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A Continuous Spectrophotometric Assay for APS Reductase Activity with Sulfite-Selective Probes

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Abstract

Mycobacterium tuberculosis (*Mtb*) adenosine 5'-phosphosulfate (APS) reductase (EC number 1.8.4.10), (APR) catalyzes the first committed step in sulfate reduction for the biosynthesis of essential reduced sulfur-containing biomolecules, such as cysteine, and is essential for survival in the latent phase of TB infection. Despite the importance of APR to *Mtb*, and other bacterial pathogens, current assay methods depend on use of [³⁵S]-labeled APS or shunt AMP to a coupled-enzyme system. Both methods are cumbersome and require the use of expensive reagents. Here we report the development of a continuous spectrophotometric method for measuring APR activity by using novel sulfite-selective colorimetric or "off-on" fluorescent levulinate-based probes. The APR activity can thus be followed by monitoring the increase in absorbance or fluorescence of the resulting phenolate product. Using this assay, we determined Michaelis-Menten kinetic constants (K_m , k_{cat} , k_{cat}/K_m) and apparent inhibition constant (K_i) for adenosine 5'-diphosphate (ADP), which compared favorably to values obtained in the gold-standard radioactive assay. The newly developed assay is robust and easy to perform with a simple spectrophotometer.

Keywords

Sulfate assimilation; Adenosine 5'-phosphosulphate (APS); Levulinate; Sulfite Sensor; Adenosine 5'-phosphosulphate reductase (EC number 1.8.4.10;APR)

Introduction

Tuberculosis (TB) is a contagious, and often lethal infection, caused by *Mycobacterium tuberculosis* (*Mtb*). The disease begins when the inhaled mycobacterial bacilli reach alveoli of the lungs. In turn, host macrophages trigger a pro-inflammatory response, recruiting T-cells and neutrophils to form a granular structure around the mycobacteria, known as the granuloma. The granules present a hostile environment, producing high levels of reactive oxygen/nitrogen species (ROS/RNS) in an attempt to neutralize the virulent bacilli. To survive and persist in the host, mycobacteria must neutralize the oxidative assault [1].

In response to oxidative stress, starvation and dormancy adaptation, recent studies demonstrate that *Mtb* up-regulates genes within the sulfate assimilation pathway, whose sulfide product is used in the biosynthesis of cysteine, methionine, and other essential

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reduced sulfur-containing co-factors [1]. Adenosine 5'-phosphosulfate (APS) reductase (APR) catalyzes the first committed step in this reductive pathway in *Mtb* and many other human pathogens, such as *Pseudomonas aeruginosa* [2; 3]. APR is a validated target to develop new anti-tubercular agents, particularly against latent TB infection [4–6]. As shown in Figure 1, this essential enzyme catalyzes the reduction of APS to sulfite (SO_3^{2-}) and adenosine-5'-monophosphate (AMP) with reducing equivalents from a protein co-factor, thioredoxin (Trx).

A significant hurdle to detailed biochemical investigations of *Mtb* APR is the absence of a facile and direct assay. Brunold and coworkers reported an assay that measures ^{35}S -sulfite production as acid-volatile radioactivity formed in the presence of [^{35}S]-APS, APR, Trx and dithioerythritol (DTT). This method requires the use of sulfuric acid (2M) and a large excess of nonradioactive sulfite to quench the reaction and produce volatile ^{35}S -sulfoxide gas. The radioactive gas is then trapped inside a sealed vial containing an organic base, such as octylamine, and analyzed through scintillation counting [7; 8]. Unfortunately, this method is hazardous, difficult to perform, and prone to large errors. Subsequently, Carroll *et al.* reported a charcoal-binding assay based on the idea that [^{35}S]-APS would bind to activated charcoal, but not the [^{35}S]- SO_3^{2-} product [9]. This assay avoids the production of a radioactive gas, but still requires the synthesis and use of [^{35}S]-APS. Then, in 2006, Sun and coworkers reported a coupled-enzyme system that monitors the 5'-AMP by-product of the APR reaction. In this method, adenylate kinase is utilized to convert the 5'-AMP to adenosine 5'-diphosphate (ADP) which is shunted to the NADH-dependent pyruvate kinase/lactate dehydrogenase coupling system [10]. Although this method represented an improvement over the earlier, radioactive assay, it requires three coupling enzymes and excess ATP. In 2009, Chung *et al.* developed an assay monitors recycling of oxidized Trx (a third product of the APR reaction (Figure 1), by NADPH-dependent Trx reductase (TrxR) [11]. This assay requires fewer auxiliary enzymes; nonetheless, it is a “signal decrease assay” and all coupled enzyme assays share potential artifacts from off-target inhibition of the enzymes used to couple the reaction to a detectable product. More recently, in 2012 Brychkova *et al.* reported an assay for plant APR using the magenta dye, fuchsin to detect sulfite with glutathione (GSH) as an electron donor [12]. However, acidic media and formaldehyde (required to generate the sulfite-reactive Schiff base on fuchsin) are not compatible with continuous enzymatic assay.

