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THE MECHANISM OF ACTION AND MODE OF INHIBITION OF DIHYDROOROTATE DEHYDROGENASE

A QUANTUM CHEMICAL STUDY

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Abstract—Semiemperical quantum chemical calculations have been applied to study the reaction mechanism and mode of inhibition of dihydroorotate dehydrogenase. The structure of substrate, intermediate, product and various inhibitors of dihydroorotate dehydrogenase were optimized using MNDO method and the geometry, heat of formation and the net atomic partial charges of optimized molecules, as well as the energy of the reaction path were calculated. This study shows that the carbanion intermediate of this reaction is rather stable (heat of formation = −134.5 kcal) and readily forms upon nucleophilic attack by groups such as hydroxyl ion. There is a good correlation between electronic properties and the biological activities of various inhibitors of this enzyme and the geometry of the most active inhibitor resembles closely that of the intermediate of the reaction. Therefore, it is concluded that the enzymatic oxidation of dihydroorotate dehydrogenase proceeds via formation of an intermediate and the inhibitors bind to the active site of this enzyme in the place of this intermediate.

Dihydroorotate dehydrogenase (DHODase‡), the fourth enzyme of the *de novo* pyrimidine biosynthetic pathway, catalyses the oxidation of dihydroorotate to orotic acid [1]. It has been suggested that the reaction proceeds via elimination of *trans* 5-pro-*S* and 6-protons of the dihydroorotate (Fig. 1) [2–5]. The activity of DHODase has been found to decrease in hepatocarcinoma *in vitro* [6, 7] and during hepatocarcinogenesis *in situ* [8]. This decrease in DHODase activity would therefore provide a potential rate-limiting reaction in the *de novo* pyrimidine pathway and a potential site for selective tumor inhibition. The naphthoquinone antibiotics, lapochol and dichloroallyllawsone, which have received clinical trial as anti-cancer drugs, act via inhibition of DHODase through interruption of the transfer of electrons [9–11]. The novel anticancer drug candidate brequinar sodium also inhibits DHODase [12]. DHODase inhibitors also have potential as selective antimalarial agents, as malarial parasites lack the salvage pathway and thus are dependent upon the *de novo* biosynthetics pathway for pyrimidines [13].

Several analogues of orotic acid has been synthesized and tested as potential inhibitors of DHODase [4]. This study shows while L-DHO is the enzyme substrate, its D-enantiomer is a weak competitive inhibitor. The product of the reaction, orotic acid, as well as its analogues, is a competitive inhibitor of this enzyme. Similarly it has been reported that DHOX to be a potent inhibitor for both the mammalian [13–15] and the protozoan [13] enzymes. Therefore, the knowledge of the mechanism of reaction catalysed by this enzyme and the mode of its inhibition is of great interest and could provide bases for the rational design of new anti-tumor and anti-malarial drugs. In the present study, we have applied quantum chemical calculation to shed light on these points.

‡ Abbreviations: DHODase, dihydroorotate dehydrogenase; MNDO, modified neglect of differential overlap; DHOX, 5-aza 5,6-dihydroorotate.

MATERIALS AND METHODS

Optimization of geometry and calculation of atomic charges. The structures of the substrate, intermediate and the product of the enzymatic reaction (Fig. 1) as well as inhibitors (Table 1) [4] were optimized using the MOPAC program [16] starting with initial geometries constructed using standard bond length and angles [17]. These initial geometries were used as input to the semiemperical all valence electron molecular orbital method, MNDO [18]. Totally unconstrained geometry optimization was performed for all compounds. For each compound both ketonic and enolic forms were considered. The net atomic charges (Mulliken population analysis) on every atom of each compound were calculated for the optimized structures of both neutral and anionic species by MNDO method [18].

Calculation of the reaction path energy. It has been postulated that the enzymatic reaction proceeds by a nucleophilic attack on the axial hydrogen of C5 and the formation of a carbanion intermediate [4]. This reaction was simulated using MNDO method. In the place of nucleophil group a hydroxy group was placed at distance of 5 Å above the axial hydrogen of C5. The distance was reduced in steps of 0.1 Å, the energy of the system was calculated and the minimum energy reaction path was determined.

