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PREDICTING INHIBITION OF DEHYDROQUINATE SYNTHASE

L. T. Piehler[§], J. -L. Montchamp[§], J. W. Frost[§*] and Charles J. Manly[‖*]

§Department of Chemistry, Purdue University, West Lafayette, IN 47907
‖DowElanco, Greenfield, IN 46140

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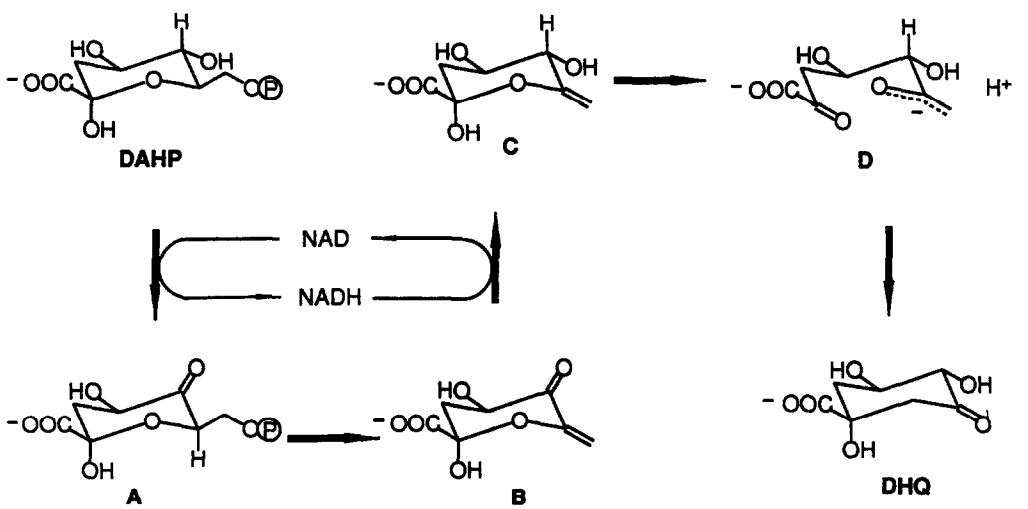
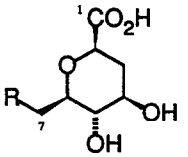
ABSTRACT: Computational molecular superimposition studies predicted that a carbaphosphonate would be a likely inhibitor of 3-dehydroquinate synthase prior to the molecule's synthesis. Mechanistic implications of the enzyme inhibition subsequently observed are discussed.

Development of computational methods suitable for the prediction of enzyme inhibition by a substrate analog before its chemical synthesis is an important goal, given the time and effort involved in synthesis. An approach which exploits computational molecular superimposition has now been developed to evaluate putative inhibitors of 3-dehydroquinate synthase. This enzyme has been the focus of an impressive amount of research due to its position in aromatic amino acid biosynthesis and as a consequence of the unique chemical transformation which the enzyme catalyzes (Scheme I).[1] Despite this attention, 3-dehydroquinate synthase is still an enzymological enigma. The superimposition studies successfully predicted the inhibition of dehydroquinate synthase by a substrate analog possessing structural features which would not, on first inspection, be considered conducive to enzyme inhibition. This inhibition and its successful prediction offer insights into the role DHQ synthase plays in catalyzing conversion of substrate 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) to product 3-dehydroquinate (DHQ).

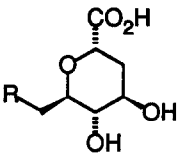
SCHEME I.



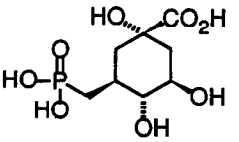
The mechanism originally proposed[2] and since largely verified for DHQ synthase begins with oxidation of the C-5 alcohol of DAHP to the carbonyl of intermediate A (Scheme I). Resulting acidification of the proton attached to C-6 expedites elimination of phosphate thereby generating the cyclic enol ether of intermediate B. The C-5 carbonyl is then reduced yielding intermediate C followed by ring opening which unmasks the C-2 carbonyl and enol(ate) found in intermediate D. The enol(ate) and C-2 carbonyl react in an intramolecular aldol condensation which results in formation of the bond between C-1 and C-2 of DHQ. Chemists have long been impressed by the apparent ability of relatively small (MW 40,000 - 44,000) DHQ synthase to catalyze the aforementioned multitude of steps during each turnover of substrate to product. However, the true role of DHQ synthase in the conversion of DAHP to DHQ has been questioned.[3,4] Dehydroquinate synthase may be nothing more than an oxidoreductase which catalyzes the initial oxidation of the alcohol at C-5 of DAHP and the subsequent reduction of the C-5 carbonyl of intermediate B. The remainder of the steps required for conversion of substrate to product are catalyzed by functional groups contained in the substrate or directed by stereochemical/structural features possessed by the substrate.