In an effort to address these issues, we have developed a new assay to monitor APR through sulfite-selective cleavage of a levulinate-protected chromophore or fluorophore, as shown in Figure 2. The inspiration for our strategy was derived from earlier reports of the sulfite sensors, resorufin levulinate [12] and boron-dipyrromethenelevulinyl ester [13]. Although selective for sulfite, these highly conjugated aromatic probes are unstable in aqueous buffer, resulting in high background signal [14]. To overcome this issue, we designed and synthesized three new levulinate-based probes by reacting the hydroxyl groups of p-nitrophenol, 7-hydroxy 4-methyl coumarin and rhodol with levulinic acid (Figure 3). Herein, the stability, selectivity, and sensitivity of these probes under the conditions of the APR assay have been evaluated. The details of the kinetic assay developed for *Mtb* APR using these new probes is also presented.

Materials and Methods

Reagents

All chemicals were purchased from Sigma-Aldrich. All solvents were purchased from Fischer Scientific. APS (>95% pure) was obtained from Biolog Life Sciences Institute (Bremen, Germany). ^1H NMR (400 MHz) and ^{13}C NMR (400 MHz) spectra were obtained on a Bruker NMR spectrometer and referenced to the residual solvent signal. Spectra were

recorded on a Cary 300 UV-visible spectrophotometer (Agilent), Cary Eclipse fluorescence spectrophotometer (Agilent), or 6120 Quadrupole LC/MS system (Agilent). Kinetic data was plotted and analyzed with KaleidaGraph software.

Mutagenesis and Protein Expression

The construction of the expression vector encoding wild-type *Mtb* APR (EC number 1.8.4.10) cloned into the vector pET24b (Novagen) has been described previously [9]. The Cys249Ala plasmid was prepared using QuikChange site-directed mutagenesis (Stratagene). Wild-type and mutant *Mtb* APR were over expressed and purified to homogeneity according to published procedures using nickel affinity and gel filtration column chromatography [9; 15; 16].

Synthesis of Lev-PNP

To a suspension of levulinic acid (500 mg, 4.3 mmol) in dichloromethane (20 mL) was added oxalyl chloride (0.82 mL, 8.6 mmol) and DMF (15 μ L). The reaction mixture was stirred at room temperature for 4 h and then the volatiles were evaporated under reduced pressure and subsequently dried with vacuum pumping. The residue was dissolved in a small amount of dry dichloromethane. The solution was slowly added into the dispersed dichloromethane solution (50 mL) containing *p*-nitrophenol (PNP; 180.7 mg, 1.3 mmol) and N, N-diisopropylethylamine (DIPEA; 0.64 mL, 3.9 mmol). After stirring for 12 h, the reaction mixture was filtered and the solution was treated with water. The organic phase was separated and washed with 1 M sodium bicarbonate solution and water, and then evaporated to obtain a solid residue. The product was purified by column chromatography using silicagel as stationary phase and 50% ethyl acetate in hexanes as eluent. The product was obtained as yellow color solid. Yield, 75%; ^1H NMR (400 MHz, CDCl_3) δ 8.23 (d, J = 6.5 Hz, 2H), 7.55(d, J = 6.8 Hz, 2H), 2.89 (m, 2H), 2.83 (m, 2H), 2.23 (s, 3H); ^{13}C NMR (400 MHz, CDCl_3) δ 201.3, 157.4, 144.7, 122.5, 125.3, 171.1, 207.7, 39.5, 37.6, 27.3; m/z calculated for $\text{C}_{11}\text{H}_{11}\text{NO}_5$ is 237.2087 found ($\text{M}+\text{Hd}$) = 238.19.

