Acta Biochimica Polonica

Vol. 56 No. 3/2009, 447–454

on-line at: www.actabp.pl

Regular paper

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

Sergey Molchanov and Adam Gryff-Keller✉

Faculty of Chemistry, Warsaw University of Technology, Warszawa, Poland

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 bleaching of concurrent 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

✉Corresponding author: Adam Gryff-Keller, Faculty of Chemistry, Warsaw University of Technology, S. Noakowskiego 3, 00-664 Warszawa, Poland; phone: (48) 22 234 5103; e-mail: agryff@ch.pw.edu.pl

★Preliminary communication: Central European School on Physical Organic Chemistry — Energetics in Chemistry. Karpacz, Poland, June 2007.

Abbreviations: 5C and 6C, five and six coordinate iron species; BSSE, basis sets superposition error; DFT, density functional theory; E–S, enzyme–substrate (complex); E–I, enzymE–Inhibitor (complex); FAH, fumaroylacetoacetate hydrolase; HPP, 4-hydroxyphenylpyruvate; HG, homogentisate; HPPD, 4-hydroxyphenylpyruvate dioxygenase; MCD, magnetic circular dichroism; MP2, Møller-Plesset second order perturbation method; NIR, near infrared; NTBC, 2-[2-nitro-4-(trifluoro methyl)benzoyl]-1,3-cyclohexanedione.

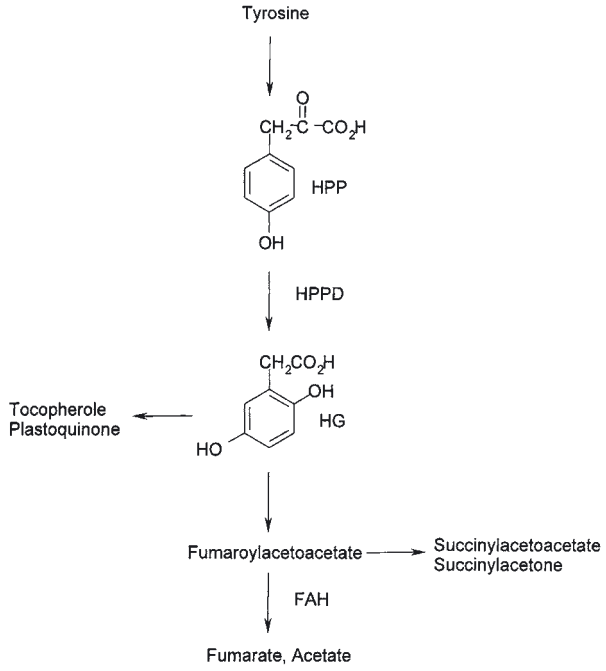

Figure 1. Tyrosine catabolism in plants and animals. HPP, 4-hydroxyphenylpyruvate; HPPD, 4-hydroxyphenylpyruvate dioxygenase; HG, homogentisate; FAH, fumaroylacetoacetate hydrolase.

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 fulfils 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

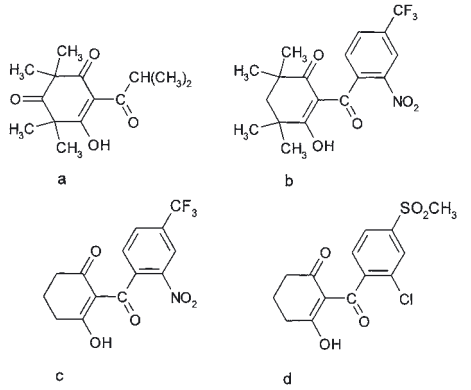

Figure 2. Examples of natural and synthetic herbicides. a, Leptospermon; b, tetramethyl-NTBC; c, NTBC; d, sulcotrione.

