the final product and leaves the active site of the enzyme (Borowski et al., 2004; Bassan et al., 2006). Thus, it seems that our calculation overestimated the \(\Delta E_3\) energy. We suppose that this inaccuracy is largely

Table 1. Relative total energies [kJ/mol] calculated for the enzyme active site, enzyme–Inhibitor and enzyme–substrate complexes in the form of 5C species, for various spin multiplicities.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(S = 0)</th>
<th>(S = 1)</th>
<th>(S = 2)</th>
<th>(S = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[HPPD–Fe(II)–2·H_2O]^+</td>
<td>114.74</td>
<td>59.61</td>
<td>0.0</td>
<td>337.7(^a)</td>
</tr>
<tr>
<td>[HPPD–Fe(II)–HPP]</td>
<td>117.14</td>
<td>80.95</td>
<td>0.0</td>
<td>125.66</td>
</tr>
<tr>
<td>[HPPD–Fe(II)–NTBC]</td>
<td>57.16</td>
<td>71.27</td>
<td>0.0</td>
<td>125.66</td>
</tr>
</tbody>
</table>

\(^a\)During geometry optimization a “dissociation” of one of hydrogen atoms occurs.
Inhibition of HPPD by NTBC

cancelled during the calculation of the difference of the overall energetic effect of HPP and NTBC binding — the final result of this work.

Enzyme–Inhibitor complex

The structure of the HPPD complex with NTBC was studied by crystallography after preparation of the complex in a crystalline form (Brownlee et al., 2004). It has been confirmed that in the complex NTBC is bidentately bound to the ferrous ion which also has two histidines and a glutamate in its coordination sphere, while the sixth ligand, a water molecule, is relatively loosely bonded (Fig. 6). The high affinity of NTBC to HPPD was also investigated using CD/MCD spectroscopic and DFT theoretical methods (Neidig et al., 2005).

As it was mentioned, our theoretical calculations have shown that in the ground state the 5C E–I complex is a quintet species (Table 1). Furthermore, calculations involving molecular geometry optimization for a model of the appropriate 6C complex result in the same conclusions as in the case of the E–S complex: the formation of the 6C type E–I complex (Eqn. 4) is strongly exothermic ($\Delta E_4 = -396.1$ kJ/mol), while the 6C $\rightarrow$ 5C transformation (Eqn. 5) is endothermic ($\Delta E_5 = 41.5$ kJ/mol).

$$[\text{HPPD–Fe(II)}–2\cdot\text{H}_2\text{O}]^+ + [\text{NTBC}]^- \rightarrow \text{HPPD–Fe(II)} – (–\text{H}_2\text{O})–\text{NTBC} + \text{H}_2\text{O} \quad (4)$$

$$\text{HPPD–Fe(II)}(–\text{H}_2\text{O})–\text{NTBC} \rightarrow \text{HPPD–Fe(II)}–\text{NTBC} + \text{H}_2\text{O} \quad (5)$$

It is possible that the 5C enzyme–Inhibitor complex can coordinate a dioxygen molecule. One cannot exclude that this process is important for NTBC oxidation that occurs in vivo (Szczeciński et al., 2008). On the other hand, for obvious reasons, this O$_2$-involving complex cannot rearrange in the manner the appropriate S–E complex does, and thus its formation seems to be of little importance for the inhibition mechanism.

Additionally, Brownlee et al. (2004) noticed that in the E–I crystal the substituted phenyl ring of NTBC forms a sandwich with two phenyl rings of phenylalanines of the enzyme polypeptide chain (Fig. 7). They concluded that the discovered stacking interaction between the enzyme and the inhibitor, although relatively weak, could play an important role in the E–I complex stabilization. Indeed, according to our evaluation, this stabilizing effect should be 46.9 kJ/mol.