1a R = PO_3H_2
1b R = OPO_3H_2



2a R = PO_3H_2
2b R = OPO_3H_2



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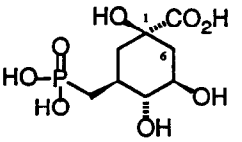

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During this time, work also focused on using inhibitors of DHQ synthase as probes of lethal metabolic perturbations in plants.[5] Circumvention of the facile conversion of some DAHP analogs into furan derivatives was of particular interest. The strategy ultimately employed to stabilize the DAHP analogs was to lock the pyranosyl form of DAHP analogs into place so that ring opening and subsequent furan formation was obviated. 2,6-Anhydro organophosphonate analogs 1a and 2a were synthesized and their inhibition of DHQ synthase examined.[5] β-(2,6-Anhydro-3-deoxy-D-*arabino*-heptulopyranosid)onate 7-phosphonate 1a was not an inhibitor while α-(2,6-anhydro-3-deoxy-D-*arabino*-heptulopyranosid)onate 7-phosphonate 2a was inhibitory. The α- and β-2,6-anhydro phosphate analogs 1b and 2b of DAHP were also synthesized.[5,6] DHQ synthase was inhibited by the β-2,6-anhydro phosphate 1b, although the α-2,6-anhydro phosphate 2b was a much better inhibitor. At the same time, the α-analog 2b was not a substrate for DHQ synthase while inorganic phosphate was eliminated from the β-analog 1b.

An alternate strategy to stabilize DAHP analogs is to substitute a carbon for the pyranosyl ring oxygen. This would remove the possibility of ring opening and subsequent furan formation without the penalty of losing enzyme active site interactions due to loss of a hydroxyl group. Substitution of a ring oxygen with carbon is, however, not a benign modification since carbocyclic analogs have been documented to lead to disappointing enzyme inhibition.[15] DHQ synthase is a

noteworthy exception with the work of Knowles demonstrating carbaphosphonate 3 to be an exceptionally good inhibitor of DHQ synthase.[4a,c] Synthesis of epimeric carbaphosphonate 4 therefore becomes of immediate interest. Given that the α-2,6-anhydro phosphonate and phosphate analogs of DAHP were better inhibitors of DHQ synthase relative to the β-analogs, epimeric carbaphosphonate 4 might likewise be an improved inhibitor relative to the already synthesized carbaphosphonate 3.

RESULTS and DISCUSSION

Before the synthesis of epimeric carbaphosphonate 4 could be justified, the potential of this α-carboxylate organophosphonate to achieve enzyme inhibition required appraisal. Inhibition of DHQ synthase by the α-carboxylate anhydro analogs 2a and 2b was, after all, quite unexpected. The stereochemical configuration at the carbon to which the carboxylate is attached in the DAHP anomer presumably bound by DHQ synthase and the same asymmetric carbon in carbaphosphonate 3 would suggest that the β-2,6-anhydro analogs 1a and 1b but not the α-analogs 2a and 2b should be inhibitory. These paradoxical results seemed ill suited as a basis for confident prediction of epimeric carbaphosphonate 4 inhibition. However, enzyme inhibition afforded by different structures provides a unique opportunity for computational methods to identify structural elements in the inhibitors which are important to enzyme inhibition that might otherwise be overlooked. This certainly has proven to be the case with dehydroquinate synthase.

