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Brief Article

NMR Studies Uncover Alternate Substrates for Dihydrodipicolinate Synthase and Suggest That Dihydrodipicolinate Reductase Is Also a Dehydratase

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Despite extensive effort, the drug target dihydrodipicolinate synthase (DHDPS) continues to evade effective inhibition. We used NMR spectroscopy to examine the substrate specificity of this enzyme and found that two pyruvate analogues previously classified as weak competitive inhibitors were turned over productively by DHDPS. Four other analogues were confirmed not to be substrates. Finally, our examination of the natural product of DHDPS and its degradation revealed that dihydrodipicolinate reductase (DHDPR) possesses previously unrecognized dehydratase activity.

Introduction

Dihydrodipicolinate synthase (DHDPS,[a] EC 4.2.1.52) and dihydrodipicolinate reductase (DHDPR, EC 1.3.1.26) are two enzymes central to the diaminopimelate pathway for lysine biosynthesis. Bacteria and plants are able to biosynthesize lysine, but mammals lack this pathway, so the enzymes of this pathway have been proposed as targets for development of antibiotics and herbicides.[1−3] Despite significant effort, discovery of effective active-site targeting compounds for either of these enzymes has so far met with only limited success.[4,5] To improve the prospects of future studies, we have undertaken an extensive NMR spectroscopic study of this enzyme couple, which clarifies the chemical nature of the reactions catalyzed by both enzymes and sheds new light on the substrate specificity of these two potential drug targets.

Three different assay systems for DHDPS have been described in the literature.[6] The first relies on the DHDPS product reacting with *o*-aminobenzaldehyde to quantitatively form an as yet unidentified purple chromophore. The second relies upon measurement of the absorbance at 270 nm when the reaction is carried out in an imidazole buffer. Again, the exact nature of the compound causing the change in absorbance is unknown, but it is known to involve a chemically formed derivative of the initially formed product. Both the *o*-aminobenzaldehyde and imidazole assays have another major disadvantage of displaying a significant lag period before entering a linear phase. The third, and most commonly used, is a coupled assay system utilizing excess DHDPR, which catalyzes the reduction of the DHDPS reaction product with the concomitant oxidation of NAD(P)H, causing an easily monitored reduction in absorbance at 340 nm. This assay has the advantage that reaction progress can be observed in real time and without any lag phase, greatly simplifying kinetic analysis. Furthermore, the same system can be used for study of DHDPR by simply ensuring that this enzyme is limiting and DHDPS is in excess, and in fact this coupled assay is the only system that has been used to determine the kinetic parameters of DHDPR.[4,7−11] Combined, these assays allow successful monitoring of any purification of a DHDPS enzyme and complete kinetic characterization of the purified protein. However, they are all specific for the natural product of the enzyme and cannot readily be used to assess the ability of DHDPS (and DHDPR) to accept alternate substrates.

This reliance on biochemically specific, but chemically indistinct, assay methods has fostered a lack of understanding of the chemistry that takes place between the enzymes DHDPS and DHDPR. Blickling et al. have shown, using NMR spectroscopy, that the product of DHDPS is not dihydrodipicolinate (DHDP) as originally proposed, but in fact (4*S*)-4-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinic acid (HTPA).[12] This has widely been assumed to dehydrate spontaneously in solution to DHDP,[12] which is then reduced by DHDPR, but this assumption has, until now, never been tested.

In this paper, we have used NMR methods to examine the substrate specificity of DHDPS for pyruvate analogues and found that the enzyme is able to accept a broader range of substrates than has previously been suggested. We have also inspected the detailed chemistry of the product of DHDPS and the substrate of DHDPR and suggest that this well characterized reductase also has a previously unrecognized dehydratase activity. These results will inform future efforts at inhibiting these two important enzymes and are likely to improve prospects in this area.

Results and Discussion

DHDPS is a putative antibiotic target for which, to date, no potent inhibitor has been found; thus in order to better

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[a]Abbreviations: DHDPS, dihydrodipicolinate synthase; DHDPR, dihydrodipicolinate reductase; DHDP, dihydrodipicolinate; HTPA, (4*S*)-4-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinic acid.

