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Redox Switching of Adenosine-5′-phosphosulfate Kinase with Photoactivatable Atomic Oxygen Precursors

Miao Zhang, Geoffrey E. Ravilious,[†] Leslie M. Hicks,[‡] Joseph M. Jez,[†] and Ryan D. McCulla*

Department of Chemistry, Saint Louis University, 3501 Laclede Avenue, Saint Louis, Missouri 63103, United States

[†]Department of Biology, Washington University, One Brookings Drive, Campus Box 1137, Saint Louis, Missouri 63130, United States

[‡]Donald Danforth Plant Science Center, 975 North Warson Road, Saint Louis, Missouri 63132, United States

Supporting Information

ABSTRACT: Adenosine-5′-phosphosulfate kinase (APSK) catalyzes the phosphorylation of adenosine-5′-phospho-sulfate (APS) to 3′-phospho-APS (PAPS). In plants, this enzymatic activity is biochemically regulated through an intersubunit disulfide bond between Cys86 and Cys119 in the N-terminal loop of APSK. To examine if $O(^3P)$ generated by the photodeoxygenation of 2,8-dihydroxymethyldibenzothiophene *S*-oxide could specifically oxidize APSK at its regulatory site, APSK was irradiated in the presence of 2,8-dihydroxymethyldibenzothiophene *S*-oxide. Near-quantitative alteration of APSK from the enzymatically active monomeric form to the inhibited dimeric form was achieved. The photoinduced increase of dimeric APSK was strongly implicated to arise from the formation of the Cys86–Cys119 disulfide bond.

Atomic oxygen ($O(^3P)$) is the putative oxidant generated during the photoinduced deoxygenation of dibenzothiophene *S*-oxide and other similar heterocycles.[1−8] Recently, the photodeoxygenation of 2,8-dihydroxymethyldibenzothiophene *S*-oxide (**1**) in water was demonstrated to generate an oxidant with a reactivity profile consistent with $O(^3P)$.[9] However, the direct identification of $O(^3P)$ in solution remains a difficult challenge. Photodeoxygenation of **1** in water resulted in the quantitative oxidation of 3-mercaptobenzoic acid to the corresponding disulfide (Figure 1). In the gas phase, $O(^3P)$ rapidly reacts with thiols to form sulfenic acids.[10] In the absence of stabilizing interactions, sulfenic acids react rapidly with thiols to form disulfides or undergo further oxidation to higher sulfur oxides.[11] Because of the apparent thiophilicity of the putative $O(^3P)$, it was postulated that **1** could be exploited to photoinduce the oxidation of protein thiols in high yields.

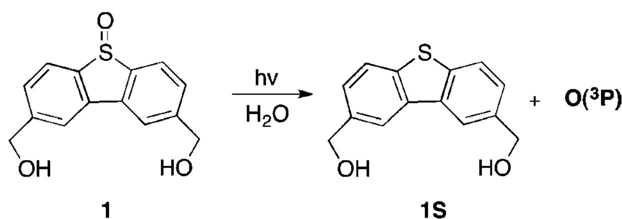

Figure 1. Photodeoxygenation of 2,8-di-hydroxymethyldibenzothiophene *S*-oxide (**1**).

Sulfur assimilation differs in various plants and microbes.[12−18] In fungi, yeast and enterobacteria, including *Escherichia coli*, sulfate is incorporated into adenosine-5′-phosphate (APS), then converted to 3′-phospho-APS (PAPS) for the incorporation of sulfur into bioactive compounds.[16,17] Other sulfate-assimilating bacteria, such as *Pseudomonas aeruginosa*, and plants use APS for sulfur assimilation with conversion of APS to PAPS by adenosine-5′-phophosulfate kinase (APSK) providing a sulfur-donor for the modification of a variety of metabolites.[16−18] In plants, APSK is essential for normal development and competes with APS reductase to partition sulfate between the primary and secondary branches of the sulfur assimilatory pathway.[19,20] Recently, it was reported that *Arabidopsis thaliana* APSK is regulated through an intersubunit disulfide bond located at the N-terminal of the enzyme (Figure 2).[21] The crystal structure of the *A. thaliana* APSK dimer revealed that Cys86 is located on the N-terminal

