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A direct spectropolarimetric assay of arabinose 5-phosphate isomerase

Todd M. Kijek [a,1], Joel A. Bozue [b], Rekha G. Panchal [a], Vladislav A. Litosh [c], Ronald W. Woodard [d], S. Ashraf Ahmed [a,*]

[a] Systems and Structural Biology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, 21702, USA
[b] Bacteriology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, 21702, USA
[c] Medical Countermeasures Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, 21702, USA
[d] Department of Chemistry, University of Michigan, Ann Arbor, MI, 48109-1065, USA

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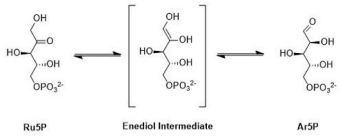
ABSTRACT

Arabinose 5-phosphate isomerase (API) catalyzes the reversible isomerization of Ribulose 5-phosphate (Ru5P) to Arabinose 5-Phosphate (Ar5P) for the production of 3-deoxy-2-octulosonic acid 8-phosphate (KDO), a component of bacterial lipopolysaccharide (LPS) of gram-negative bacteria. API is an attractive target for therapeutic development against gram-negative bacterial pathogens. The current assay method of API activity utilizes a general reaction for keto sugar determination in a secondary, 3-h color development reaction with 25 N sulfuric acid which poses hazard to both personnel and instrumentation. We therefore aimed to develop a more user friendly assay of the enzyme. Since Ru5P absorbs in the UV region and contains at least 2 chiral centers, it can be expected to display circular dichroism (CD). A wavelength scan revealed indeed Ru5P displays a pronounced negative ellipticity of 30,560 mDeg $M^{-1}cm^{-1}$ at 279 nm in Tris buffer pH 9.1 but Ar5P does not have any CD. API enzymatic reactions were monitored directly and continuously in real time by following the disappearance of CD from the Ru5P substrate, or by the appearance of CD from Ar5P substrate. The CD signal at this wavelength was not affected by absorption of the enzyme protein or of small molecules, or turbidity of the solution. Common additives in protein and enzyme reaction mixtures such as detergents, metals, and 5% dimethylsulfoxide did not interfere with the CD signal. Assay reactions of 1–3 min consistently yielded reproducible results. Introduction of accessories in a spectropolarimeter will easily adapt this assay to high throughput format for screening thousands of small molecules as inhibitor candidates of API.

1. Introduction

Arabinose 5-phosphate isomerase (API) catalyzes the reversible isomerization of Ribulose 5-phosphate (Ru5P) to Arabinose 5-Phosphate (Ar5P). Ar5P is the precursor of 3-deoxy-2-octulosonic acid 8-phosphate (KDO), a component of bacterial lipopolysaccharide (LPS) of gram-negative bacteria [1]. API and other enzymes of the KDO pathway are sufficiently conserved among gram-negative bacteria and are essential for growth of the bacteria. Recent investigations in our laboratory have established that API is essential for *Francisella tularensis* virulence [2].



Isomerization of Ar5P by API

F. tularensis is a gram-negative facultative intracellular bacterial pathogen and is the causative agent of tularemia that infects many mammals including humans. Because of its ability to cause a lethal infection, low infectious dose, and aerosolizable nature, *F. tularensis* subspecies *tularensis* is considered a potential biowarfare agent [3]. There is a critical need for additional therapeutic development against this organism. API is an attractive target for such therapeutic

* Corresponding author.
E-mail address: syed.a.ahmed.civ@mail.mil (S.A. Ahmed).
[1] Present Address: 2769 PGA Blvd, Navarre FL, 32566, USA.

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development [2,4–7]. The current approach to therapeutic development against most pathogens heavily relies on high throughput screening assay of the enzymes and other targets in the presence of thousands of small organic molecules.