The correlation of the biological activities of various inhibitors and their physico-chemical parameters. The biological activity (measured as $\log 1/K_i$) of

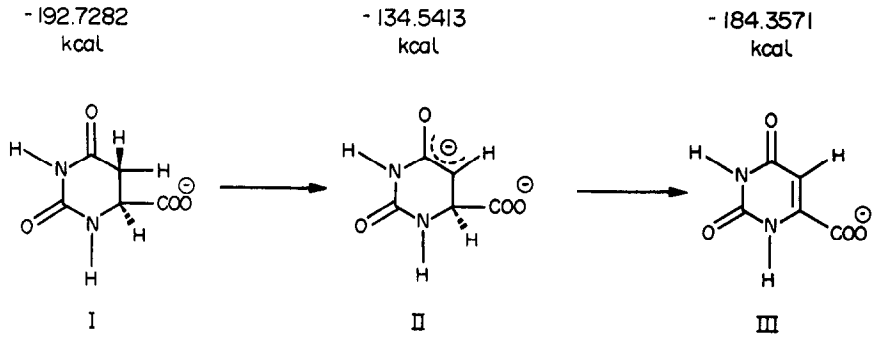
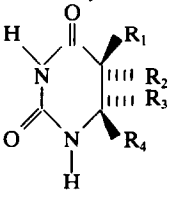
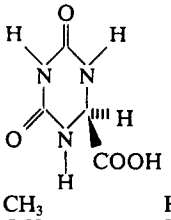
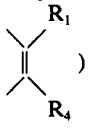


Fig. 1. The pathway of oxidation of dihydroorotate catalysed by dihydroorotate dehydrogenase. I, substrate (dihydroorotate); II, intermediate; and III, product (orotate).

Table 1. The structure and biological activity of the several inhibitors of DHODase [4]



No.	R_1	R_2	R_3	R_4	K_i
1	H	H	H	COOH	K_m = 12.5 μM
2	H	H	COOH	H	2.5 μM
3					9 μM
4	CH_3	H	H	COOH	694 μM
5	C_2H_5	H	H	COOH	3.9 mM
6	CH_2O^-	H	H	 (as lactone with R_1)	915 μM
7	H	CH_3	H	COOH	684 μM
8	H(C_5,C_6= )			COOH	13 μM
9	Br			COOH	56 μM
10	NH_2			COOH	296 μM
11	CH_3			COOH	18 μM

various C5 substituted analogues of orotic acid (Table 2) were correlated with various physicochemical parameters [19] using standard linear regression analysis [20]. Similarly, the net atomic charges on the various atoms of DHODase inhibitors (Table 3) were correlated with their biological activity. The optimized structure of the most active inhibitor (DHOX) was superimposed on the optimized structures of substrate, intermediate and product and the root mean square error (r.m.s.e.) were calculated as a measure of similarity [21].

RESULTS

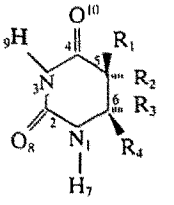
Reaction pathway

MNDO calculation shows that the ketonic forms

Table 2. Physico-chemical parameters of orotate analogues (for structure see Table 1)

No.	R	π	σ_m	σ_p	$\mathcal{F}$	$\mathcal{R}$	MR	L	B1
8	H	0.0	0.0	0.0	0.0	0.0	1.03	2.06	1.0
9	Br	0.86	0.39	0.23	0.44	−0.17	8.88	3.83	1.95
10	NH_2	−1.23	−0.16	−0.66	0.02	−0.68	5.42	2.93	1.5
11	CH_3	0.56	−0.07	−0.17	−0.04	−0.13	5.65	3.0	1.52

Table 3. The biological activity and partial atomic charges on various atoms of several DHODase inhibitors (for compounds see Table 1)



No.	Log $1/K_i$	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10
2	2.620	−0.339	0.483	−0.399	0.379	0.010	0.036	0.168	−0.418	0.195	−0.382
3	5.046	−0.384	0.469	−0.404	0.475	−0.366	0.218	0.202	−0.424	0.199	−0.418
4	3.159	−0.372	0.477	−0.418	0.363	−0.039	0.083	0.213	−0.434	0.194	−0.378
5	2.410	−0.366	0.473	−0.416	0.363	−0.021	0.079	0.210	−0.433	0.194	−0.378
6	3.039	−0.385	0.478	−0.405	0.373	−0.102	0.122	0.209	−0.348	0.224	−0.310
7	3.165	−0.351	0.466	−0.413	0.365	−0.041	0.072	0.195	−0.429	0.193	−0.380
8	4.886	−0.326	0.477	−0.412	0.423	−0.284	0.167	0.217	−0.425	0.193	−0.401
9	4.252	−0.321	0.476	−0.411	0.436	−0.309	0.205	0.222	−0.417	0.198	−0.375
10	3.529	−0.328	0.476	−0.406	0.410	−0.256	0.219	0.221	−0.421	0.197	−0.408
11	4.745	−0.324	0.476	−0.412	0.432	−0.317	0.186	0.219	−0.425	0.196	−0.398