Synthesis of Lev-Cou

To a suspension of levulinic acid (500 mg, 4.3 mmol) in dichloromethane (20 mL) was added oxalyl chloride (0.82 mL, 8.6 mmol) and DMF (15 μ L). The reaction mixture was stirred at room temperature for 4 h and then the volatiles were evaporated under reduced pressure and subsequently dried with vacuum pumping. The residue was dissolved in a small amount of dry dichloromethane. The solution was slowly added into the dispersed dichloromethane solution (50 mL) containing 7-hydroxy 4-methy coumarin (229 mg, 1.3 mmol) and DIPEA (0.64 mL, 3.9 mmol). After stirring for 12 h, the reaction mixture was filtered and the solution was treated with water. The organic phase was separated and washed with 1 M sodium bicarbonate solution and water, and then evaporated to obtain a solid residue. The product was purified by column chromatography using silicagel as stationary phase and 50% ethyl acetate in hexanes as mobile phase. The product was obtained as cream color powder. Yield, 65%; ^1H NMR (400 MHz, CDCl_3) δ 7.81 (d, J = 6.3 Hz, 1H), 7.39(d, J = 6.6 Hz, 1H), 7.25 (m, 1H), 6.23 (s, 1H), 2.72 (m, 2H), 2.72 (s, 3H), 2.42 (s, 3H), 2.13 (s, 3H); ^{13}C NMR (400 MHz, CDCl_3) δ 207.7, 160.8, 153.7, 152.7, 171.1, 118.3, 116.4, 112.5, 110.1, 37.6, 29.5, 27.3; m/z calculated for $\text{C}_{11}\text{H}_{11}\text{NO}_5$ is 274.2687 found ($\text{M}+\text{H}$) = 275.32.

Synthesis of Lev-Rhol

(a) Rhodol 2-(4-Diethylamino-2-hydroxybenzoyl)benzoic acid (1.26 g, 4.00 mmol) and resorcinol (443 g, 4.0 mmol) were added to a heavy-walled pressure flask and dissolved in 15 mL of trifluoroacetic acid. The reaction contents were heated to 90 $^\circ\text{C}$ for 12 h, then

cooled to room temperature, and evaporated to dryness. The crude material was purified by column chromatography using silicagel as stationary phase and dichloromethane 45%: ethyl acetate 45%: methanol 10% as mobile phase. The product Rhodol was isolated as a red-brown solid (1.1 g, 75% yield). ^1H NMR ($\text{CDCl}_3/10\% \text{CD}_3\text{OD}$, 400 MHz): δ 8.19 (1H, d, $J = 7.2$ Hz), 7.59 (2H, quartet, $J = 7.2$ Hz), 7.11 (1H, d, $J = 7.2$ Hz), 6.86–6.95 (3H, m), 6.68 (2H, dd, $J = 2.0, 9.2$ Hz), 6.64 (1H, d, $J = 2.0$ Hz), 3.44 (4H, q, $J = 7.2$ Hz), 1.16 (6H, t, $J = 7.2$ Hz); ^{13}C NMR ($\text{CDCl}_3/10\% \text{CD}_3\text{OD}$, 100 MHz): δ 163.5, 153.4, 152.4, 150.9, 128.9, 127.3, 126.7, 126.2, 126.0, 124.4, 113.2, 110.0, 109.7, 108.7, 98.6, 92.3, 41.8, 8.3. Calculated m/z for $\text{C}_{24}\text{H}_{21}\text{NO}_4$ 388.1549, found (M+H) 389.21. (b) Lev-Rhol. To a suspension of levulinic acid (500 mg, 4.3 mmol) in DMF (40 mL) was added Hydroxybenzotriazole (HOBT; 1.3 g, 8.6 mmol), *O*-Benzotriazole-*N,N,N',N'*-tetramethyluronium-hexafluoro-phosphate (HBTU; 3.2 g, 8.6 mmol), Rhodol (229 mg, 1.3 mmol) and DIPEA; 0.77 mL, 4.3 mmol) under nitrogen atmosphere. After stirring for 24 h, the reaction mixture was evaporated to dryness at the pump and the contents were solubilized in water and ethyl acetate. The organic phase was separated and washed with 1 M sodium bicarbonate solution and water, and evaporated to obtain a solid residue. The product was purified by column chromatography using silicagel as stationary phase and 5% dichloromethane in methanol as the mobile phase. The product was obtained as red solid. Yield, 52%; ^1H NMR (400 MHz, CDCl_3) δ 7.7 (d, $J = 6.4$ Hz, 1H), 7.59 (m, 1H), 7.49 (m, 2H), 7.29 (d, 6.3Hz, 1H), 6.96 (m, 2H), 6.39 (m, 2H), 3.41(q, 8.2Hz, 4H), 2.72 (s, 4H), 2.72 (s, 3H), 2.13 (s, 3H); ^{13}C NMR (400 MHz, CDCl_3) δ 202.4, 169.5, 151.7, 151.4, 150.3, 149.4, 127.2, 125.8, 124.1, 128.8, 115.2, 111.4, 106.5, 104.6, 47.1, 37.6, 29.5, 27.3, 12.9; m/z calculated for $\text{C}_{29}\text{H}_{27}\text{NO}_6$ is 485.5278 found (M+H) =486.61.