The main hurdle to this approach is to devise a convenient assay method for API enzymatic activity. The current assay method relies on the sulfuric acid conversion of the ketosugar Ru5P into furfural followed by its prolonged incubation with cysteine and carbazole in the presence of 25 N sulfuric acid to produce a color adduct [7]. With an extinction coeffient ε_{549} of 250,200, $M^{-1}cm^{-1}$ the method is sufficiently sensitive [8]. However, the method is not specific for Ru5P but is a general method for measuring almost all ketosugars. In addition, use of such high concentration of sulfuric acid is health, workplace, and environmental hazard. Moreover, it takes as much as 3 h for the color development alone [7]. A direct UV-spectrophotometric assay of Ru5P should eliminate these drawbacks. Although Ru5P absorbs at 280 nm, it has a very low extinction coefficient ε_{280} of 50–60 $M^{-1}cm^{-1}$ [4,9] which makes it extremely insensitive, and thus less useful. Besides, many small organic molecules strongly absorb at 280 nm to obscure the absorption changes due to enzymatic activity determined by absorbance. Therefore, we explored for an alternative approach to API activity assay.

Assay of many amino acid racemases takes the advantage of a chiral center in either of its D- or L-substrate which display specific but opposite optical rotation at one or more wavelengths in a polarimeter [10–16]. Like most sugars, Ru5P or Ar5P can also be expected to display optical rotation of polarized light. As such, Ru5P having more than one chiral center is also expected to display circular dichroism (CD) [17]. We conducted a CD wavelength scan of Ru5P and found it displays a pronounced negative ellipticity centered at 279 nm. In contrast, Ar5P did not display any CD at any wavelength. We found that the API enzymatic reactions can be monitored directly and continuously in real time for as little as 1–3 min by following the disappearance of CD from the Ru5P substrate, or the appearance of CD from the conversion of Ar5P substrate into RuP product. The CD signal was not affected by absorbance or fluorescence of small molecules, or turbidity of the reaction mixture. These advantages coupled with a very short assay time could be conveniently applied to screen large batch of small molecule inhibitors of API activity.

2. Materials and methods

Francisella tularensis API was purified from *E. coli* harboring a multicopy recombinant plasmid as previously described [7]. The substrates Ar5P, Ru5P, and most common reagents used were purchased from Sigma-Aldrich. Enediol compounds were custom synthesized and purified by New England Discovery Partners (23 Business Park Drive, Branford Drive, CT 06404, USA).

CD spectra were recorded in 1 mm light path quartz cuvettes at 20 °C or as indicated with a Jasco 718 spectropolarimeter equipped with a Jasco Peltier-temperature controller. An average of 5 scans at 20 nm/min scan speed with a response time of 8 s was recorded to increase signal-to-noise ratio. In all spectral measurements, a buffer blank was recorded separately and subtracted from sample recordings. Time course of enzyme reactions were measured by following the ellipticity changes at 30 °C or as indicated.

Unless and otherwise mentioned, all enzyme reaction mixtures contained 5 mM Ru5P and 1 mM EDTA in 50 mM Tris-HCl pH 9.1 and variable amounts of API in a total volume of 200 μl, that was incubated at 30 °C. Data collection was started within 10 s of addition of 1–5 μl API to start the reaction. Unless mentioned otherwise, all results are average of at least two assays.

NMR identification of product of D-arabinose 5-phosphate isomerization. 2-[^{13}C]-D-Arabinose-5-monophosphate was obtained by enzyme-driven selected phosphorylation as described [18] of commercially available 2-[^{13}C]-D-arabinose (Omicron Biochemicals, Inc.). A 500 μl solution 50 mM 2-[^{13}C]-D-Arabinose-5-monophosphate in D_2O was placed into a 2 mm NMR tube. 10 μl of 10 mM Tris buffer was added, and ^{13}C NMR spectrum with no ^{13}C–^{1}H or ^{13}C–^{31}P was recorded on the 500 MHz Bruker Avance III NMR spectrometer (NCI branch at Fort Detrick, MD). After recording the initial spectrum, 5 μl of 81 μM solution of the API enzyme was added. The ^{13}C NMR spectra were recorded at 5 min interval for 30 min, followed by 10 min interval for 100 min, at 420 min and 1260 min since commencement of the reaction.