Epimeric carbaphosphonate inhibition would depend on whether the molecule can interact with the same amino acid residues which stabilize reactive intermediates present during substrate DAHP conversion to product DHQ. Reactive intermediate functional groups which likely interact with active site amino acid residues were first identified. Qualitative overlays of the functional groups in the reactive intermediates were then attempted with various functional groups in the epimeric carbaphosphonate 4. None of the cyclic intermediates A, B, or C (Scheme I) provided satisfactory overlays. The main impediment was the carbaphosphonate 4 carbon to which the carboxylate is attached. This asymmetric center was opposite to the stereochemical configuration of the same carbon in the cyclic, reactive intermediates. This offending asymmetric center is, however, notably absent in acyclic intermediate D where the C-2 carbonyl and enol(ate) have become unmasked immediately prior to intramolecular aldol condensation. Computational molecular superimposition studies then focused on determining the feasibility of overlays between epimeric carbaphosphonate 4 and the carboxylate, C-2 carbonyl, and C-4/C-5 asymmetric region of acyclic intermediate D.

Molecular superimposition studies for intermediate D and carbaphosphonate 4 were performed using the neutral species in order to reduce the computational complexity and to avoid potential problems interpreting conformational energy differences based on unsolvated anion calculations. Structures were relaxed using the Sybyl force field and were submitted to quantum mechanical calculations using the PM3 Hamiltonian with full geometry optimization in cartesian coordinate space. The quantum mechanically derived partial atomic charges and optimized geometries were used in subsequent molecular mechanics calculations using the Sybyl force field with inclusion of the electrostatic term.

TABLE 1.

Calculated Energy Values (kcal) for the Low Energy Conformations of Carbaphosphonate 4 and Acyclic Intermediate D Before Molecular Superimposition

	Sybyl	PM3	CHARMm
intermediate D	7.3	-243	0.89
carbaphosphonate 4	-21.6	-423	-26.3

Calculated Energy Values (kcal) for the Low Energy Conformations of Carbaphosphonate 4 and Acyclic Intermediate D After Molecular Superimposition

	Sybyl	PM3	CHARMm
intermediate D	9.1	-223	7.14
carbaphosphonate 4	-20.9	-398	-16.8

A systematic search was performed on both carbaphosphonate 4 and intermediate D in Sybyl including all significant rotatable bonds. Several low energy conformations (Sybyl force field) were identified from each search. Energy minimization (Sybyl) through molecular mechanics geometry optimization of these conformations allowed the selection of low energy reference conformations for each molecule. In the case of intermediate D, the geometry of this reference was an extended conformation. Entries in Table 1 show Sybyl, PM3, and CHARMm energies for the superimposition conformation (Sybyl geometry) and conformations showing geometries relaxed under each method. The higher cost estimated from the PM3 energy for these two matched conformations is reduced considerably when acyclic intermediate D is allowed to relax slightly from the Multifit conformation. Allowing all atoms except for those important to the molecular superimposition to relax under geometry optimization with PM3, the calculated energy for intermediate D drops to -236 kcal from -223 kcal.

The molecular superimposition of Figure 1 was generated using Sybyl Multifit while allowing both molecules to relax. The atom pair superimpositions specified were carbaphosphonate C-4 hydroxyl oxygen with acyclic intermediate's C-5 hydroxyl oxygen, carbaphosphonate C-5 hydroxyl oxygen with acyclic intermediate's C-4 hydroxyl oxygen, carbaphosphonate C-1 hydroxyl oxygen with acyclic intermediate's doubly bonded carboxylate oxygen, carbaphosphonate doubly bonded carboxylate oxygen with acyclic intermediate's C-2 carbonyl oxygen, and carbaphosphonate singly bonded carboxylate oxygen with acyclic intermediate's singly bonded carboxylate oxygen. As seen from inspection of the molecular superimposition results of Table 1 and Figure 1, the cost of attaining the superimposed conformations for these two molecules is small. Epimeric carbaphosphonate 4 was thus predicted to be an inhibitor of DHQ synthase.