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understand the capacity of the active site to bind substrate analogues, we used NMR spectroscopy to explore the substrate specificity of DHDPS. In contrast to previous studies on bromopyruvate, which is an irreversible inhibitor of DHDPS,[13,14] we were interested in identifying pyruvate analogues that were still substrates, especially those with modifications at the reacting centers. Knowledge of the tolerance to substitution at these centers will inform downstream design of mechanism-based inhibitors which could be expected to exhibit both high specificity and high efficacy through irreversible inhibition. To this end, we used ^{1}H NMR spectroscopy to investigate a series of pyruvate analogues for reactivity in the DHDPS system.

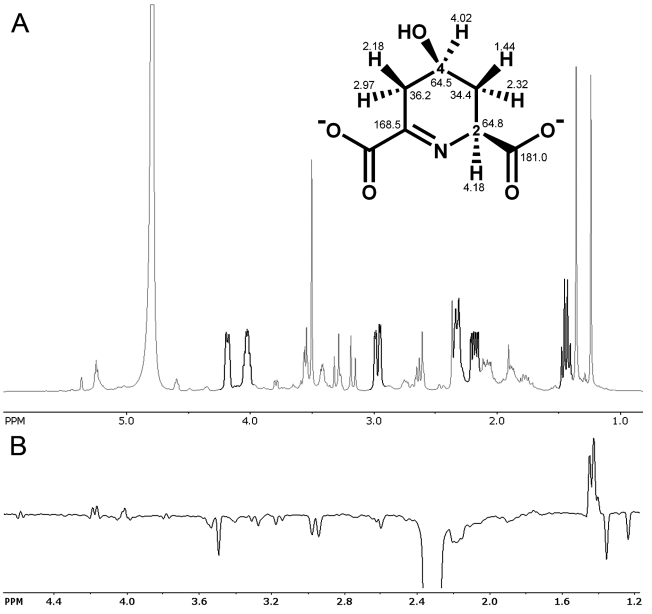

Figure 1. (A) ^{1}H NMR spectrum of HTPA with contaminating signals due to buffer components, pyruvate (and pyruvate dimer[21]), and (*S*)-ASA greyed out and (B) 1D slice of the NOESY spectrum showing key through space correlations from $H3_{ax}$ to H2 and H4. This ^{1}H NMR spectrum is of the same sample used to obtain the NOESY and 2D NMR spectra (see SI). A cleaner, optimized ^{1}H NMR spectrum is presented in the SI.

Before examining pyruvate analogues, it was necessary to optimize the reaction conditions and spectrum acquisition parameters with the natural substrates. It was found that the DHDPS reaction product degraded relatively rapidly at the reaction pH of 8, but that it was sufficiently stable at pH 12 to allow time-consuming 2D-NMR experiments to be carried out (see Supporting Information (SI) for spectra). These spectra allowed the full characterization of the DHDPS reaction product, which we can confirm is HTPA, as described by Blickling et al.[12] We are able to assign chemical shifts to all protons and carbons of the molecule except one carboxylate group (Figure 1). NOESY experiments (Figure 1) and analysis of proton−proton coupling constants revealed that, in solution, HTPA adopts a chairlike conformation, as might be expected, with the substituents all placed equatorially. This results in the 4-hydroxy group having no antiperiplanar oriented proton for facile elimination via the E2 mechanism, and thus raised the question of whether in fact the dehydration of this compound is the spontaneous process that has been previously suggested.[12]

The dehydration of HTPA was investigated using time-course experiments at pH 8.0, which showed that HTPA clearly degrades in solution, although no clear single degradation product was observed, suggesting that the degradation pathway consists of a mixture of imine hydrolysis, imine/enamine tautomerism, and associated reactions. No signals consistent with those that would be expected for DHDP could be detected at any point during HTPA degradation (Figure 2), which suggested to us that the true substrate for DHDPR may in fact be HTPA rather than DHDP.

To test this hypothesis, we added varying quantities of DHDPR to preformed HTPA in the presence of excess NADPH and monitored the rate of HTPA disappearance by ^{1}H NMR spectroscopy. The data clearly show that rate of HTPA consumption is dependent on DHDPR concentration (Figure 3) and thus, by implication, that DHDPR actually accepts HTPA, rather than DHDP, as a substrate.