of all tested compounds are the more stable forms. Also, it has been reported that dihydroorotate and orotic acid are bound to the enzyme in the form of anionic species [4]. Therefore, the keto-anionic forms of the substrate, intermediate, product and inhibitors (except inhibitor with lacton ring) were considered for the rest of the calculations. Calculation shows that the carbanion intermediate [4] (II) of the reaction catalysed by DHODase is a rather stable compound (heat of formation = −134.5 kcal/mol) and barriers of 58.18 and 49.82 kcal/mol exist between this intermediate and substrate (I) and product (III), respectively (Fig. 1). It has been found that of two C5 hydrogens of the substrate, the axial hydrogen is the more acidic as its net atomic charge is +0.06 eV compared to +0.03 eV of equitorial hydrogen. Simulation of the enzymatic reaction shows that nucleophilic groups such as hydroxy anion can eliminate the axial hydrogen of the C5 and form intermediate as a stable species (Fig. 2). It has been noted that the C6 hydrogen of this intermediate has a negative charge (−0.07 eV).

Structure–activity relationship of inhibitors

The regression analysis shows that there is a good correlation between the electronic parameter of ($\mathcal{R}$) of the substituent at C5 of the pyrimidine ring and the biological activities of various inhibitors (Table 2) as shown by Eqn 1 (Fig. 3)

$$Y = \log 1/K_i = 4.8341 + 1.9643 \times \mathcal{R} \qquad (1)$$

where N = 4, $r = 0.958$, $s = 0.216$, $F(1,2) = 22.28$, $P < 0.01$.

No significant correlation was observed for the other parameters.

It also has been found that there is a good correlation between the net atomic charges on the atom No. 5 (Q5) of the pyrimidine ring and the biological activities of various inhibitors (Table 3) according to Eqn 2 (Fig. 4)

$$Y = \log 1/K_i = 2.623 - 6.157 \times Q5 \qquad (2)$$

where N = 10, $r = 0.928$, $s = 0.385$, $F(1,8) = 11.26$, $P < 0.01$.

No significant correlation was found between the charges on the other atoms and the biological activities. Comparing of the structure of the most active inhibitor (DHOX) with substrate, intermediate and product of the reaction shows that this compound is the most similar to the intermediate of the reaction (r.m.s.e. = 0.182, Fig. 5) compared to substrate (r.m.s.e. = 1.030) or product (r.m.s.e. = 0.352).

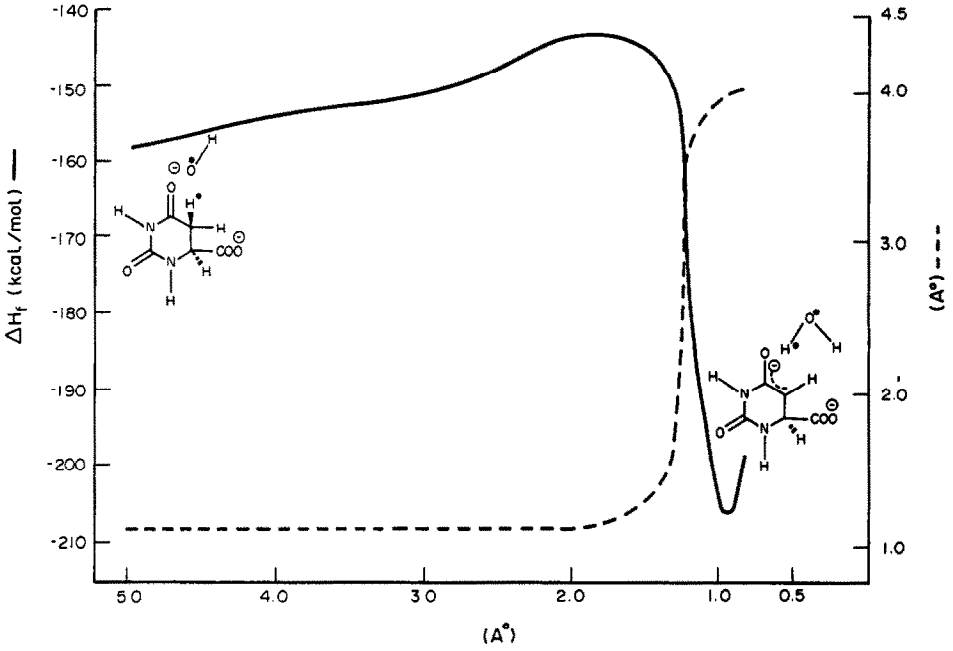


Fig. 2. The plot of minimum energy reaction path for oxidation of dihydroorotate to hydroorotate carbanion (———), and also distance between C5 and H* (A°) (– – –) versus distance between O* and H* (A°).