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

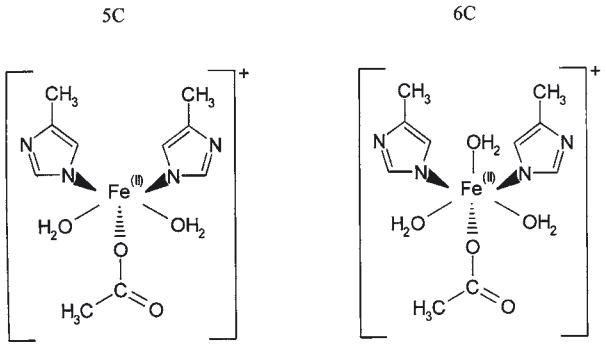

Figure 3. Structure of the active site of 4-hydroxyphenylpyruvate dioxygenase (HPPD).
Model of 5C complex — optimized molecular geometry for S = 2 species, and model of 6C complex — molecular geometry according to the crystal structure (Fritze *et al.*, 2004).

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 molecule (6C species) into the appropriate water-free complexes (5C species) (Figs. 4 and 6), are dissociation type reactions: AB $\rightarrow$ 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$\rightarrow$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)(–H_2O)–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 (Kavana & 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

also in the natural biological environment the resting enzyme active centers exist as a mixture of 5C and 6C species in a dynamic equilibrium:

$$[HPPD–Fe(II)–3{\cdot}H_2O]^+ \leftrightarrows [HPPD–Fe(II)–2{\cdot}H_2O]^+ + H_2O \quad (1)$$

The results of our theoretical calculations performed for the enzyme model are in agreement with the above findings. The molecular structures of the $[HPPD–Fe(II)–2{\cdot}H_2O]^+$ 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 is a quintet species (Table 1).

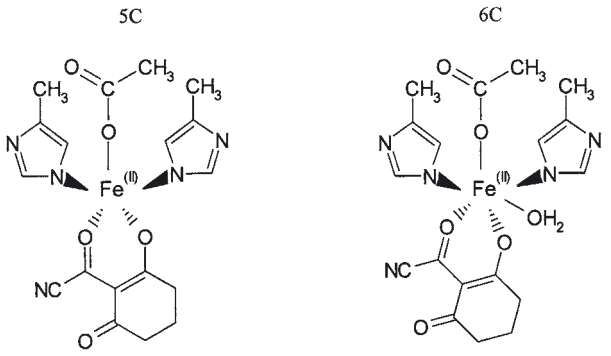

Figure 4. Molecular models of 5C and 6C forms of the enzyme (4-hydroxyphenylpyruvate dioxygenase, HPPD) — substrate (4-hydroxyphenylpyruvate, HPP) complexes. The structures are based on optimized geometries of appropriate S = 2 species.

The formation of the E–S complex is accompanied by pushing at least one water molecule out of the Fe(II) coordination sphere:

$$[HPPD–Fe(II)–2{\cdot}H_2O]^+ + [HPP]^- \rightarrow HPPD–Fe(II)(–H_2O)–HPP + H_2O \quad (2)$$

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 (ΔE_2 = 400.0 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 (ΔE_3 = 36.4 kJ/mol).

$$HPPD–Fe(II)(–H_2O)–HPP \leftrightarrows HPPD–Fe(II)–HPP + H_2O \quad (3)$$

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 CO_2 (Fig. 5), which readily releases 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 ΔE_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.

Optimized molecular geometries for S = 2 species can be found in supplementary materials.

Compound	S = 0	S = 1	S = 2	S = 3
[HPPD–Fe(II)–2·H2O]$^+$	114.74	59.61	0.0	337.7[a]
[HPPD–Fe(II)–HPP]	117.14	80.95	0.0	125.66
[HPPD–Fe(II)–NTBC]	57.16	71.27	0.0	125.66

[a]During geometry optimization a "dissociation" of one of hydrogen atoms occurs.

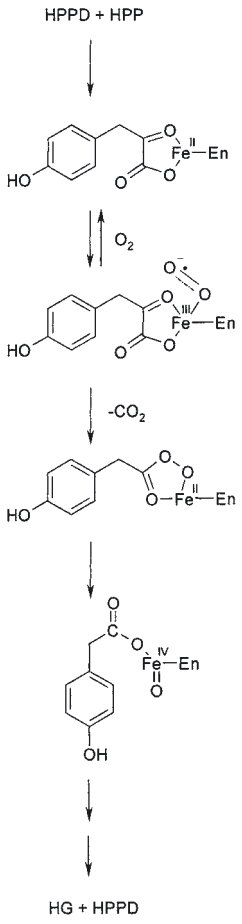

Figure 5. HPPD-catalyzed transformation of 4-hydroxyphenylpyruvate (HPP) into homogentisate.