![Figure 5. HPPD-catalyzed transformation of 4-hydroxyphenylpyruvate (HPP) into homogentisate.](image1)

![Figure 6. Molecular models of 5C and 6C forms of the enzyme (4-hydroxyphenylpyruvate dioxygenase, HPPD)-inhibitor (2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione, NTBC) complexes.](image2)

The structures are based on optimized geometries of appropriate S = 2 species.

![Figure 7. Molecular model of the sandwich formed in the enzyme (HPPD)-inhibitor (NTBC) complex owing to π-stacking interaction between the arene ring of the inhibitor and rings of two phenylalanines of the enzyme polypeptide chain.](image3)
Mechanism of E–I complex formation

Thanks to the stability of the HPPD–NTBC complex it was possible to investigate the kinetics of its formation (Ellis et al., 1995; Kavana & Moran, 2003). The latter authors have shown that this deceptively simple reaction proceeds in three steps, the first two being NTBC concentration-dependent, as opposed to the third one. Moreover, it has been established that the rates of the second and the third step are not influenced by the solution acidity in the 6–8 pH range, but both exhibit a remarkable H/D isotope effect. The mechanism proposed by Kavana and Moran (2003) assumes that a pre-equilibrium binding step is followed by the bidentate association of the neutral NTBC enol molecule with the active site metal ion. Finally, in the third step, the irreversible conversion of the bound enol to the enolate ion occurs, assisted by the Fe(II) ion — a Lewis acid.

The assumption about participation of a neutral NTBC molecule, rather than its anion, in reaction 4, however, seems to be a serious weakness of the proposed mechanism. NTBC is a relatively strong acid of \( pK_a = 3.1 \) (Lee et al., 1998) and the abundance of its neutral form at physiological pH is very low (Szcześciński et al., 2006). Moreover, the enolate anion is certainly a stronger ligand than the neutral enol molecule. In our opinion, an alternative explanation of the experimental results of Kavana and Moran (2003) is that NTBC enolate anion participates in the reaction from the very beginning and that the third step involves reaction 5 leading to an equilibrium between 6C and 5C complexes and/or formation of a sandwich of the NTBC aromatic ring and the enzyme phenylalanines. Formally, both reactions 4 and 5 include removing only one water molecule each, but, actually, they are certainly accompanied by much more complicated rearrangements of the solvation sphere of the species involved. Such processes could be a reason of the observed kinetic isotope effects.

It is worthwhile to devote some attention to the problem of reversibility/irreversibility of the E–I formation. We feel that this point is crucial for understanding the inhibition mechanism considered. It was concluded on the basis of specially designed experiments that reaction 4 was practically irreversible (Kavana & Moran, 2003). On the other hand, another group of investigators (Ellis et al., 2005; 2006) claimed that NTBC as well as some other compounds (Fig. 2) were reversible tight-binding inhibitors of HPPD. The rapid inactivation of the enzyme was due to the formation of an E–I complex which dissociated extremely slowly with partial recovery of the enzyme activity. The divergence between the two above opinions concerning reversibility/irreversibility of the blocking of HPPD by NTBC may be, however, only apparent. Firstly, the investigated HPPD samples originated from different biological sources, and secondly, a very slow dissociation of the E–I complex can very well mimic the “practical irreversibility” of its formation. In this context it is perhaps noteworthy that NTBC used as medicine in thyrosinemia type I is administered permanently several times a day, as its level in plasma decreases in time (Hall et al., 2001; Pohorecka et al., 2008). On the other hand, it was shown that the effects of a single dose administration of NTBC to healthy volunteers disappeared only after several weeks (Hall et al., 2001).