3. Results

3.1. CD properties of substrates & API

We collected CD data of both Ar5P and Ru5P over a wavelength range of 180–900 nm at pH 6.83 and 20 °C. While Ar5P did not display any significant CD, Ru5P showed only one intense CD signal centered at 279 nm (Fig. 1A). This signal was not affected by changing the temperature from 5 °C to 50 °C or by adding 5%DMSO (see later, Fig. 5), or 0.05% Triton X-100 or 0.05% Tween-20, or turbidity of the solution (data not shown). The signal was not changed by the presence of 1 mM metal ions, EDTA or DTT, the common reagents used in enzyme assays. These results suggested that an enzyme assay by following CD at 279 nm should not be affected due the changes in the Ru5P substrate properties by these common reagents. Fig. 1B shows the far-UV CD spectra of 5.6 μM API indicating a typical helical secondary structure [17], but having no signal at 260 nm, and above using 1 mm path length cuvettes (not shown). Therefore, an enzymatic assay following CD changes at 279 nm will not be affected by the enzyme itself.

The CD of Ru5P is not adversely affected by its concentration (Fig. 2A). In addition, data generated from 0.2 mM to 10 mM Ru5P perfectly follows a straight line at pH < 1 (0.5 N HCl), 6.8 (water) and 9.1 (50 mM Tris buffer) (Fig. 2B). Linearity of the straight line was maintained up to 30 mM Ru5P (not shown), the maximum concentration used in these assays. These results show that depending on the purpose of the assay and availability, very low (0.2 mM) to very high (10 mM) substrate concentration can be employed without any compromise to the CD signal (Fig. 2B). From the slope of the straight lines in Fig. 2B, it is evident that the intensity of the CD signal decreases with pH. However, retention of more than 70% of the maximum CD signal at the low pH of 1 or below will be ideal to follow enzyme reactions that are stopped by acidifying the reaction mixtures (see later). We calculated a CD of 30,560 $M^{-1}cm^{-1}$ for a molar solution of Ru5P at pH 9.1 in 50 mM Tris-HCl buffer. This value is over 60 times more sensitive than the molar absorbance of Ru5P at 280 nm (ε_{280} of 50–60 $M^{-1}cm^{-1}$) [4,9] but almost an eighth of the cysteine-carbazole color adduct (ε_{549} of 250,200 $M^{-1}cm^{-1}$) [8]. The nearly perfect straight lines in Fig. 2B are also advantageous because absorbance of all chromophoric solutions decrease with the increase in concentration of the chromophore generating a curved line according to the Bear-Lambert's Law. As such, a measured absorbance above 2.0 usually is not accepted as valid. CD measurement is thus free from the substrate (or product) concentration effect.

At pH 0.1–1.0 (0.5 N HCl) intensity of the negative peak at 279 nm is reduced by 28% (Fig. 2B), but it does not change further over time (data not shown). Should a reaction be terminated by acidifying the reaction mixture, a standard curve must be constructed at that pH. We calculated a molar ellipticity of Ru5P in 0.5 N HCL as 22,200 $M^{-1}cm^{-1}$. At pH 14 (1 N NaOH) the CD signal of Ru5P gradually disappeared (not shown), most likely due to its non-enzymatic conversion to Ar5P or another sugar (Lobry De van Bryn-Ekestein Reaction) that display no ellipticity (Fig. 1A) [19]. However, by leaving the Ru5P solution at pH 9.5 for several hours did not decrease the CD signal (data not shown). But the molar ellipticity of Ru5P is highest (Fig. 2B).