Analysis of Lev-Probe-Sulfite Reactions

(a) Selectivity: In a 1 mL clear quartz cuvette, Hepes (10 mM) pH 7.5 buffer, Lev-Probe (5, 10 or 20 μM) was added and a reference absorbance/fluorescence spectrum was obtained. Then sulfite (500 μM) or sulfite with other nucleophiles (500 μM) was added to the cuvette, and absorbance/fluorescence was recorded every 3 min at rt. (b) pH Dependence: In a 1 mL clear quartz cuvette, with respective pH buffer, Lev-probe (5, 10 or 20 μM) was added and a reference absorbance/fluorescence spectrum was obtained. Next, sulfite (500 μM) was added and absorbance/fluorescence was recorded every 3 min at rt. (c) Stability: In a 1 mL clear quartz cuvette, with respective pH buffer, Lev-Probe was added and the absorbance/fluorescence spectrum was recorded every h for 24 h. The resulting data were fit to pseudo-first order exponential decay equation $[A] = [A]_0 e^{-kt}$ to obtain the observed rate constant (k_{obs}), which was then converted into half life ($t_{1/2}$) using the equation ($\ln 2/k_{\text{obs}}$). (d) Sensitivity. In a 1 mL clear quartz cuvette, Hepes (10 mM) pH 7.5 buffer, Lev-Probe (5, 10 or 20 μM) was added and a reference absorbance/fluorescence spectrum was obtained. Then, sulfite (0–80 μM) was added and absorbance/fluorescence was recorded every 3 min at rt. Parallel reactions were conducted in the absence of sulfite and the change in absorbance/fluorescence of “probe only” reactions were subtracted to report standard sulfite sensitivity plots.

APR Michaelis-Menten Kinetic Analysis

(a) Using Lev-probes: In a 1 mL clear quartz cuvette, Hepes (10 mM) pH 7.5 buffer, DTT (25 μM), *E. coli* Trx (10 μM), APS (1, 3, 6, 12, 24, 48, 96, 192 or 384 μM) and Lev-probe (concentration given in legends) was added. Reactions were initiated by adding wild-type (100 nM) or C249A *Mtb*APSR (200 nM). Parallel reactions were conducted without APR and subtracted as background. The reactions were monitored by recording the absorbance/fluorescence every 3 min at rt. The first 15% of the reactions were taken into account to calculate the net initial velocity, v_0 . The initial velocity was plotted versus [APS] to obtain the Michaelis-Menten plot using the equation $v_0 = V_{\text{max}}[S]/\{[K_m] + [S]\}$. (b) Using ^{35}S -

APS: The reactions were carried out at rt and contained Hepes (10 mM) pH 7.5, DTT (25 μ M), *E. coli* Trx (10 μ M), APS (1, 3, 6, 12, 24, 48, 96, 192 or 384 μ M) and 35 S-APS (3 nM). Reactions were incubated for 5 minutes prior to the initiation by the addition of APR (100 nM). At each time point, 10 μ L of the reaction mixture was quenched with charcoal solution (2% w/v) containing Na₂SO₃ (20 mM). The suspension was vortexed, clarified by centrifugation, and an aliquot of the supernatant containing the radio labeled sulfite product was counted in scintillation fluid. Kinetic constants were calculated as above.