There are three conceivable mechanisms for an overall replacement of a hydroxyl group with a hydride that are not distinguishable at this point: elimination (presumably to give a conjugated diene) followed by reduction of the newly formed double bond; S_N2 type nucleophilic displacement of the hydroxyl by the incoming hydride, and S_N1 type reaction

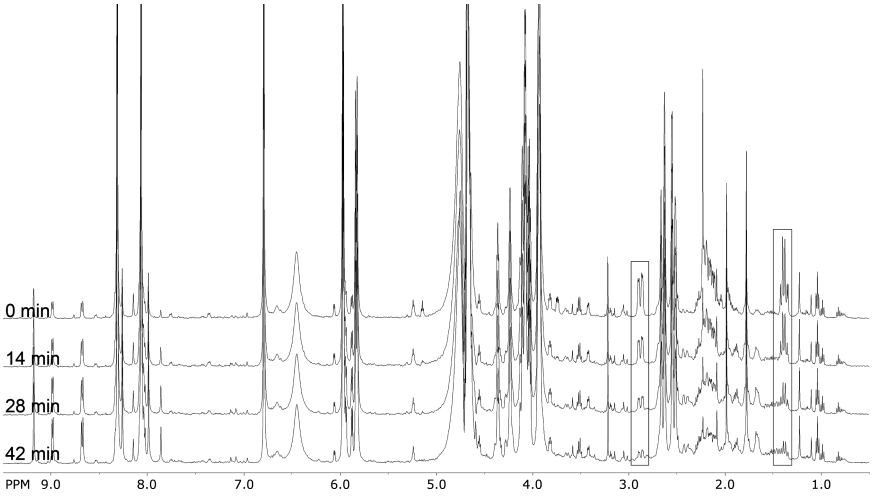

Figure 2. Stacked spectra of HTPA (time of acquisition after reaction initialization is shown on the left of the traces) showing nonspecific decomposition, and the absence of olefinic protons that would be characteristic of DHDP. The characteristic signals of HTPA which are not obscured by signals from NADPH or enzyme buffer are boxed.

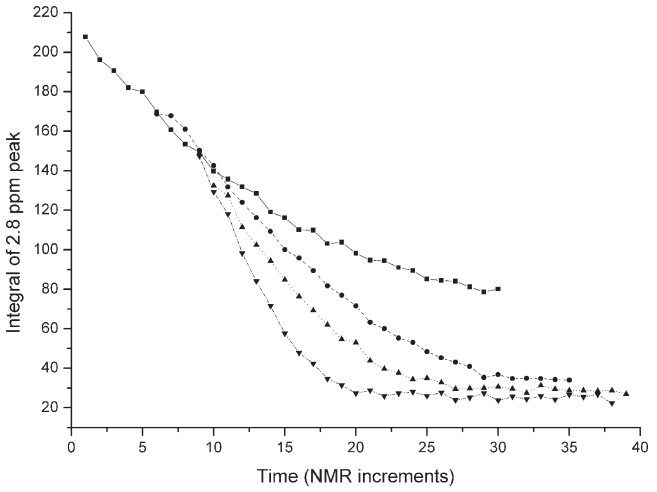

Figure 3. Loss of HTPA in the presence of differing amounts of DHDPR (− ■ − 0 μg DHDPR; -- ● -- 5 μg DHDPR; · · · · ▲ · · · · 10 μg DHDPR; − · − ▼ − · − 20 μg DHDPR). The horizontal offset in starting points is due to reaction progression during NMR setup time prior to data acquisition.

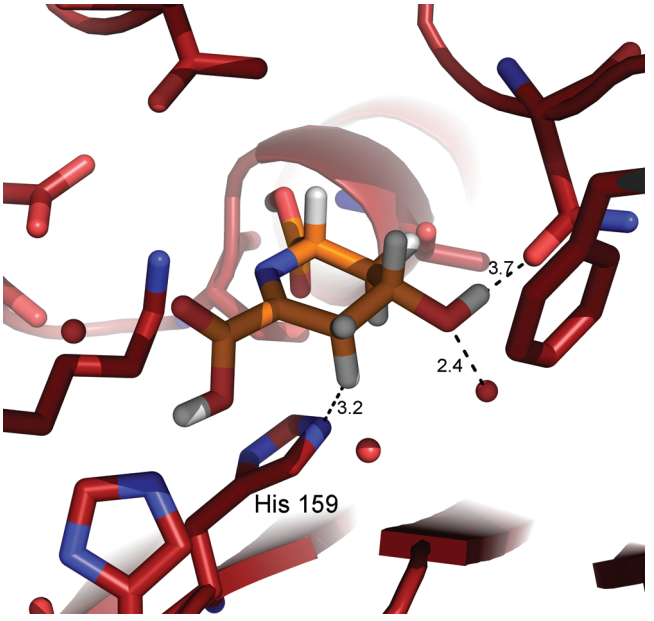

Figure 4. Structure of the active site of *Escherichia coli* DHDPR (PDB ID: 1ARZ[16]) with HTPA (orange) manually modeled in place of the pyridinium dicarboxylate from the PDB. Conserved active site residues are shown as sticks and selected distances between active site residues (or water) and HTPA are indicated.