3.2. Isomerization of Ru5P & Ar5P by API

Incubation of Ru5P with API resulted in gradual decrease in

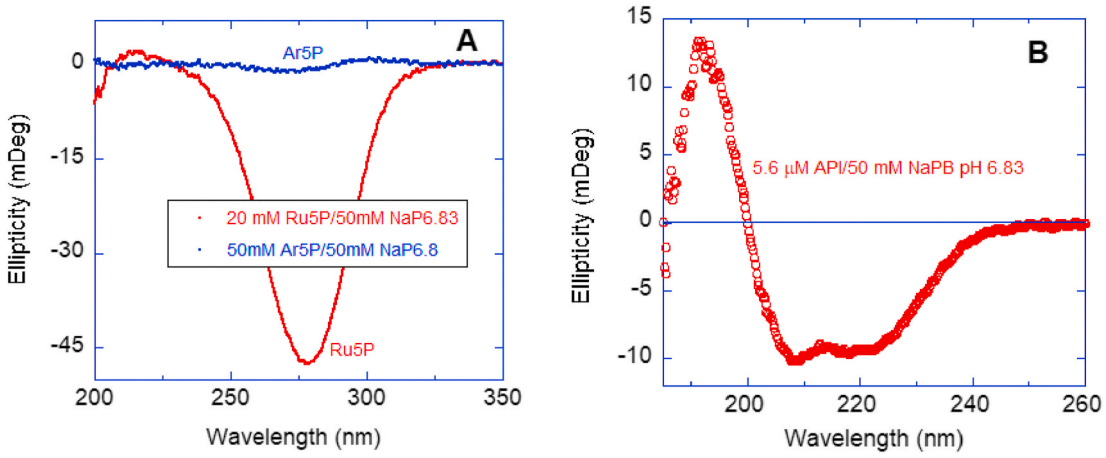

Fig. 1. Circular dichroism spectra of the substrate Ru5P (20 mM) and Ar5P (50 mM) (A), and 5.6 µM API from *Francisella tularensis* (B) in 50 mM NaPB pH 6.83 at 20 °C. Spectra were collected from 900 nm to 180 nm in 1 mm path length quartz cuvettes. Only the portions of the wavelength range displaying noticeable ellipticity are shown.

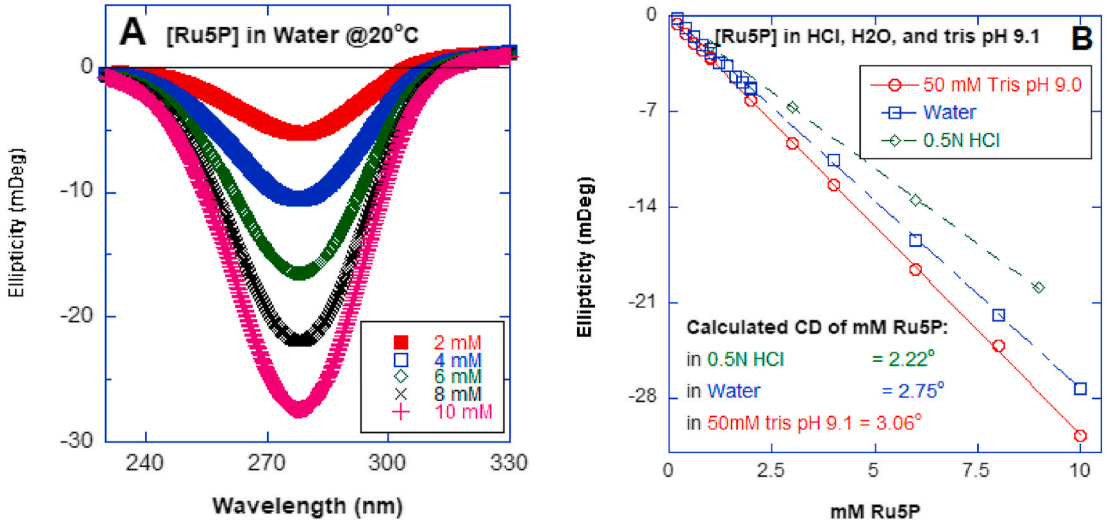

Fig. 2. CD spectra of various concentrations of Ru5P in water at 20 °C (A), and standard curve of Ru5P concentrations in water, HCl, and buffer versus CD at 279 nm (B). The data points in B were taken from figures similar to A. Each spectrum in A is an average of 5 scans.

ellipticity (Fig. 3A and B), presumably due to its conversion to Ar5P which does not show any ellipticity. Although Ar5P does not show CD, incubation of this substrate with API resulted in appearance of a negative CD at 279 nm, again most likely due to its conversion into Ru5P (Fig. 3A).

In order to obtain proof that the products identified in our newly established CD assay for API are indeed Ru5P and Ar5P, we enzymatically synthesized ^{13}C-labelled Ar5P from D-arabinose and ATP and purified by chromatography. We verified its authenticity by MS and ^{13}C NMR in D_2O. By CD we have verified that this labelled 10 mM substrate in D_2O is converted into Ru5P by API in a time dependent fashion. We then recorded the ^{13}C NMR spectra of 35 mM [2–^{13}C]Ar5P before and after 2–1250 min of reaction with 1.5 µM API in Tris.HCl buffer pH 9.0 at 20 °C in a 500 MHz Bruker Avance III NMR spectrometer (NCI, Fort Detrick, MD). A signal at 81 ppm corresponding to Ar5P gradually decreased with the concomitant increase in a new signal at 208 ppm that was unambiguously identified as belonging to Ru5P (data not shown). This result confirmed that the API enzymatic product that we are measuring by the continuous CD assay method is indeed Ru5P.