ADP Inhibition of APSR

(a) Using Lev-probes: In a 1 mL clear quartz cuvette, Hepes (10 mM) pH 7.5 buffer, DTT (25 μ M), *E. coli* Trx (10 μ M), APS (1, 3, 6, 12, 24, 48, 96, 192 or 384 μ M), respective ADP (0, 40, 80, 160, or 320 μ M) and probe was added. Reactions were incubated for 5 min prior to initiation by the addition of APR (100 nM). Parallel reactions were conducted without APR and subtracted as background. The reactions were monitored by recording the absorbance/fluorescence every 3 min at rt. The first 15% of the reactions were used to calculate the initial velocity. The initial velocity was plotted versus [APS] to obtain K_m and K_m^{app} using the equation $v_0 = V_{max}[S]/\{[K_m] + [S]\}$. The K_i for ADP was calculated using the equation $K_m^{app} = K_m(1+[I]/K_i)$. The inverse of the initial velocity was plotted versus the inverse [APS] at each ADP concentration to obtain the Lineweaver–Burk plot. (b) Using 35 S-APS: The reactions were carried out at rt and contained HEPES (10 mM) pH 7.5, DTT (25 μ M), *E. coli* Trx (10 μ M), APS (1, 3, 6, 12, 24, 48, 96, 192 or 384 μ M), 35 S-APS and respective ADP (0, 40, 80, 160, or 320 μ M). Reactions were initiated by the addition of APR (100 nM). At each time point, 10 μ L of the reaction mixture was quenched with charcoal solution (2% w/v) containing Na₂SO₃ (20 mM). The suspension was vortexed, clarified by centrifugation, and an aliquot of the supernatant containing the radio labeled sulfite product was counted in scintillation fluid. The first 15% of the reactions were used to calculate the initial velocity. The initial velocity was plotted versus [APS] to obtain K_m and K_m^{app} using the equation $v_0 = V_{max}[S]/\{[K_m] + [S]\}$. The K_i of ADP is calculated using the equation $K_m^{app} = K_m(1+[I]/K_i)$. The inverse of the initial velocity was plotted versus the inverse of the [APS] for each ADP concentration to obtain the Lineweaver–Burk plot.

Results and Discussion

At the outset of this project, we envisioned an APR activity assay based on selective detection of the primary sulfite product. A variety of methods been reported for sulfite quantitation: electrochemistry [17–19], chromatography [20], chemiluminescence [21; 22], electrochemical and enzymatic techniques [18; 19]. However, most conventional methods either suffer from poor selectivity, are time consuming, or are expensive and utilize complex procedures. To improve on these methods, turn-on fluorescent probes were subsequently developed. For instance, probes based on the reaction of sulfite with aldehyde[23] or with glyoxal[24] have been reported. Nonetheless, substantial cross reactivity of these probes with simple thiols, like cysteine and DTT, meant they could not be used to measure APR activity.

An important contribution in this regard is the discovery that the levulinyl *O*-protecting group could be cleaved by sulfite under neutral conditions to give the free hydroxyl [25]. Based on this chemistry, resorufin levulinate[12] and BODIPY levulinate[13] probes have been reported. Although selective for sulfite, these highly conjugated aromatic probes are unstable in aqueous buffer, leading to high background signal and also have poor water solubility [14]. To develop turn-on sulfite detection probes, which would be stable in aqueous buffer and enable continuous monitoring of APR activity, we screened many levulinate-protected chromophore and fluorophore chromophores (data not shown). Of these, Lev-PNP, Lev-Cou and Lev-Rhol had optimal qualities and were prepared in high

yield through the coupling of *p*-nitrophenol (PNP), 7-hydroxy 4-methyl coumarin(Cou) or Rhodol (Rhol) to levulinic acid (Lev), as shown in Figure 3.

In initial experiments, Lev-functionalized probes were tested for their chromogenic or fluorogenic properties upon reaction with sulfite in Hepes (10 mM) pH 7.0 buffer. In the absence of sulfite, Lev-PNP showed an absorbance of less than 0.01 at 400 nM. However, the addition of sulfite (100 eq.) was accompanied by intense absorption at this wavelength (Figure 4a, *top*). Other common bio-functional groups present in the APS reaction (*i.e.*, thiols, alcohols, amines) were nonresponsive when incubated (100 eq.) with Lev-PNP; however, addition of sulfite to these reactions restored the absorbance (Figure 4a, *bottom*). Next, the fluorogenic reaction of Lev-Cou or Lev-Rhol (Figure 4b and c) and sulfite was evaluated in the absence (Figure 4b and c, *top*) and presence (Figure 4b and c, *bottom*) of potentially interfering functional groups present in the APR assay. Lev-Cou displayed a high selectivity for sulfite, while the more conjugated Lev-Rhol was slightly responsive to thiols; nevertheless, the fluorescence enhancement factor (F/F_0) observed for sulfite at 552 nm was large (500-fold) when compared to the enhancement factor (F/F_0) observed for thiols (100-fold) using the Lev-Rhol probe.