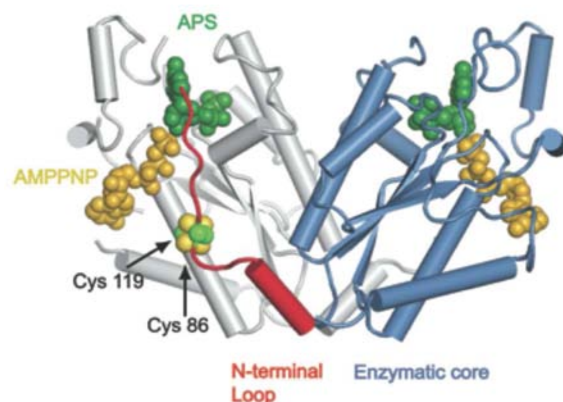

Figure 2. Crystal structure of *A. thaliana* APSK.[21] Overall structure of APSK in complex with the ATP-analogue AMPPNP (gold) and APS (green). The N-terminal region (residues 78−98) is shown in red, while the enzymatic core of the same monomer is shown in blue (residues 99−276). The disulfide bond formed by Cys86−Cys119 is shown as green (carbon) and gold (sulfur) spheres. Note two conformations of the disulfide were crystallographically observed.[21].

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loop (Figure 2, red, residues 78−98) and forms a disulfide with Cys119 in the core of the adjoining monomer. Comparative kinetics of reduced and oxidized APSK revealed that reduced APSK had a higher catalytic efficiency and was less sensitive to substrate inhibition by APS.[21,22] It was postulated that the critical redox switch (i.e., the Cys86−Cys119 disulfide) of *A. thaliana* APSK would be efficiently oxidized by $O(^3P)$ to provide a convenient photochemical means to control enzymatic activity.

To investigate this hypothesis, APSK and **1** were photolyzed (350 nm) in a buffered solution by the following procedure. Compound **1** was prepared as previously described.[9] Recombinant *A. thaliana* APSK lacking the N-terminal chloroplast localization sequence (residues 1−77) was expressed using an *E. coli* expression system and purified to homogeneity as previously described.[21,22] This previous work also demonstrated that the change in oxidation state that affects activity is specifically linked to formation of the Cys86−Cys119 disulfide in *Arabidopsis* APSK.[21] APSK in buffered solution (25 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) pH 7.5, 200 mM KCl, 5% glycerol (v/v) and 5 mM 2-mercaptoethanol (BME)) was supplemented with 10 mM dithiothreitol (DTT) and incubated (30 min; 35 °C). Then, the low molecular weight reducing agents (DTT, BME) were removed using a Sephadex G-25 column. All photolysis solutions were prepared to begin with 10 μM APSK. Irradiations were carried out by dissolving **1** in the APSK solution and irradiating with a 350 nm light source. The reported molecular weight of APSK monomer (reduced) and dimer (oxidized) are 22 and 44 kDa, respectively.[21] After photolysis, the sample was analyzed by SDS-PAGE to separate reduced APSK (monomer) from oxidized APSK (dimer). Figure 3 displays a gel image from a sample containing 10 μM

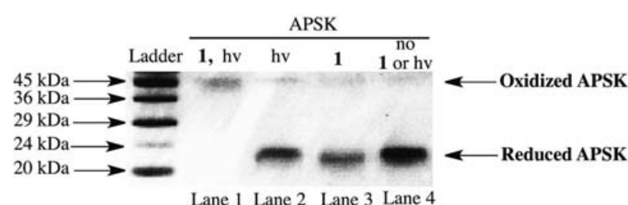

Figure 3. SDS-PAGE of aerobic photolysis of *A. thaliana* APSK with **1**. Right arrows label the oxidized APSK (dimeric) and reduced APSK (monomeric) forms. Lane 1, 10 μM APSK that had been irradiated for 45 min in the presence of **1** (100 μM) under an ambient atmosphere at 25 °C; lane 2, APSK irradiated 45 min in the absence of **1**; lane 3, APSK and **1** kept in the dark; lane 4, APSK kept in the dark.

APSK that had been irradiated for 45 min in the presence of **1** (100 μM) under ambient atmosphere. Irradiation of APSK in these conditions resulted in nearly complete oxidation of the monomer to the dimer (lane 1, Figure 3). In contrast to lane 1, less than 7% of oxidized APSK was observed in control samples that included irradiation without **1** (lane 2), no irradiation (lane 3), and APSK that was not exposed to **1** or light (lane 4).