Ellipticity of Ru5P incubated with API never reached zero, and incubation of an equimolar Ar5P with API never equilibrated to 50% of maximum Ru5P ellipticity (Fig. 3B). For an isomerase reaction with an equilibrium constant of 1, the CD values from both substrates should meet at midpoint. However starting with 10 mM of either substrate, CD values from both incubations became almost superimposable that was in equilibrium after overnight incubation (Fig. 3B). With an ellipticity of −7.5° at equilibrium (Fig. 3A), we calculated approximately 76% of the sugar exist as Ar5P, and the remaining as Ru5P. This yields an equilibrium constant ([Ru5P]/Ar5P]) of 0.24 favoring Ar5P. This compares with the *E. coli* API equilibrium constant of 0.3, greatly favoring Ar5P [20]. Fig. 3C shows a typical linearity of the progress curve of the reaction of 5 mM Ru5P with API at the initial stage of the reaction. Thus the rate of the API reaction can be measured by following as little as 1–2 min. This compares with a total of more than 3 h needed for the cysteine-carbazole reaction for assaying API [7]. Fig. 3D shows linearity of the reaction rate of 1 mM Ru5P with varying concentration of the API enzyme. The range of concentration of the enzyme used in these assays (40–240 nM) are well within 200 nM employed in the cysteine-carbazole assay of API [7].