As proposed by Ono *et al.*, the chromogenic and fluorogenic signal from these probes is due to sulfite-induced selective deprotection of levulinic acid from Lev-PNP, Lev-Cou and Lev-Rhol to exposure the phenolate of the chromophore or fluorophore (Figure 2). In this reaction, cleavage of levulinate is initiated by attack of sulfite at the terminal carbonyl of levulinate, with formation of a tetrahedral intermediate, and intramolecular cyclization at the ester carbonyl carbon leading to cleavage of the ester and exposure of the corresponding anion [12; 25]. To further confirm this mechanism of action (beyond the observation of sulfite-dependent absorbance/fluorescence signal), the products of the reaction between the Lev-probes and sulfite were verified by LC-MS and $^1\text{H-NMR}$ analyses (data not shown).

Next, we evaluated the stability of the Lev-probes in aqueous buffer at pH 6.0, 6.5, 7.0, 7.5 and 8.0. The resulting data, presented as half-lives in Table 1, indicate excellent stability for Lev-PNP ($t_{1/2} \sim 13$ h) and Lev-Cou ($t_{1/2} \sim 5$ h) at pH 7.5; Lev-Rhol was less robust ($t_{1/2} \sim 2$ h), but sufficiently stable for experiments of 30 minutes or less at this pH. Since the hydrolysis of each probe increases at higher pH, we then determined the pH optimum that would maximize signal-to-noise (*i.e.*, chromogenic properties and reagent stability; Figure 5). This analysis indicates that Lev-PNP and Lev-Cou probes show a maximum response with sulfite at pH 8.0, whereas this value for Lev-Rhol was pH 7.5. Taking the pH dependence of APR activity into account [26], we reasoned that pH 7.5 would be suitable for conducting the assay. The limit of sulfite detection was then determined at pH 7.5 for each probe: Lev-PNP (3 μM), Lev-Cou (1 μM), and Lev-Rhol (0.25 μM) (Figure 6). These detection limits for sulfite compare favorably to earlier levulinate-based probes based on resorufin (49 μM)[12] and boron-dipyrromethene (58 μM)[13].

With these results in hand, we tested whether these probes could effectively monitor APR-dependent sulfite production in a reaction that included Hepes buffer pH 7.5, Trx, DTT (to recycle oxidized Trx), APS and various amounts of enzyme. These data show a linear relationship between APR concentration and sulfite production (as evidenced by the increase in absorption/fluorescence; SI Figure 1). Of note, the rate of reduction of thioredoxin- S_2 by DTT is $1650 \text{ M}^{-1} \text{ s}^{-1}$ at neutral pH [27]. Using initial rates, v_0 , of the APR reaction (*i.e.*, the first 5% - 15% of reaction) we confirmed that these rates were essentially identical with 25 – 250 μM DTT, suggesting that our kinetic constants are not reporting (or limited by) thioredoxin regeneration.

Next, the initial velocity (v_0) was determined at multiple APS concentrations using the Lev-probes or radioactive assay (Figure 7). The resulting data fit well to the Michaelis-Menten model and could, therefore, be used to obtain steady-state kinetic parameters (Table 2). Control experiments conducted with the catalytically inactive *Mtb* C249A APR [16] showed no significant increase in sulfite production, as expected (Figure 7).

Michaelis constants (K_m) for APS were in good agreement among all assays, ranging between 15 and 20 μM . Likewise, values for $V_{\max}$ (0.12 – 0.78 $\mu\text{M}/\text{min}$), k_{cat} (1.2 – 7.8 min^{-1}) and k_{cat}/K_m ($3.2 - 3.3 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$) compared favorably between the Lev-Cou/Rhol and ^{35}S -APS assays. Indeed, the only significant deviation from the radioactive assay was observed in $V_{\max}$, k_{cat} , and k_{cat}/K_m values (~ 4 -fold lower) obtained using the Lev-PNP probe. One explanation for this discrepancy is that the *p*-nitrophenol is not in the fully deprotonated state under the conditions of the assay ($\text{pH} = 7.5$; note that the $\text{p}K_a$ of the PNP phenol group is 7.2), thereby decreasing the already modest molar extinction coefficient of this chromophore. However, when the pH of the reaction was increased to 8.0, the net reaction rate only increased by 2-fold (data not shown). Of note, the detection limit of Lev-PNP for sulfite is lower than that of our fluorescent probes. To compensate for the lower limit of detection, we increased the concentration of Lev-PNP from 10 μM to 50 μM in APR assays. Although $V_{\max}$ was increased by ~ 4 -fold, it was still lower than $V_{\max}$ obtained from the ^{35}S -APS assay (see Table 1). Despite this limitation, our data clearly indicate that Lev-PNP can be used to obtain accurate K_m and K_i values. Finally, we tested the ability of the Lev-probes to monitor inhibition of *Mtb* APR by the competitive inhibitor, adenosine 5'-diphosphate (ADP). Each probe displayed an apparent inhibition constant (K_i) in good agreement with the value obtained using the radioactive assay (83 – 86 μM ; Figure 8 and Table 3).

