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THE BASIS FOR SLOWLY REVERSIBLE INHIBITION OF DEHYDROQUINATE SYNTHASE: A CASE OF MISTAKEN IDENTITY?

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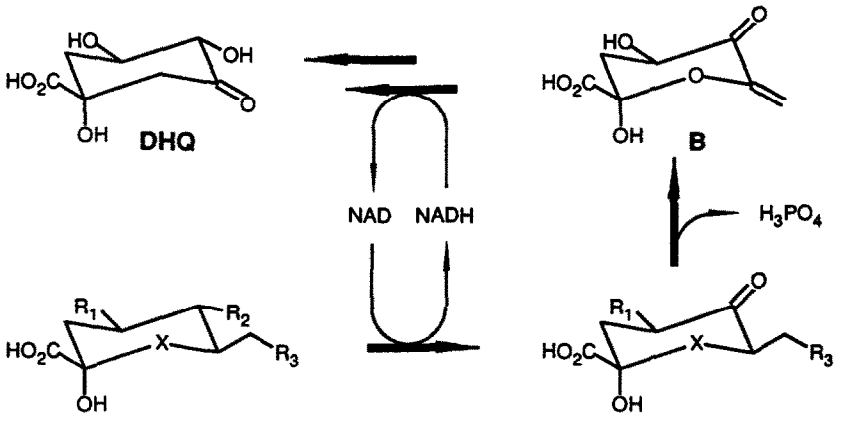
Abstract: Kinetic parameters have been determined for inhibition of DHQ synthase by [1R-(1α,4α,5β]-1,4-dihydroxy-5-(phosphonomethyl)cyclohexane-1-carboxylic acid (C-3 deoxycarbaphosphonate) and [1S-(1α,3β,5β]-1,3-dihydroxy-5-(phosphonomethyl)cyclohexane-1-carboxylic acid (C-4 deoxycarbaphosphonate). Comparison of these parameters suggests a new interpretation for slowly reversible inhibition of DHQ synthase.

Nanomolar-level, slowly reversible inhibition of 3-dehydroquinate (DHQ) synthase by carbaphosphonate **1a** (Scheme I) is observed to coincide with accumulation of enzyme-bound NADH.[1] Formation of NADH indicates that, like substrate 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP), the inhibitor's C-4 hydroxyl group is being oxidized. The resulting enzyme-bound ketocarbaphosphonate[2] **1b** (Scheme I) is an analogue of the reactive intermediate **A** (Scheme I) derived from enzyme-catalyzed oxidation of substrate DAHP. Strong interactions between the enzyme active site and the in situ-generated reactive intermediate analogue **1b** are the suggested basis for the potent inhibition of DHQ synthase by carbaphosphonate **1a**.[1b] Is this interpretation correct? Questions are now raised and an alternate interpretation proposed as a consequence of measured parameters for C-3 deoxycarbaphosphonate **2a** and C-4 deoxycarbaphosphonate **3** inhibition of DHQ synthase.[3]

SCHEME I



DAHP R_1 = OH, R_2 = OH, R_3 = OPO_3H_2, X = O
1a R_1 = OH, R_2 = OH, R_3 = PO_3H_2, X = CH_2
2a R_1 = H, R_2 = OH, R_3 = PO_3H_2, X = CH_2
3 R_1 = OH, R_2 = H, R_3 = PO_3H_2, X = CH_2

A R_1 = OH, R_3 = OPO_3H_2, X = O
1b R_1 = OH, R_3 = PO_3H_2, X = CH_2
2b R_1 = H, R_3 = PO_3H_2, X = CH_2

The deoxycarbaphosphonates were synthesized in order to ascertain the contribution to active site binding for each secondary alcohol in carbaphosphonate **1a**. Both of the deoxycarbaphosphonates were discovered to be slowly reversible inhibitors of DHQ synthase (Table I). Accumulation of enzyme-bound NADH was detected during inhibition of DHQ synthase by the C-3 deoxycarbaphosphonate. However, the inhibition constant (K_i) for C-3 deoxycarbaphosphonate **2a** indicated a fortyfold drop in potency of inhibition relative to carbaphosphonate **1a**. A hundredfold drop in K_i was observed for the C-4 deoxycarbaphosphonate **3**. Observed variation in inhibition constants is primarily dictated by the large difference in the rate constant (k_{on}) for carbaphosphonate **1a** association with DHQ synthase relative to the associatation rate constants (k_{on}) for deoxycarbaphosphonates **2a** and **3**.