A synthesis was subsequently developed by which both carbaphosphonate diastereomers were efficiently derived from quinic acid.[7] The kinetics associated with carbaphosphonate 3 slow binding inhibition of DHQ synthase were first reexamined. In the presence of epimeric carbaphosphonate 4, the rate of product formation reflected time dependent behavior indicative of slow binding

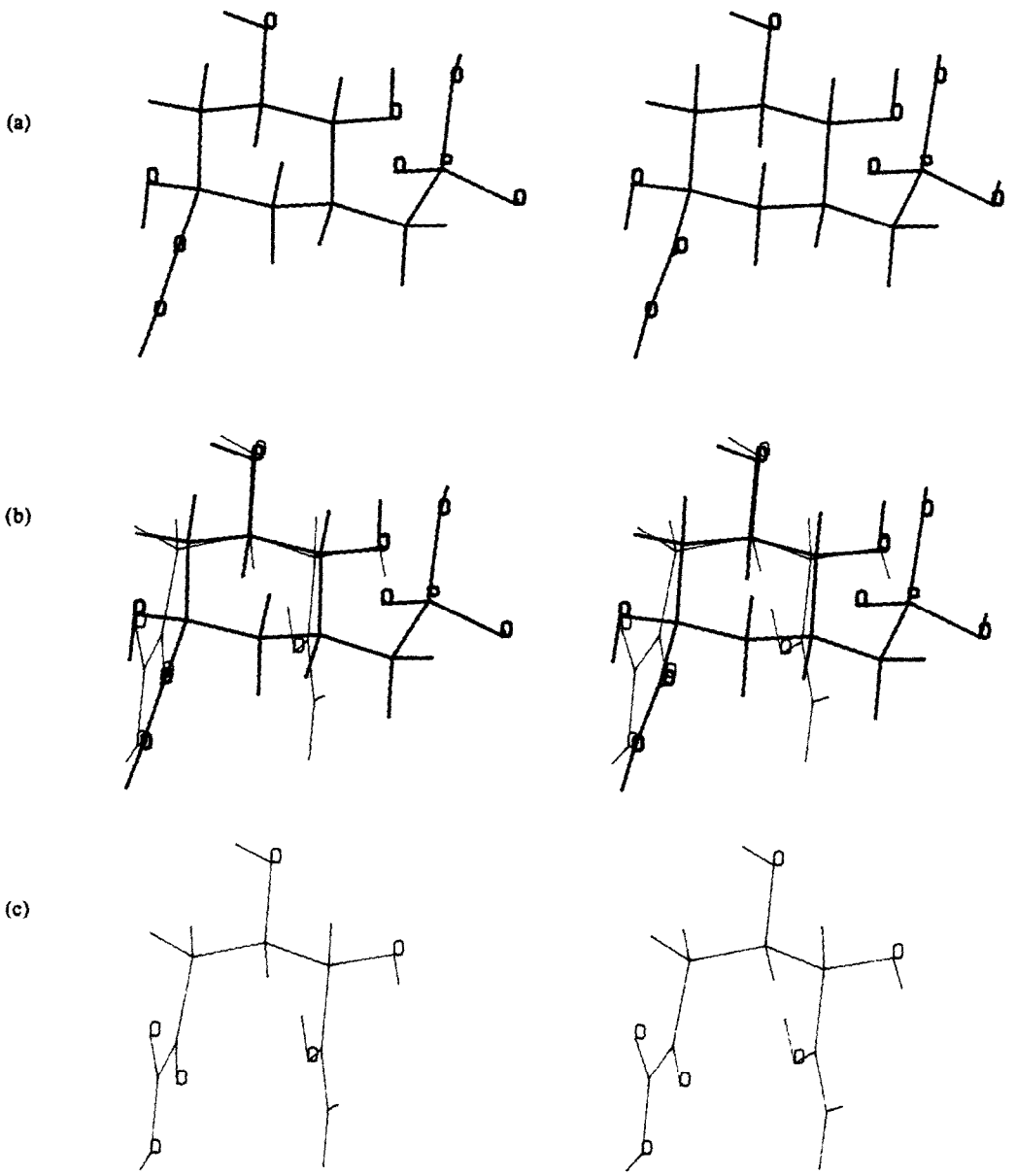


FIGURE 1. Stereoviews of the energy minimized Sybyl Multifit conformers of: (a) carbaphosphonate 4, (b) carbaphosphonate 4 superimposed with acyclic intermediate D, and (c) acyclic intermediate D.

inhibition of DHQ synthase.[14] Subsequent experiments established the kinetic parameters of this inhibition for epimeric carbaphosphonate 4 to be k_{on} = 1.5 x 10^4 $M^{-1}s^{-1}$, k_{off} = 1.1 x 10^{-4} s^{-1}, and an inhibition constant K_i = 7.3 x 10^{-9} M. The same kinetic parameters for carbaphosphonate 3 inhibition were k_{on} = 1.4 x 10^5 $M^{-1}s^{-1}$, k_{off} = 7.5 x 10^{-4} s^{-1}, and K_i = 5.4 x 10^{-9} M. Rather surprisingly, both carbaphosphonates had similar inhibition constants although epimeric 4 was bound and released by the enzyme much more slowly than carbaphosphonate 3.