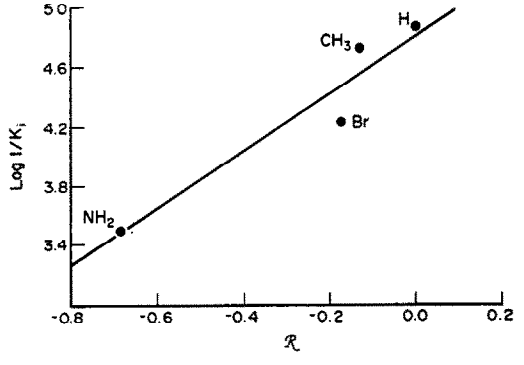


Fig. 3. The relationship between the electronic parameters ($\mathcal{R}$) of C5-substituted orotic acid analogues and their biological activity. The biological activity is expressed as $\log 1/K_i$.

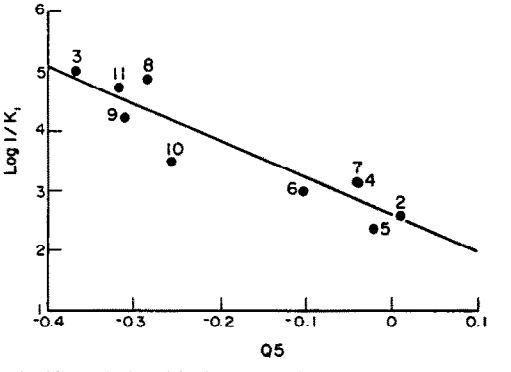


Fig. 4. The relationship between the net atomic charges on C5 of the various inhibitors of DHODase and their biological activity. The biological activity is expressed as $\log 1/K_i$ (for numbers see Table 1).

DISCUSSION

There is good evidence from work on both the bovine [2] and crithidial [3] DHODase to suggest that substrate oxidation involves proton abstraction from the C5-pro-*S*-position of dihydroorotate together with delivery of two electrons and a proton (perhaps as hydride) from C6 of the substrate to flavine. Then the two electrons transfer to ubiquinone cofactor by the electron transfer system. The removing of these two *trans* hydrogens energetically favors elimination [4] (Fig. 1). Work on the crithidial DHODase, in part on the basis of kinetic isotope effects and in part on solvent-exchange reactions, shows that dehydrogenation may proceed by a stepwise process with intermediate formation of a C5 carbanion [3]. The result of double isotope substitution studies and analysis for substrate isotope exchange with solvent in bovine DHODase point toward a concerted mechanism for oxidation of dihydroorotate [21].

However, it has been pointed out [22] that a stepwise reaction may proceed at such a speed that the experimental methods would fail to detect the

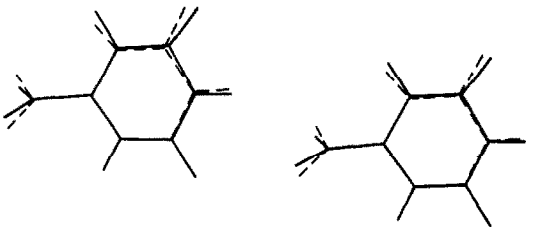


Fig. 5. The superposition of the most potent inhibitor DHOX (- - -) with hydroorotic acid carbanion intermediate (———). For structures see Table 1.

existence of a stable intermediate. Then, theoretical calculations of the structure of the transient species, as well as the energy of reaction pathway, may provide much useful information on this matter [22]. The results presented here point out that the intermediate of the reaction is a stable species and forms readily upon the nucleophilic attack from dihydroorotic acid (Fig. 2). Therefore, according to the following reasons, it can be concluded that the reaction proceeds with the formation of an intermediate as has been suggested previously [3].

1. Formation of a stable intermediate after nucleophilic attack by OH anion (Fig. 2).

2. The most potent inhibitor is the most structurally similar to the intermediate of the reaction (Fig. 5) and both have an electron pair with SP2 hybridation on the atom No. 5 of the pyrimidine ring. It should be mentioned that this inhibitor is active against both bovine and crithidial enzymes [13–15], which points to a similar mechanism of inhibition in these enzymes.

3. Also the activity of the inhibitors increases with the electronic effect ($\mathcal{R}$) of the substituents on the C5 (Eqn 1 and Fig. 3) and the net atomic charge on this atom (Eqn 2 and Fig. 4). This points to the fact that the activity of the inhibitors increases as the electronic structure becomes more similar to the intermediate.

Therefore, one can conclude that the oxidation of dihydroorotic acid is carried out via formation of a carbanion intermediate. Furthermore, these results suggest that the inhibitors of DHODase bind to the active site of this enzyme in place of the intermediate, and therefore act as transition state analogues.

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