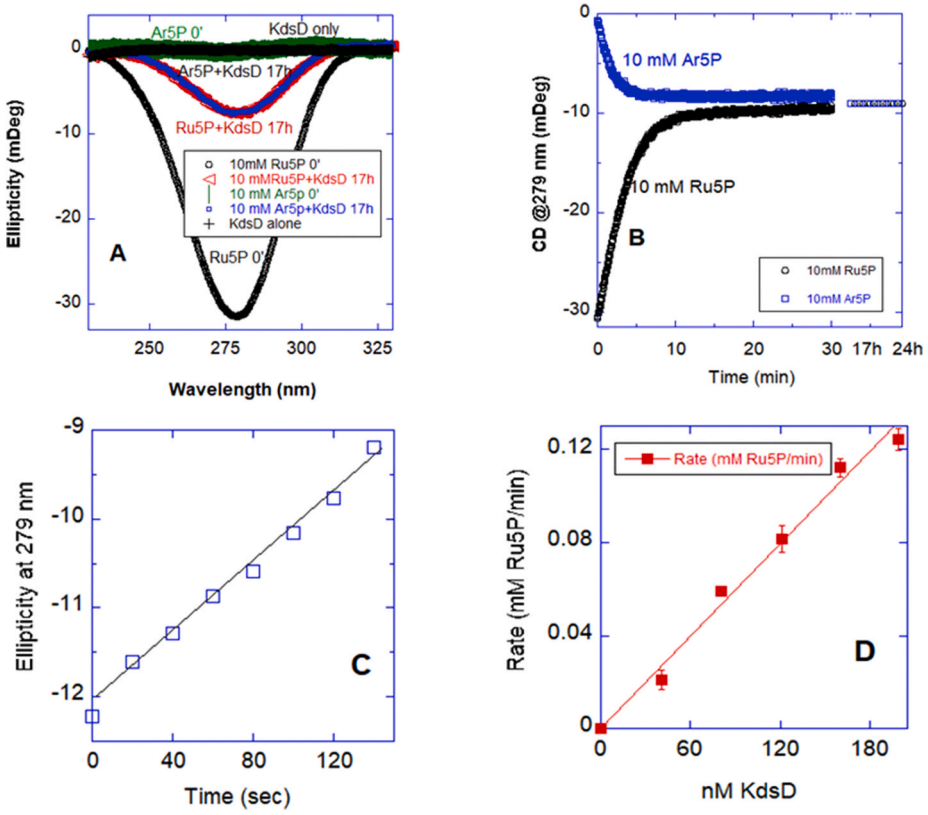

Fig. 3. (A) and (B): (A), CD spectra of 10 mM Ru5P and 10 mM Ar5P alone both in 50 mM Tris.HCl pH 8.1, 20 °C is compared with those of the substrates at equilibrium in the presence of 0.81 μM API in 1 mm path length quartz cuvettes. Ellipticity values at 279 nm were continuously collected upon addition of API. After initial incubation of the substrates with 0.81 μM API at 30 °C for 30 min (as shown in Fig. 3B), the reaction mixtures were incubated for additional 16.5 h at 20 °C. Almost superimposable spectra of the two reaction mixtures after this long incubation indicate that they are at equilibrium. (B), Time course of CD changes at 279 nm for enzymatic reactions of 10 mM Ar5P or 10 mM Ru5P in 50 mM Tris.HCl pH 8.1, 20 °C. C and D: C, Linearity of API reaction rate with time. 5 mM Ru5P was incubated with 2 μM API in 50 mM Tris.HCl pH 8.1. D, Linearity of the reaction rate with increasing concentration of API. 1 mM Ru5P was incubated in triplicate with the indicated concentrations of API in 50 mM Tris.HCl containing 1 mM EDTA (see later). Initial rates of reactions were calculated from plots similar to those shown in C at each API concentration.

3.2. Effects common additives on the activity of API

We investigated the effect of several common metal salts and reagents on the reactions of Ru5P with API as measured by monitoring ellipticity at 279 nm (Fig. 4). Some of these reagents, such as detergents, dithiothreitol (DTT) and ethylenediamine tetraacetate (EDTA) are frequently used with enzymes and enzyme reactions [21]. None of them had any effect on either the intensity or the shape of the CD spectra (data not shown). Some of these however, had profound effect on the API activity (Fig. 4). Divalent metal salts of mercury and zinc almost completely abolished the activity, while those of magnesium, calcium, manganese, cobalt, nickel, and copper increasingly inhibited the activity in this order. That the counterion sulfate has very little effect is demonstrated by the fact that magnesium chloride and magnesium sulfate has very similar effect on the enzyme activity (Fig. 4).

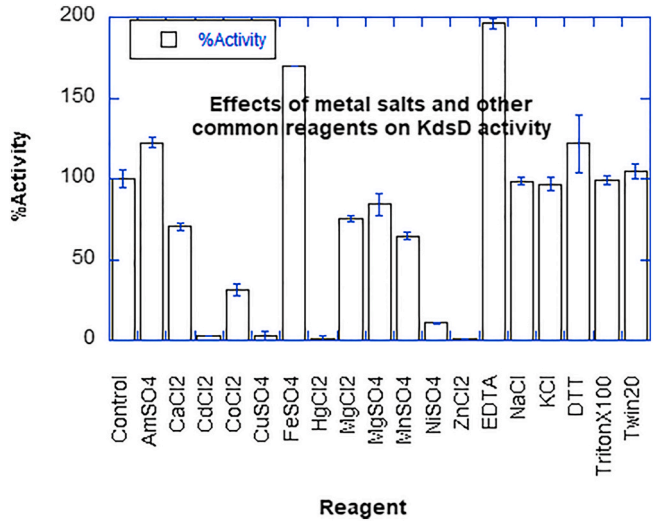

Fig. 4. Effect of divalent metal salts and other common reagents on the catalytic activity of API followed by monitoring ellipticity at 279 nm. The reaction mixtures contained 10 mM Ru5P as a substrate and 2 μM API and 50 mM Tris.HCl pH 9.1. Final concentration of the salts and EDTA was 1 mM, and of Tween-20 or triton X-100 was 0.05%. Activities were calculated from initial rates of 3–4 reactions in each case. Solid bars represent standard deviations.