TABLE I

Inihibitor	type of inhibition	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_i (M)
1a	slowly reversible	1.4×10^5	7.5×10^{-4}	5.4×10^{-9}
2a	slowly reversible	1.5×10^3	3.2×10^{-4}	2.2×10^{-7}
3	slowly reversible	1.0×10^3	5.4×10^{-4}	5.4×10^{-7}

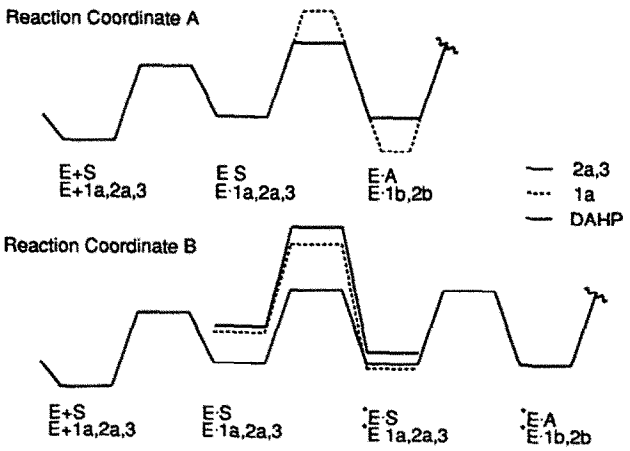
These parameters for deoxycarbaphosphonate inhibition provide intriguing insights into the underlying basis for inhibition of DHQ synthase by carbocyclic substrate analogues. Oxidation of DAHP to reactive intermediate A establishes an internal equilibrium constant between enzyme-bound DAHP and enzyme-bound intermediate A. Removal of the pyranosyl oxygen as with carbaphosphonate **1a** has been suggested[1b] (Reaction Coordinate A, Scheme II) to displace the internal equilibrium constant towards enzyme-bound reactive intermediate analogue **1b** relative to enzyme-bound carbaphosphonate **1a**. This follows the precedented[4] displacements of equilibrium constants in favor of oxidized products for alcohol to carbonyl conversions upon removal of electronegative groups vicinally situated to the oxidized alcohol

Removal of the secondary alcohol vicinally situated to the oxidized alcohol might lead to further displacement of the internal equilibrium constant in favor of reactive intermediate analogue **2b** and improved inhibition of DHQ synthase by the C-3 deoxycarbaphosphonate **2a**. Accumulation of enzyme-bound NADH is observed during C-3 deoxycarbaphosphonate inhibition. However, C-3 deoxycarbaphosphonate is a less potent inhibitor than carbaphosphonate **1a**. The reduced potency of DHQ synthase inhibition by the C-3 deoxycarbaphosphonate could indicate that lost interactions between the active site and the C-3 alcohol override any improved thermodynamics for C-4 alcohol oxidation

Inhibition of DHQ synthase by the C-4 deoxycarbaphosphonate **3** is more difficult to explain. Even though this deoxycarbaphosphonate completely lacks the C-4 alcohol whose oxidation has been suggested to be important to DHQ synthase inhibition, C-4 deoxycarbaphosphonate **3** is still (Table I) a slowly reversible inhibitor whose inhibition constant (K_i) is similar in magnitude to that determined for the C-3 deoxcarbaphosphonate **2a**. Most

importantly, the rate constant for release (k_{off}) of the C-4 deoxycarbaphosphonate **3** is quite similar to the rate constant for release of the C-3 deoxycarbaphosphonate **2a** *and* the rate constant for release of carbaphosphonate **1a**. From the data of Table I, it is clear that oxidation of the C-4 alcohol of carbaphosphonate **1a** is not the central feature dictating potent, slowly reversible inhibition of DHQ synthase.