with loss of the hydroxyl group to form a carbocation at the 4-position, which is then attacked by the incoming hydride. Of these possible mechanisms, the elimination−reduction mechanism seems the most likely, with the dehydration step possibly proceeding via an enamine precursor, analogous to the mechanism outlined for deoxy sugar biosynthesis in granaticin biosynthesis.[15]

Previous mutagenesis studies have revealed the critical role played by residue His 159 in the catalytic mechanism of DHDPR (Figure 4).[16] Replacement of this residue with an alanine or a glutamine was reported to result in a 150−200-fold reduction in catalytic rate as well as a 6-fold increase in K_M. His 159 has been proposed to act as a general acid during catalysis, providing the proton required after hydride addition

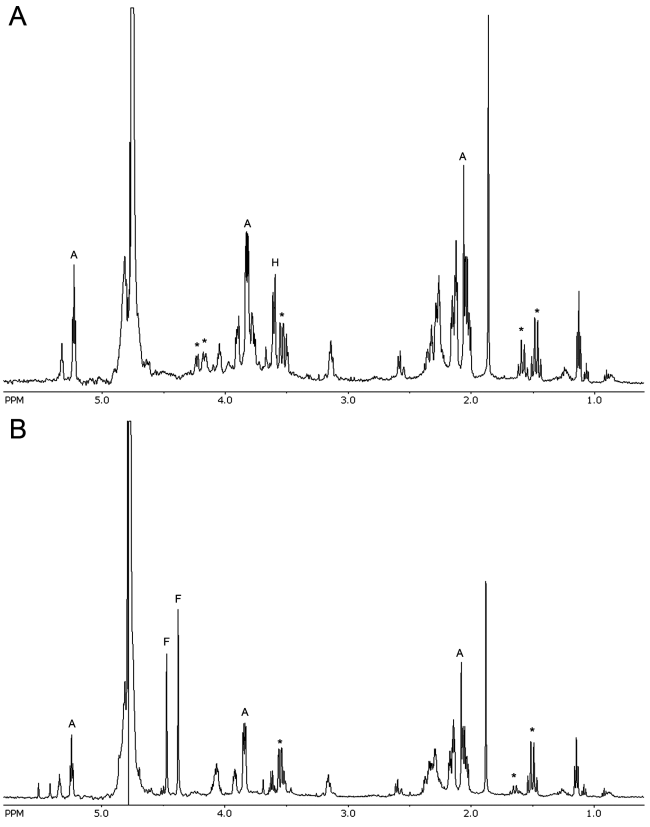

Figure 5. ^{1}H NMR spectra of reaction catalyzed by DHDPS in the presence of (*S*)-ASA and (A) β-hydroxypyruvate and (B) 3-fluoropyruvate. Peak annotations are: A, (*S*)-ASA; H, β-hydroxypyruvate; F, 3-fluoropyruvate and * product. Product formation is most easily identified by the quartet-like signals at ~1.4 ppm.

to the double bond of DHDP. It seems reasonable to speculate that this residue could also play a role in an initial dehydration step by acting as a base, deprotonating C5 of HTPA prior to hydroxide loss to form DHDP, which would then be reduced according to the previously proposed mechanism.

With methodology for examining DHDPS reaction products established, we turned to investigating the substrate specificity of DHDPS and found that oxobutyrate, oxamic acid, oxaloacetate, and α-ketoglutarate are not accepted as substrates by DHDPS but both β-hydroxypyruvate and 3-fluoropyruvate react with (*S*)-ASA when DHDPS is present, albeit very slowly (Figure 5). Previous reports have identified oxobutyrate, α-ketoglutarate, β-hydroxypyruvate, and 3-fluoropyruvate as competitive inhibitors of DHDPS. The last two are now revealed to be competitive substrates.

β-Hydroxypyruvate formed a pair of diastereomeric products (based on observation of two quartet-like resonances at 1.39 and 1.51 ppm) with little preference (integral ratios of 1.1:1). The formation of a product with 3-fluoropyruvate was significantly slower than with β-hydroxypyruvate, although low level NMR signals of product could be seen in the spectrum after 10 min of reaction time. The sample was incubated at room temperature for an hour before acquiring further spectra, which showed a clear accumulation of product. In contrast to the β-hydroxypyruvate reaction product, the product of 3-fluoropyruvate reaction was heavily biased in favor of a single diastereoisomer (integral ratios of 3:1).