In sum, our method to monitor APSR activity exploits new sulfite-selective colorimetric and “off-on” fluorescent levulinate-based probes. APR activity can thus be followed by monitoring the increase in absorbance or fluorescence of the resulting phenolate product. Using this assay, we determined Michaelis-Menten kinetic constants (K_m , k_{cat} , k_{cat}/K_m) and apparent inhibition constant (K_i) for adenosine 5'-diphosphate (ADP), which compared favorably to the values obtained in the standard radioactive assay. The new assay is therefore robust and easy to perform with a simple spectrophotometer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Lev-PNP	4-nitrophenyl 4-oxopentanoate
Lev-Cou	4-methyl-2-oxo-2H-chromen-7-yl 4-oxopentanoate
Lev-Rhol	3'-(diethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl 4-oxopentanoate
PNP	<i>p</i> -nitrophenol
Cou	7-hydroxy-4-methyl-2H-chromen-2-one

Rhol	Rhodol, 3'-(diethylamino)-6'-hydroxy-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one
APS	Adenosine 5'-phosphosulfate
APR	Adenosine 5'-phosphosulfate reductase
DTT	Dithiothreitol
Trx	Thioredoxin

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Figure 1.

APR catalyzes the reduction of APS to sulfite and AMP with reducing equivalents from Trx. In turn, Trx is recycled by the small-molecule reductant, DTT.

Figure 2.
Mechanism of sulfite detection by Lev-protected probes.

Figure 3.

A) Synthesis of the Lev-PNP and Lev-Cou probes. i) $(\text{COCl})_2$, CHCl_3 , catalytic DMF ii) *p*-nitrophenol or iii) 7-hydroxy 4-methyl coumarin, DIPEA, DMF. **B)** Synthesis of the Lev-Rhol probe. iv) Resorcinol, TFA, 90 °C v) Levulinic acid, HOBT, HBTU, *N,N*-DIPEA, DMF.

Figure 4.

Spectral properties and selectivity of Lev-probes with sulfite. Top: Wavelength spectra of Lev-probes in the absence (—) or presence (---) of sulfite: A) Lev-PNP, B) Lev-Cou, C) Lev-Rhol. Bottom: Selectivity of Lev-probes: A) Lev-PNP B) Lev-Cou C) Lev-Rhol. Conditions: Hepes (10 mM) pH 7.5 buffer, sulfite (500 μ M) incubated in the absence or presence of β -mercaptoethanol (BME; 500 μ M), DTT (500 μ M), glutathione (GSH; 500 μ M), lysine (Lys; 500 μ M) or benzyl alcohol (BA; 500 μ M). Probe concentration and reaction time for each probe are: Lev-PNP (10 μ M, 15 min), Lev-Cou (5 μ M, 20 min), Lev-Rhol (5 μ M, 12 min). All reactions were conducted at rt and were corrected with the appropriate blank spectra. The experiments described here were performed in at least two independent trials; representative examples of the data are shown.

Figure 5.

The pH dependence of the sulfite reaction with Lev-probes. A) Lev-PNP B) Lev-Cou C) Lev-Rhol. Conditions: reaction at pH 6.0 and 6.5 was measured in Bis-Tris (10 mM) buffers; reaction at pH 7.0, 7.5, and 8.0 was measured in Hepes (10 mM) buffers; sulfite (500 μ M). Probe concentration and reaction time were: Lev-PNP (10 μ M, 15 min), Lev-Cou (5 μ M, 20 min), Lev-Rhol (5 μ M, 12 min). All reactions were conducted at rt and were corrected with the appropriate blank spectra. The experiments described here were performed in at least two independent trials; representative examples of the data are shown.

Figure 6.