SCHEME II



What then is the basis for improved inhibition of DHQ synthase upon substitution of a methylene group for the pyranosyl oxygen? One possibility (Reaction Coordinate B, Scheme II) is that an enzyme conformational change occurs after formation of the initial Michaelis complex. This altered conformational form of the enzyme (*E) precedes and is essential to oxidation of enzyme-bound substrate or inhibitor. Inhibitors **1a**, **2a**, and **3** would only be loosely bound by enzyme (E) in the initial Michaelis complex, but much more tightly bound by the conformationally altered form of the enzyme (*E) As a result, the ground state free energies of enzyme-bound inhibitors would likely be quite similar for the loosely bound Michaelis complex while the ground state free energies for enzyme-bound inhibitors would be quite different after the enzyme conformational change. Furthermore, it is reasonable to propose that the rate constant for the conformational change which transforms E into *E may be diminished upon substitution of a methylene group for the pyranosyl oxygen in the carbaphosphonate and may be even further reduced for the deoxycarbaphosphonates due to removal of the individual secondary alcohols. Combination of each of these features provides one possible explanation for slowly-reversible inhibition, similar dissociation rate constants (k_{off}), and different association rate constants (k_{on}) for carbaphosphonate and the deoxycarbaphosphonates.

Designing enzyme inhibitors to structurally mimic reactive intermediates has proven to be a highly effective strategy for achieving potent enzyme inhibition.[5] Inhibition of DHQ synthase by carbaphosphonate **1a** is, upon first inspection, an alluring example of potent enzyme inhibition attendant with in situ generation of a reactive intermediate analogue. However, the central role assigned to the ketocarbaphosphonate may be a case of mistaken identity. A different interpretation consistent with DHQ synthase inhibition by C-3 deoxycarbaphosphonate **2a**

and C-4 deoxycarbaphosphonate **3** suggests that potent, slowly reversible inhibition of DHQ synthase is not due to selective *stabilization* of an enzyme-bound reactive intermediate analogue. Instead, nanomolar-level, slowly reversible inhibition may be the consequence of selective *destabilization* of an activation barrier separating two enzyme internal states.

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References and Notes

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2. This molecule has been synthesized and its inhibition of DHQ synthase examined: Montchamp, J.-L.; Frost, J. W. *J. Am. Chem. Soc.* **1991**, *113*, 6296.

3. Substrate DAHP and DHQ synthase were obtained as previously described.[1c] Determination of the rate constants (k_{on}) for the deoxycarbaphosphonates as well as detection of enzyme-bound NADH also followed literature protocols.[1c] A different method was employed for determining the rate constant (k_{off}) for release of C-4 deoxycarbaphosphonate from DHQ synthase. This method began with incubating 2 mL of a solution containing carbaphosphonate (500 μM), DAHP (500 μM), MOPS (50 mM), cobalt (II) chloride (0.25 mM), NAD^+ (0.25 mM), and DHQ synthase (0.1μM) at pH 7.5 and 15°C for 3 h. An aliquot (1.5 mL) of this solution was then dialyzed at 4°C against 500 mL of a pH 7.5 solution containing MOPS (50 mM), cobalt (II) chloride (0.25 mM), and NAD^+ (0.25 mM). The buffer solution was changed after 3 h and dialysis continued for another 21 h. An aliquot (0.5 mL) of the dialyzed enzyme-containing solution was then added to a pH 7.5 solution containing DAHP (0.1 mM), MOPS (50 mM), cobalt (II) chloride (0.25 mM), and NAD^+ (0.25 mM). The progress curve was followed at 15 °C by quantitating formation of inorganic phosphate. Subsequent derivation of k_{off} used literature procedures.[1c] Linear portions of the progress curves used to determine k_{on} for the C-4 deoxycarbaphosphonate provided steady state velocity (V) values. Plots of 1/V against [I] indicated a value for C-4 deoxycarbaphosphonate's inhibition constant (K_i) of 2.2×10^{-7}M.

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