Competition between HPP and NTBC

In order to explain the exceptional effectiveness of NTBC as an HPPD inhibitor, theoretical calculations for model structures of NTBC, HPP and their complexes with HPPD were performed (Neidig et al., 2005). Unexpectedly, it was found that the complexation energy for NTBC was somewhat lower than the complexation energy for HPP. We obtained a similar result for our models of both 5C and 6C type. Neidig et al. (2005) hypothesized that the energetic effect of the stacking interaction overcompensated the complexation energy difference. Indeed, as it was mentioned above, the stacking interaction stabilizes the NTBC complex by about \( \Delta E_{3,1} = 47 \text{ kJ/mol} \), while the apparent energy of the stacking interaction in the analogous sandwich of HPP should not exceed the binding energy of the benzene trimer, estimated to be \( \Delta E_{4,5} = 24 \text{ kJ/mol} \) (Tauer & Sherrell, 2005). Actually, it remains unknown whether in the case of HPP the molecular geometry allows the sandwich formation at all. Thus, the complexation energy difference \( \Delta \Delta E \) would be:

\[
\Delta \Delta E = \Delta E_4 + \Delta E_5 + \Delta E_{3,1} - \Delta E_2 - \Delta E_3 - \Delta E_{4,5}
\]

\[
= 13.9 \text{ kJ/mol}
\]  

(6)

It is to be remembered, however, that the estimated complexation energy difference is of a limited precision. First of all, this is so because the calculations are performed for model systems rather than for the real molecules participating in the biotransformations and, by necessity, the result is dependent on the selected model. Moreover, non-specific solvation phenomena also influence the complexation energies, especially in the case of charged species. In such a situation it is difficult to judge whether the stacking interaction energy is really sufficient to compensate for the apparent higher affinity of HPP to HPPD, or not. In the case of tight-binding inhibitors, however, explanation of their effectiveness does not demand the assumption that the affinity of the
inhibitor to the enzyme is higher than that of the natural substrate. The most important thing is that formation of the E–I complex is hardly reversible and that subsequent transformations of this complex are much more difficult than transformations of the E–S complex. Products of NTBC metabolism have been discovered only recently (Szczeciński et al., 2008) and it is believed that during NTBC administration as a drug against thyrosinemia type I its substantial part is excreted in urine in an unchanged form (Hall et al., 2001; Pohorecka et al., 2008).

Nevertheless, it is evident that the stacking interaction between the enzyme and the inhibitor, although relatively weak, increases the stability of the E–I complex. It can be deduced that the effectiveness of such a stabilization should increase with increasing electron-deficiency of the aromatic ring occupying the central position in the sandwich (Beg et al., 2008). On the other hand, bulky substituents may diminish the strength of this interaction. Thus, this non-bonded interaction seems to explain, at least partially, the empirical observations concerning the effectiveness of various triketones as herbicides (Lee et al., 1997; 1998).

The role of the stacking interaction, however, is not always so important for stabilization of NTBC complexes. Recently it was shown that NTBC is an effective inhibitor of hydroxymandelate synthase (Conrad & Moran, 2008). The inhibition occurs despite significant differences between the active sites of HPPD and this enzyme. These differences preclude sandwich formation with the aromatic ring of the inhibitor molecule.

**SUMMARY**

Results of our calculations as well as the above discussion point out that HPPD reacts with the enolate anion of NTBC forming a 6C species (Fig. 6). Then, an equilibrium between 6C and 5C complexes is established (Fig. 6), and in the final step of the E–I complex formation a sandwich of aromatic rings of the inhibitor and two enzyme phenyl alanines is formed (Fig. 7). NTBC fulfills both preconditions for an HPPD inhibitor: (i) it exhibits high affinity to this enzyme forming a stable E–I complex and (ii) this complex cannot be decomposed in a subsequent reaction, analogous to the reaction of HPP, the natural HPPD substrate. In view of the practical irreversibility of the E–I complex formation, the small difference of the complexation energies of the enzyme with NTBC and HPP seems to be of little importance. It also seems that the stacking interaction mentioned above is responsible for differentiating the effectiveness of various 2-benzoyl-1,3-cyclohexanedione inhibitors.

**Acknowledgements**

The authors are thankful to Professor Maria Bretner for her valuable remarks concerning presentation of the results of this work.

This work was financially supported by the State Committee for Scientific Research (grant No. 1295/T09/2005/29).

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