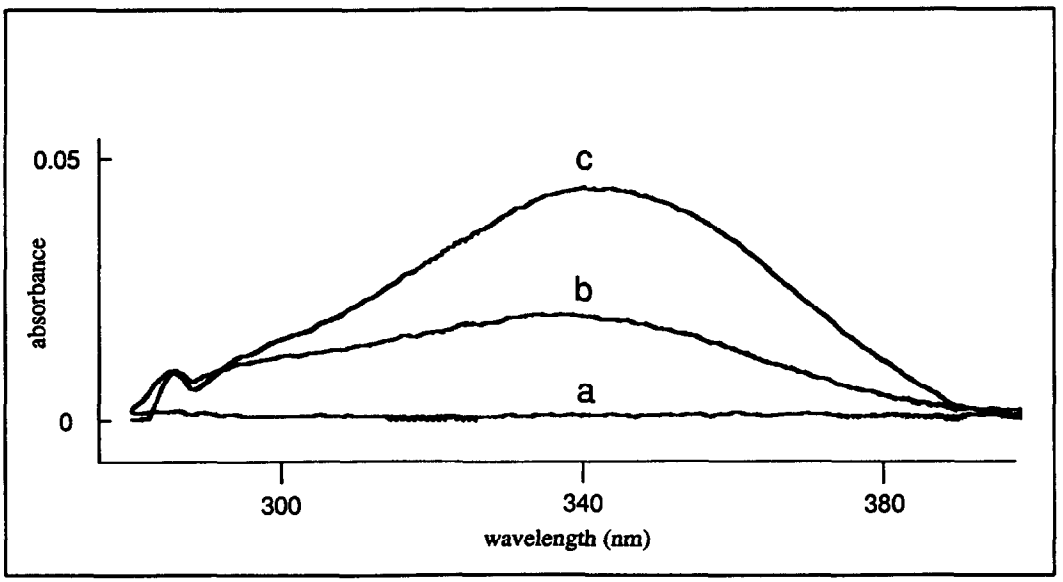


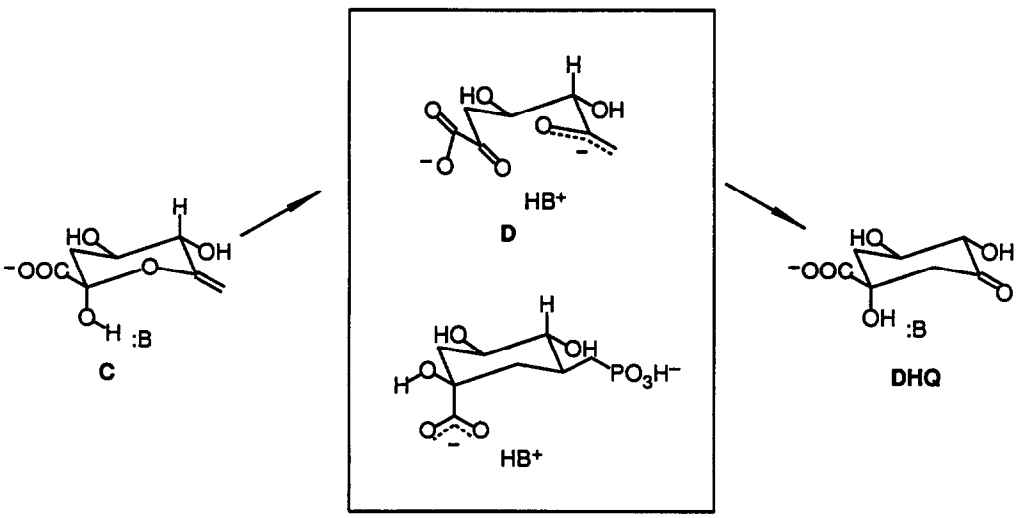
FIGURE 2. Difference spectra obtained during incubation of epimeric carbaphosphonate 4 with DHQ synthase: (a) DHQ synthase (20 μM) in assay buffer before addition of carbaphosphonate 4, referenced to baseline; (b) One minute after addition of epimeric carbaphosphonate 4 (30 μM); (c) One hour after addition of carbaphosphonate 4.