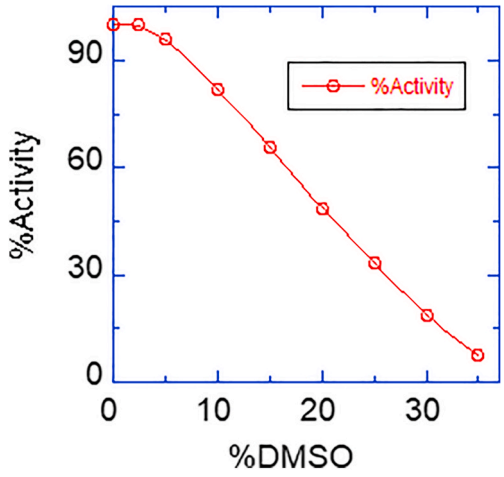

Fig. 5. Effect of increasing concentrations of DMSO on the API enzymatic activity. Activities were calculated from initial rates of reactions containing 10 mM Ru5P, 1 mM EDTA and 2 μM API in 50 mM Tris.HCl pH 9.0.

In a simulated 3-D model, two histidine residues at the active site of the API have been implicated to participate in catalysis [22], as was found in the *Pseudomonas aeruginosa* enzyme ([23] by Mark Olson (USAMRIID, personal communication). The divalent metal induced inhibition observed in Fig. 4 may be due to the metal-histidine interactions

at the active site. The metal chelator EDTA increased the activity twofold. Inhibition by divalent metal salts and activation by the metal chelator EDTA strongly suggest that our API preparation contained metal ions. In fact, the enzyme was purified from Ni-NTA affinity column [7]. The protein might have picked up nickel from the column during the purification step, and our results described here show that $NiSO_4$ strongly inhibited the API. Therefore, all further enzyme activity reaction mixtures included 1 mM EDTA. The divalent iron of ferrous sulfate on the other hand stimulated the activity remarkably. A structural role of iron in API would be an important aspect of further investigation.

3.3. Low concentrations of dimethyl sulfoxide has no effect on CD of Ru5P and on API activity

API is an attractive target for therapeutic development against tuleramia and other gram-negative infections [2]. Since drug-like organic enzyme inhibitors are often insoluble in aqueous solvents, dimethyl sulfoxide (DMSO) is frequently used to solubilize those [24]. Thus in many enzyme reactions, low concentrations of DMSO inadvertently get introduced that may adversely affect the enzyme activity. Determination of the threshold of DMSO concentration is a prerequisite for small molecule inhibitor screening [24]. We therefore looked at the effects of increasing concentrations of DMSO on API activity (Fig. 5). DMSO at 5% or lower concentration had an insignificant effect on the enzyme activity. Above 5%, the solvent progressively inhibited the enzyme activity. At 10% DMSO, the enzyme retained more than 80% activity. Therefore we used 5–10% DMSO in the assay mixture as a control (with no inhibitor) to keep the inhibitors in solution.

4. Discussions

Rotation of polarized light is a unique property of molecules containing one or more asymmetric carbon atoms. Rotation of plane polarized light by such molecules has been routinely used for some racemase enzyme assays [10–16]. On the other hand, extensive application of circularly polarized light, such as CD, is used in protein secondary structure determination [17]. CD of a molecule is the difference in the absorbance of left circularly polarized light and right circularly polarized light. In a limited extent, it has also been used for polysaccharide secondary structure determination [25]. However, compared to UV–Vis absorption spectrophotometry, the utility of CD for routine enzyme activity assays has remained largely unexplored. Only a handful of examples are available. CD-based assays of ornithine decarboxylase [26], mandelate racemase [14], triose phosphate isomerase [27], and amino acid racemase [28] have been used in addition to other more conventional methods. These CD based assays are direct, without the need for additional coupling enzyme or chemical reaction, and can easily be adapted to initial velocity measurements. The basic principle of this assay is that either the reactant or the principal product, but not both, of the enzymatic reaction has a CD ellipticity at a particular wavelength. Progress of the enzyme reaction is accompanied by an increase or decrease of the CD ellipticity. Because no other reactant(s) is necessary, the technique is a direct measure of the enzyme activity and should be a preferred method.

The reaction of API described here uses one substrate Ru5 and produces one product Ar5P. Because the reaction does not require an extraneous energy source, such as ATP, the reaction is also reversible. In other words the product can also serve as a substrate. In this paper, we have shown that Ru5P has a strong negative ellipticity at 279 nm of 30,560 $M^{-1}cm^{-1}$, while Ar5P has no ellipticity (Fig. 1A). The lack of ellipticity of the latter is due to the fact that it does not have any noticeable absorbance at 279–280 nm. The difference in CD properties of these two compounds formed the basis of development of our API assay.