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 com-

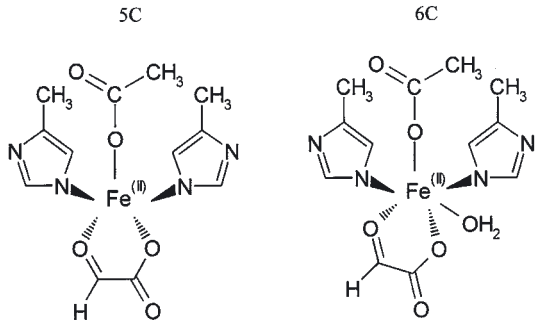

Figure 6. Molecular models of 5C and 6C forms of the enzyme (4-hydroxyphenylpyruvate dioxygenase, HPPD)-inhibitor (2-[2–nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione, NTBC) complexes.
The structures are based on optimized geometries of appropriate S = 2 species.

plex (Eqn. 4) is strongly exothermic (ΔE_4 = –396.1 kJ/mol), while the 6C → 5C transformation (Eqn. 5) is endothermic (ΔE_5 = 41.5 kJ/mol).

$$[HPPD–Fe(II)–2{\cdot}H_2O]^+ + [NTBC]^- – HPPD–Fe(II) – (–H_2O)–NTBC + H_2O \quad (4)$$

$$HPPD–Fe(II)(–H_2O)–NTBC \leftrightarrows HPPD–Fe(II)–NTBC + H_2O \quad (5)$$

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.

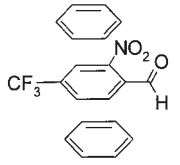

Figure 7. Molecular model of the sandwich formed in the enzyme (HPPD)-inhibitor (NTBC) complex owing to π-stacking interaction between the arene ring of the inhibitor and rings of two phenylalanines of the enzyme peptide chain.

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 (Szczeciński *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 $\Delta E_{s,I}$ = 47 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 $\Delta E_{s,B}$ = 24 kJ/mol (Tauer & Sherrill, 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:

$$\Delta\Delta E = \Delta E_4 + \Delta E_5 + \Delta E_{s,I} - \Delta E_2 - \Delta E_3 - \Delta E_{s,S} = 13.9 \text{ kJ/mol} \quad (6)$$

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 fulfils 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).

REFERENCES

Bassan A, Borowski T, Siegbahn PEM (2004) Quantum chemical studies of dioxygen activation by mononuclear non-heme iron enzymes with the 2-His-1-carboxylate facial triad. *Dalton Trans* 3153–3162.

Bassan A, Blomberg MRA, Borowski T, Siegbahn PEM (2006) Theoretical studies of enzyme mechanisms involving high-valent iron intermediates. *J Inorg Biochem* **100**: 727–743.

Beg S, Waggoner K, Ahmad Y, Watt M, Lewis M (2008) Predicting face-to-face arene-arene binding energies. *Chem Phys Lett* **455**: 98–102.

Borowski T, Bassan A, Siegbahn PEM (2004) 4-Hydroxyphenylpyruvate dioxygenase: A hybrid density functional study of the catalytic reaction mechanism. *Biochemistry* **43**: 12331–12342.

Boys SF, Bernardi F (1970) The calculations of small molecular interaction by the difference of separate total energies-some procedures with reduced error. *Mol Phys* **19**: 553–566.

Brownlee JM, Johnson-Winters K, Harrison DHT, Moran GR (2004) Structure of the ferrous form of (4-hydroxyphenyl)pyruvate dioxygenase from *Streptomyces avermitilis* in complex with the therapeutic herbicide NTBC. *Biochemistry* **43**: 6370–6377.

Conrad JA, Moran GR (2008) The interaction of hydroxymandelate synthase with the 4-hydroxyphenylpyruvate dioxygenase inhibitor: NTBC. *Inorg Chim Acta* **361**: 1197–1201.

Ellis MK, Whitfield AC, Gowans LA, Auton TR, Provan WM, Lock EA, Smith LL (1995) Inhibition of 4-hydroxyphenylpyruvate dioxygenase by 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione and 2-(2-chloro-4-methanesulfonylbenzoyl)-cyclohexane-1,3-dione. *Toxicol Appl Pharm* **133**: 12–19.