Perhaps the most interesting trademark of slow binding inhibition of DHQ synthase by carbaphosphonate 3 is the enzyme-mediated oxidation of the inhibitor.[4a,c] Oxidation of epimeric carbaphosphonate 4 and formation of enzyme-bound NADH could thus be used to evaluate whether this carbaphosphonate fits in the enzyme active site in a fashion which is similar to that of carbaphosphonate 3. The diastereomeric relationship between the two carbaphosphonates might argue against a similar fit. Nonetheless, incubation of DHQ synthase with epimeric carbaphosphonate results in an observed increase in optical density at 340 nm (Figure 2) which is indicative of inhibitor oxidation and reduction of enzyme-bound NAD.

The molecular superimposition studies predicted that epimeric carbaphosphonate would inhibit DHQ synthase before the molecule was synthesized. Although its inhibition of DHQ synthase is not an improvement over that of carbaphosphonate 3, epimeric carbaphosphonate 4 is still a potent

inhibitor. This inhibition can be more fully viewed from the perspective of active site residues which might catalyze the intramolecular aldol condensation involving the acyclic intermediate used in the molecular superimposition studies. A basic residue (Scheme II) could remove the proton from the hydroxyl group attached to the anomeric carbon thereby catalyzing the ring opening of cyclic intermediate C. The resulting protonated base would then be capable of stabilizing the enolate as well as donating a proton back to the C-2 carbonyl oxygen as the intramolecular aldol condensation proceeds. It is the interaction of the epimeric carbaphosphonate 4 carboxylate with this "aldol catalyzing base" which is likely responsible for the observed inhibition of DHQ synthase.

SCHEME II.



This catalytic view of DHQ synthase is taken into consideration during the molecular superimposition studies with the superimposition of the carbaphosphonate α-carboxylate doubly bonded oxygen and the C-2 carbonyl of the acyclic intermediate along with the superimposition of the carboxylate singly bonded oxygens of both molecules. It is especially noteworthy that such superimpositions are achieved with minimal energy cost even when the hydroxyl oxygens attached to the asymmetric carbon centers are simultaneously superimposed. Computational molecular superimposition thus indicate that the carbaphosphonate α-carboxylate is suitably oriented in three dimensional space to take advantage of binding interactions with the putative aldol catalyzing base.

The hypothesis for why epimeric carbaphosphonate 4 binds with DHQ synthase contrasts with other researchers' views of DHQ synthase catalysis. In elegant model experiments, a protected form of intermediate C has been synthesized which generates intermediate C after photolysis in aqueous solution.[3] This reactive intermediate underwent ring opening and subsequent stereospecific

aldol condensation to generate DHQ. The observation of such a reaction occurring under nonenzymatic conditions led to the suggestion that the enzyme may not catalyze or guide the stereochemical outcome of the intramolecular aldol condensation. In addition, evidence has been amassed that a DAHP phosphate oxygen possibly catalyzes elimination of phosphate from intermediate A.[4a,c,d,6] These results have led to the proposal that DHQ synthase is really a catalytic "sheep in wolf's clothing."[4a]

If DHQ synthase plays no catalytic role after reduction of the C-5 carbonyl of intermediate B, the aldol catalyzing base is removed from consideration. This eliminates the binding interaction which the computational molecular superimposition predicts could be gained via situation of the carboxylate of the carbaphosphonate in the α-configuration. Enzyme inhibition by epimeric carbaphosphonate 4 then becomes difficult to explain. The carboxylate of DAHP is important to binding which points to an active site likely optimized for interaction with this charged residue. Given that carbaphosphonate 4 is *diastereomeric* to carbaphosphonate 3, it is unreasonable to expect that the enzyme active site would be so tolerant (in lieu of an aldol catalyzing base) as to bind carbaphosphonate 4 so tightly. Nanomolar enzyme inhibitors are, after all, not that common. It seems more reasonable to formulate the existence of an enzyme active site residue which enables DHQ synthase to play an active catalytic role during intramolecular aldol condensation of intermediate D. Therefore, although DHQ synthase may not be a catalytic "wolf," the enzyme is probably a good deal more than a catalytic "sheep."