Linearity of a measurement parameter with substrate (or product) concentration, reaction time, and enzyme concentration, are the foundations of a dependable enzyme assay [24]. We have demonstrated that the ellipticity of the API substrate (or product) Ru5P linearly decreases with its increasing concentration (Fig. 2B), ellipticity of the API substrate Ru5P increases linearly in the presence of API with the passage of time (Fig. 3B and D), and the initial rates of the API enzyme reaction are linear with the API concentration (Fig. 3D). Thus, the CD based assay of API fulfills the three essential criteria of a dependable enzyme assay.

Enzyme assays often require component reagents in addition to the substrate(s) and enzyme. We have tested several such reagents including DTT, EDTA, detergents, and metal salts to determine if these compounds might have any adverse effect on the CD property of the substrate(s) or on the enzyme itself. None of these reagents had any adverse effect on the CD of the substrate Ru5P (Fig. 4). Some Metals, such as zinc and nickel, had strong inhibitory effect on the API activity. A similar effect was also observed with the enzyme from *E. coli* [7]. The inhibitory effect of zinc can be utilized as an internal control in screening compound databases as inhibitor or drug candidates. Small organic molecules are often insoluble in aqueous solvents and require DMSO to solubilize. In most cases, DMSO is inhibitory to enzyme activity. Our demonstration in Fig. 5 that low concentrations of DMSO had no effect on Ru5P CD and API enzyme activity makes this spectropolarimetric assay an ideal tool to screen for small molecule inhibitors.

We have also probed the dependability of the CD method by comparing results of our assay with those of the literature determined by a more cumbersome yet conventional method of cysteine-carbazole method [7,8]. Although the latter method is eightfold more sensitive in detecting the substrate Ru5P than our CD method (Fig. 2B), we were able to use 0.4–1 mM substrate consistently (an example is shown in Fig. 3D), a concentration close to 0.5 mM used in the cysteine-carbazole method [7]. The 1 mM concentration of the substrate used is also above the K_m as reported for *F. tularensis* Ar5P (0.3 mM) [7], and for *E. coli* Ar5P (0.57 mM) and Ru5P (0.3 mM) [20]. Validity of our CD method is further demonstrated by the similarity of Ru5P K_m of 0.7 ± 0.1 mM determined by CD with the above mentioned reported values in literature that were determined by the cysteine-carbazole method. We determined this value by measuring the reaction rates of 0.4–2 mM Ru5P with a fixed 20 nM API concentration. Most importantly, the CD method described here eliminates the use of highly corrosive 25 N sulfuric acid in the cysteine carbazole method.

The CD method described here is thus fully compatible with the conventional cysteine-carbazole method. In addition, unlike the latter, our method is direct, avoids the toxic sulfuric acid, and at least 90 times faster. Therefore the CD method should be a routine method of choice for API enzyme assay in screening for inhibitors from large compound libraries.

Although the API assay can be accomplished in 1 min, the method described here manually handles one assay at a time. However, CD spectroplolarimeter manufactures have come up with capabilities to handle 96 samples at a time. For example, both Jasco and Bio-Logics and Hinds Instruments market CD machines that has an accessory to screen 96-well plates in 2 min. Adaptation of such instruments in the API activity assay would accelerate the discovery of small drug-like molecules against targeting API for the discovery of new antibiotics for gram negative bacterial infections.

In conclusion, the API enzymatic assay described here is a direct, rapid, and 'real time' assay that is free from health and environmental hazard, and should be in routine use for screening of compound libraries for drug discovery against *Francisella tularensis*.

In conclusion, the API enzymatic assay described here is a direct, rapid, and 'real time' assay that is free of sulfuric acid use and should be in routine use for screening of compound libraries for targets allowing CD measurements.

Author contributions

Todd M. Kijek – Data Analysis.
Joel A. Bozue – Paper writing & Project supervision.
Rekha G. Panchal – Data Analysis.
Vladislav A. Litosh – NMR experiments and results interpretation.
Ronald W. Woodard – Enzyme purification.
S. Ashraf Ahmed – Conceive method, conduct CD experiments, and write manuscript.

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