Ellis MK, Whitfield AC, Gowans LA, Auton TR, Provan WM, Lock EA, Lee DL, Smith LL (1996) Characterization of the interaction of 2-[2-nitro-4-(trifluoromethyl) benzoyl]-4,4,6,6-tetramethyl-cyclohexane-1,3,5-trione with rat hepatic 4-hydroxyphenylpyruvate dioxygenase. *Chem Res Toxicol* **9**: 24–27.

Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Montgomery Jr. JA, Vreven T, Kudin KN, Burant JC, Millam JM, Iyengar SS, Tomasi J, Barone V, Mennucci B, Cossi M, Scalmani G, Rega N, Petersson GA, Nakatsuji H, Hada M, Ehara M, Toyota K, Fukuda R, Hasegawa J, Ishida M, Nakajima T, Honda Y, Kitao O, Nakai H, Klene M, Li X, Knox JE, Hratchian HP, Cross JB, Adamo C, Jaramillo J, Gomperts R, Stratmann RE, Yazyev O, Austin AJ, Cammi R, Pomelli C, Ochterski JW, Ayala PY, Morokuma K, Voth GA, Salvador P, Dannenberg JJ, Zakrzewski VG, Dapprich S, Daniels AD, Strain MC, Farkas O, Malick DK, Rabuck AD, Raghavachari K, Foresman JB, Ortiz JV, Cui Q, Baboul AG, Clifford S, Cioslowski J, Stefanov BB, Liu G, Liashenko A, Piskorz P, Komaromi I, Martin

RL, Fox DJ, Keith T, Al-Laham MA, Peng CY, Nanayakkara A, Challacombe M, Gill PMW, Johnson B, Chen W, Wong MW, Gonzalez C, Pople JA (2003) *Gaussian 03 Revision B.05*. Gaussian Inc.: Pittsburgh PA.

Fritze IM, Linden L, Freigang J, Auerbach G, Huber R, Steinbacher S (2004) The crystal structures of *Zea mays* and Arabidopsis 4-hydroxyphenylpyruvate dioxygenase. *Plant Physiol* **134**: 1388–1400.

Grompe M (2001) The pathophysiology and treatment of hereditary tyrosinemia type 1. *Semin Liver Dis* **21**: 563–571.

Hall MG, Wilks MF, Provan WM, Eksborg S, Lumholtz B (2001) Pharmacokinetics and pharmacodynamics of NTBC (2-(2-nitro-4-fluoromethylbenzoyl)-13-cyclohexanedione) and mesotrione inhibitors of 4-hydroxyphenyl pyruvate dioxygenase (HPPD) following a single dose to healthy male volunteers. *Brit J Clin Pharmacol* **52**: 169–177.

Hausinger RP (2004) Fe(II)/alpha-ketoglutarate-dependent hydroxylases and related enzymes. *Crit Rev Biochem Mol* **39**: 21–68.

Kavana M, Moran GR (2003) Interaction of (4-hydroxyphenyl)pyruvate dioxygenase with the specific inhibitor 2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione. *Biochemistry* **42**: 10238–10245.

Krebs C, Fujimori DG, Walsh CT, Bollinger JM (2007) Non-heme Fe(IV)-oxo intermediates. *Acc Chem Res* **40**: 484–492.

Lee DL, Prisbylla MP, Cromartie TH, Dagarin DP, Howard SW, Provan WM, Ellis MK, Fraser T, Mutter LC (1997) The discovery and structural requirements of inhibitors of p-hydroxyphenylpyruvate dioxygenase. *Weed Sci* **45**: 601–609.

Lee DL, Knudsen CG, Michaely WJ, Chin HL, Nguyen NH, Carter CG, Cromartie TH, Lake BH, Shribbs JM, Fraser T (1998) The structure-activity relationships of the triketone class of HPPD herbicides. *Pestic Sci* **54**: 377–384.

Lock EA, Ellis MK, Gaskin P, Robinson M, Auton TR, Provan WM, Smith LL, Prisbylla MP, Mutter LC, Lee DL (1998) From toxicological problem to therapeutic use: The discovery of the mode of action of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) its toxicology and development as a drug. *J Inherit Metab Dis* **21**: 498–506.