Utilization of these two substrates in the coupled assay with DHDPR did not show any consumption of NADPH, thus the products of DHDPS reaction with β-hydroxypyruvate and

3-fluoropyruvate are not accepted as substrates by DHDPR. This is consistent with previous results, which indicated that DHDPS displays very high substrate specificity, not accepting any substrate analogues, although this was examined using the coupled assay.[17,18] This result highlights one of the primary limitations intrinsic to coupled assays—they are liable to provide false negative results in substrate specificity studies as the alternate substrate must be accepted by both enzymes to give a positive result.

Conclusion

It appears that the true substrate of DHDPR is in fact HTPA, suggesting that DHDPR overall catalyzes a deoxygenation reaction, likely by a dehydratase−reductase route, although further experiments will be needed to confirm this.

We have found that DHDPS has a less restrictive substrate specificity than previously thought—both β-hydroxypyruvate and 3-fluoropyruvate are turned over, although at significantly slower rates than is the natural substrate pyruvate. To be accepted as a substrate, it seems that an electronegative substituent at the 3-position is necessary, as oxobutyrate, carrying a methyl group at the 3-position, was not accepted as a substrate despite possessing a similar steric bulk to β-hydroxypyruvate. Neither of the HTPA derivatives produced by DHDPS reacting pyruvate analogues were substrates for DHDPR, which seems to have a more discerning active site than does DHDPS.

The results described here will be useful for inhibitor design studies as they indicate that pyruvate analogues carrying electron withdrawing groups at the 2-position could be suitable starting points for inhibitor development and that inhibitors of DHDPR should be based on the HTPA molecule rather than DHDP.

Experimental Section

Materials. All reagents were of the highest grade and obtained from Sigma-Aldrich Chemical Co. or Invitrogen Ltd. unless otherwise stated. (*S*)-ASA was prepared via the Weinreb amide route and was of a high purity as determined by ^{1}H NMR spectroscopy.[18]

Enzyme Overexpression and Purification. *Escherichia coli* DHDPS and DHDPR were prepared as described previously.[19,20] Coupled enzymatic assays were carried out according to established methods.[11,20]

NMR Spectroscopy. NMR spectra were obtained on a Varian INOVA spectrometer, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C detection and temperature regulated to 23 °C. Spectra obtained in H_2O were locked and shimmed on a D_2O insert and run using a presaturation pulse sequence with a satpwr of 20, satdly of 3.0 s, gain of 24, and a d1 of 0 s to reduce the intensity of the water signal. Spectra were referenced to either the water signal at δ 4.79 ppm or the methyl signal from added *t*-butanol at δ 1.24 ppm for ^{1}H and 30.3 ppm for ^{13}C.

For substrate specificity studies, reaction was initiated immediately prior to acquisition of spectra by addition of 50 μg DHDPS to an NMR tube containing (*S*)-ASA (12 mM) and pyruvate analogue (12 mM) in 100 mM sodium phosphate buffer, pH 8.0, to give a final volume of 500 μL. For each compound, a series of five spectra were obtained as described above, with a 2 min preacquisition delay between each spectrum. If there was any indication of reaction, the reaction was incubated at room temperature for 60 min before acquisition of further spectra.

HTPA degradation studies were carried out as described above, although with the addition of 0.25 mM *t*-butanol (i.e., 2.25 mM *t*-butanol methyl protons). Fifteen successive spectra were obtained and integrals measured following drift correction and normalization against the *t*-butanol peak, which was assigned an integral of 22.5 units (each unit corresponding to 0.1 mM proton concentration).

To examine the DHDPR-dependency of HTPA consumption, reactions were executed as described above, although with the further addition of 18 mM NADPH and varying amounts of DHDPR (0−20 μg) as indicated. Thirty spectra were obtained successively for each sample. Integrals were obtained following drift correction and normalization of each spectrum against the *t*-butanol peak, which was assigned an integral of 22.5 units.

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Supporting Information Available: A clean, optimized ^{1}H NMR spectrum of HTPA and 2D NMR spectra (COSY, NOESY, HSQC, and HMBC) of the DHDPS reaction product. This material is available free of charge via the Internet at http://pubs.acs.org.

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