Sensitivity of Lev-probes for sulfite detection. Conditions: Hepes (10 mM) pH 7.5 buffer, probe concentration and reaction times were: A) Lev-PNP (50 μM , 60 min), B) Leu-Cou 5 μM , 90 min), C) Lev-Rhol (5 μM , 30 min). All reactions were conducted at rt and were corrected with the appropriate blank spectra. The net absorbance/ fluorescence was plotted against respective sulfite concentration to develop the standard curve. The experiments described here were performed in at least two independent trials; representative examples of the data are shown.

Figure 7.

Michaelis-Menten kinetic plots for *Mtb* APR as assayed using Lev-probes or ^{35}S -APS. Each reaction was conducted at rt in Hepes (10 mM) pH 7.5 buffer, with DTT (25 μM), Trx (10 μM), wild-type APR (●; 100 nM) or C249A APR (●; 200 nM) and APS (1, 3, 6, 12, 24, 48, 96, 192 or 384 μM) with: A) Lev-PNP (50 μM), B) Lev-Cou (5 μM) C) Lev-rhol (20 μM) or D) ^{35}S -APS. Control reactions were also conducted without APSR. To quantify sulfite production in the APR reaction, the extinction coefficient of *p*-nitrophenol (13002 $\text{M}^{-1} \text{cm}^{-1}$ at pH 7.5) was used for Lev-PNP, whereas standard curves were generated for use with Lev-Cou or Lev-Rhol. The experiments described here were performed in at least two independent trials; representative examples of the data are shown.

Figure 8.

ADP inhibition of APR followed as assayed using Lev-probes or ^{35}S -APS. Each reaction was conducted at rt in Hepes (10 mM) pH 7.5 buffer, with DTT (25 μM), Trx (10 μM), *Mtb* APR (100 nM), APS (1, 3, 6, 12, 24, 48, 96, 192 or 384 μM), and ADP (0, 40, 80, 160 or 320 μM) with: A) Lev-PNP (10 μM), B) Lev-Cou (5 μM) C) Lev-Rhol (20 μM) or D) ^{35}S -APS. Control reactions were conducted in the absence of APR. The apparent inhibition constants, K_i , are presented in Table 3. The experiments described here were performed in at least two independent trials; representative examples of the data are shown.

Table 1Stability of Lev-probes under aqueous buffer conditions.^a

pH	Lev-PNP <i>t</i> _{1/2} (h)	Lev-cou <i>t</i> _{1/2} (h)	Lev-rhol <i>t</i> _{1/2} (h)
6.0 ^a	95.2	62.8	5.7
6.5 ^a	75.8	60.5	4.3
7.0 ^b	23.1	29.1	3.9
7.5 ^b	12.9	5.1	2.0
8.0 ^b	3.4	1.3	0.6

^a Conditions: Stabilities at pH 6.0 and 6.5 were measured in Bis-Tris (10 mM) buffers; stabilities at pH 7.0, 7.5, and 8.0 were measured in Hepes (10 mM) buffers. Probe concentrations were as follows: Lev-PNP (10 μ M), Lev-cou (5 μ M), and Lev-rhol (5 μ M). The increase in absorbance or fluorescence (indicative of ester hydrolysis) was fitted by the pseudo-first order equation to obtain the observed rate constant (k_{obs}) and converted to half-life ($t_{1/2}$).

Table 2Michaelis-Menten kinetic constants for *Mtb* APR.^a

Method	K_m (μM)	$V_{\max}$ ($\mu\text{M min}^{-1}$)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{min}^{-1}$)
Lev-PNP	17.3 ± 0.2	0.12 ± 0.007	1.2 ± 0.07	$0.7 \times 10^5 \pm 0.3 \times 10^4$
Lev-Cou	15.0 ± 1.7	0.48 ± 0.01	4.8 ± 0.1	$3.2 \times 10^5 \pm 0.7 \times 10^4$
Lev-Rhol	10.9 ± 1.0	0.35 ± 0.01	3.5 ± 0.1	$3.2 \times 10^5 \pm 0.9 \times 10^4$
³⁵ S-APS	24.3 ± 2.3	0.78 ± 0.04	7.8 ± 0.4	$3.3 \times 10^5 \pm 0.2 \times 10^4$

^aKinetic constants are presented as average values and standard deviations are from two independent measurements. Note that the APR concentration was based on the number of active molecules, determined as previously reported (SI Figure 2).

Table 3

Apparent inhibition constant, K_i , obtained for competitive inhibitor, ADP.

Method	K_i of ADP (μM)
Lev-PNP	83 ± 0.5
Lev-Cou	84 ± 0.6
Lev-Rhol	86 ± 0.5
^{35}S -APS	83 ± 0.2