Mitchell G, Bartlett DW, Fraser TEM, Hawkes TR, Holt DC, Townson JK, Wichert RA (2001) Mesotrione: a new selective herbicide for use in maize. *Pest Manag S* **57**: 120–128.

Neidig ML, Kavana M, Moran GR, Solomon EI (2004) CD and MCD studies of the non-heme ferrous active site in (4-hydroxyphenyl)pyruvate dioxygenase: Correlation between oxygen activation in the extradiol and alpha-KG-dependent dioxygenases. *J Am Chem Soc* **126**: 4486–4487.

Neidig ML, Decker A, Kavana M, Moran GR, Solomon EI (2005) Spectroscopic and computational studies of NTBC bound to the non-heme iron enzyme (4-hydroxyphenyl)pyruvate dioxygenase: Active site contributions to drug inhibition. *Biochem Biophys Res Commun* **338**: 206–214.

Neidig ML, Brown CD, Kavana M, Choroba OW, Spencer JB, Moran GR, Solomon EI (2006a) Spectroscopic and electronic structure studies of the role of active site interactions in the decarboxylation reaction of alpha-keto acid-dependent dioxygenases. *J Inorg Biochem* **100**: 2108–2116.

Neidig ML, Decker A, Choroba OW, Huang F, Kavana M, Moran GR, Spencer JB, Solomon EI (2006b) Spectroscopic and electronic structure studies of aromatic electrophilic attack and hydrogen-atom abstraction by non-heme iron enzymes. *Proc Natl Acad Sci USA* **103**: 12966–12973.

Pohorecka M, Wawer Z, Filipez M, Sykut-Cegielska J, Pronicka E (2008) Pharmacokinetics of NTBC (nitisinone) following a single dose to 5 children with tyrosinemia type 1. *J Inher Metab Dis* **31**: (Suppl 1) 020P.

Prescott AG, Lloyd MD (2000) The iron(II) and 2-oxoacid-dependent dioxygenases and their role in metabolism. *Nat Prod Rep* **17**: 367–383.

Russo PA, Mitchell GA, Tanguay RM (2001) Tyrosinemia: A review. *Pediatr Devel Pathol* **4**: 212–221.

Serre L, Sailland A, Sy D, Boudec P, Rolland A, Pebay-Peyroula E, Cohen-Addad C (1999) Crystal structure of *Pseudomonas fluorescens* 4-hydroxyphenylpyruvate dioxygenase: an enzyme involved in the tyrosine degradation pathway. *Structure* **7**: 977–988.

Siegbahn PEM, Borowski T (2006) Modeling enzymatic reactions involving transition metals. *Acc Chem Res* **39**: 729–738.

Szczeciński P, Gryff-Keller A, Molchanov S (2006) ^{1}H NMR ^{13}C NMR and computational DFT study of the structure of 2-acylcyclohexane-1,3-diones and their alkali metal salts in solution. *J Org Chem* **71**: 4636–4641.

Szczeciński P, Lamparska D, Gryff-Keller A, Gradowska W (2008) Identification of 2-[2-nitro-4-(trifluoromethyl) benzoyl]-1,3-cyclohexanedione metabolites in urine of patients suffering from tyrosinemia type I with the use of ^{1}H and ^{19}F NMR spectroscopy. *Acta Biochim Pol* **55**: 749–752.

Tanguay RM, Jorquera R, Poudrier J, St'Louis M (1996) Tyrosine and its catabolites: From disease to cancer. *Acta Biochim Polon* **43**: 209–216.

Tauer TP, Sherrill CD (2005) Beyond the benzene dimer: An investigation of the additivity of π–π interactions. *J Phys Chem A* **109**: 10475–10478.

Yang C, Pflugrath JW, Camper DL, Foster ML, Pernich DJ, Walsh TA (2004) Structural basis for herbicidal inhibitor selectivity revealed by comparison of crystal structures of plant and mammalian 4-hydroxyphenylpyruvate dioxygenases. *Biochemistry* **43**: 10414–10423.

Ye XY, Li ZH, Wang WN, Fan KN, Xu W, Hua ZY (2004) The parallel π–π stacking: a model study with MP2 and DFT methods. *Chem Phys Lett* **397**: 56–61.