EXPERIMENTAL

Materials.

DAHP was synthesized according to the procedure of Frost and Knowles[8] from methyl (methyl 3-deoxy-D-*arabino*-heptulopyranosid)onate. This starting material was obtained by the procedure elaborated by Reimer et al.[9] with the exception that *E. coli* BJ502*aroB*(pKD130A)[10] was used for the whole cell synthesis of 3-deoxy-D-*arabino*-heptulosonic acid instead of *E. coli* JB-5. Dehydroquinate synthase was isolated from *E. coli* RB791 (pJB14) according to the procedure of Frost et al.[11] High purity NAD^+ (grade V-C) was obtained from Sigma. Lower grades of NAD^+ contained a contaminant which interfered with the enzyme assay.[4b]

Methods.

Spectrophotometric assays were carried out on a Perkin-Elmer Lambda 3B Spectrophotometer. Quantitation of protein concentrations relied on Coomassie dye binding.[12] Enzyme was desalted with a Bio-Rad PD-10 desalting column prior to use. Least-squares curve fitting utilized the computer program Eureka 1.0 (Borland International). Computational molecular superimpositions were performed on VAX8800 and Cray2 platforms. The Sybil molecular modelling system, Version 5.3, was acquired through Tripos Associates. Other computations utilized the PM3 Hamiltonian (MOPAC 5.0, QCPE 455, J.J.P. Stewart, F.J. Seiler, Quantum Chemistry Program Exchange) and CHARMm (Quanta 3.0, CHARMm 21.2, Polygen Corporation).

Determination of the Rate Constant (k_{on}) for Carbaphosphonate 4 Binding to DHQ Synthase.

Assay solutions consisted of deionized, glass distilled water containing 50 mM MOPS buffer

at pH 7.5, cobalt(II) chloride (0.25 mM), NAD^+ (0.25 mM) and DAHP (0.025 - 0.2 mM). After incubation at 15 °C for 15 min, enzyme (0.05 nmol/mL) was added and aliquots (0.125 - 0.5 mL) were removed at timed intervals and quenched with 20% (w/v) trichloroacetic acid (0.1 - 0.225 mL). Product inorganic phosphate was quantitated by the Ames procedure.[13] Points on the progress curve were fitted to the equation:[14] absorbance = $a - be^{-kt}$ (where t = time and a, b, and k are adjustable parameters). Only progress curves with a final velocity much less than the initial velocity were used. The association rate constant, k_{on}, was calculated from:

$$k_{on} = \frac{(k - k_{off})\,(1 + [DAHP]/K_m)}{[\text{ carbaphosphonate } \underline{4} \text{ }]}$$

using $K_m = 18 \times 10^{-6}$ M and a value for k_{off} which was determined as described below. The calculated k_{on} values did not change with the concentration of DAHP or carbaphosphonate 4.

Determination of the Rate Constant (k_{off}) for Carbaphosphonate 4 Release from DHQ Synthase.

Carbaphosphonate 4 (3.75 μM) and DHQ synthase (0.94 or 1.25 μM) were incubated together at 15 °C for 30 min in 2 mL of a pH 7.5 solution containing MOPS (50 mM), cobalt(II) chloride (0.25 mM) and NAD^+ (0.25 mM). An aliquot (40 μL) of this solution was then added to a solution of DAHP (0.7 mM) in the same MOPS/Co(II)/NAD^+ buffer at 15 °C, and the progress of the reaction was followed by the phosphate production (for 40 min) as above. Points on the progress curves were fitted to the equation: absorbance = $a + be^{-kt} + c$.

Detection of Enzyme-bound NADH During Incubation of DHQ Synthase with Carbaphosphonate 4.

A solution of DHQ synthase (20 μM) in 50 mM MOPS (pH 7.5) containing cobalt(II) chloride (50 μM) and NAD^+ (10 μM) was placed in a quartz cuvette, and the solution was scanned from 400 nm to 250 nm in a spectrophotometer to set the baseline absorbance. A small aliquot of a solution of carbaphosphonate 4 was added to make the enzyme solution 30 μM in the carbaphosphonate. Scans were made at timed intervals until there was no further rise in absorbance at 340 nm.

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