For all experiments, SDS-PAGE analysis revealed a small amount of oxidized APSK (5−20% band percent) in the untreated sample (lane 4) despite being incubated with 10 mM DTT for 30 min. For consistent comparison of APSK oxidation across all the experiments, the change in oxidation state of APSK was normalized based on the percentage of dimeric APSK in lane 4 for a given experiment. The normalized change in oxidation for lanes 1−3 was defined as the difference of oxidized APSK for a given lane and lane 4 divided by the amount of APSK available for oxidation (100% minus oxidized APSK % in lane 4). The extent of APSK oxidation was studied at decreasing concentrations of **1**. As seen in Table 1, 30 min

Table 1. Photoinduced Oxidation of *A. thaliana* APSK by 1[a]

	normalized oxidation percentage (%)		
1 conc. (μM)[b]	lane 1 (**1**, hν)	lane 2 (hν)	lane 3 (**1**)
100	70 ± 12[c]	8.6 ± 5.1	3.6 ± 1.9
40	55 ± 13	2.8 ± 2.0	−2.0 ± 5.0
10	30 ± 15	3.3 ± 1.6	4.3 ± 4.5

[a]All reactions started with 10 μM APSK and were photolyzed or kept in the dark for 30 min under aerobic conditions at 25 °C. [b]Starting concentration of **1**. [c]95% confidence interval.

irradiations starting with 100 μM of **1** afforded the highest conversion (70%) of APSK. Starting with 40 μM **1**, 55% APSK conversion was observed, and 30% for photolysis with 10 μM **1** initially. This was consistent with the expectation that generating more $O(^3P)$ would result in increased protein oxidation.

To determine if an interaction between **1** or **1S** with APSK was influencing the extent of protein oxidation, UV spectra of **1** and **1S** in a series of solutions with an increasing concentration of APSK were obtained (Supporting Information (SI)). Compared to the spectra obtained in the absence of APSK, no change in the λ_{max} of characteristic absorbance bands of **1** or **1S** was observed as the concentration of APSK increased. This indicated there was no change in the polarity of the microenvironment for **1** and **1S** as ASPK was added, which suggests **1** and **1S** were not strongly associated with APSK for all of the experiments performed.[23]

Since **1S** is the detectable product from the deoxygenation of **1**, the amount of $O(^3P)$ can be estimated from the formation of **1S**. To investigate the relationship of oxidation of the critical cysteine residues of APSK and the amount of $O(^3P)$ generated in solution, the concentration of **1S** was determined by a previously reported HPLC method over the course of irradiation.[9] Figure 4 plots the amount of **1S** formed and the percentage of oxidized APSK (dimeric) after 15, 30, and 45 min of irradiation. A positive relationship between formation of

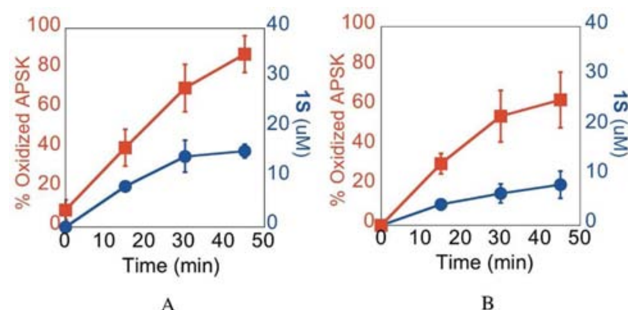

Figure 4. *A. thaliana* APSK oxidation compared to amount of photogenerated **1S**: (A) 10 μM APSK irradiated at 350 nm with 100 μM **1** for 15, 30, or 45 min; (B) 10 μM APSK irradiated at 350 nm with 40 μM **1** for 15, 30, or 45 min. Red squares: normalized oxidation percentage of APSK. Blue circles: concentration of **1S** determined by HPLC during photolysis. At least 6 experiments were performed for each time point, and the error bars represent the 95% confidence intervals.

Table 2. Aerobic and Anaerobic Oxidation of *A. thaliana* APSK and Its Truncation Mutant (APSKΔ96) with 2,8-Di-hydroxymethyldibenzothiophene *S*-oxide (1)

entry	protein type[a]	irradiation time (min)	Ar sparged[b]	normalized oxidation percentage (%)		
				lane 1 (**1**, hν)	lane 2 (hν)	lane 3 (**1**)
1	APSK	20	N	62 ± 11[c]	5.3 ± 2.8	−0.47 ± 4.3
2	APSK	20	Y	46 ± 10	9.6 ± 3.4	6.9 ± 3.4
3	Truncation mutant (APSKΔ96)[d]	30	N	13 ± 9.9	11 ± 10	4.9 ± 7.2
4	Truncation mutant (APSKΔ96)	45	N	8.7 ± 4.3	11 ± 3.1	0.33 ± 2.0
5	Truncation mutant (APSKΔ96)	45	Y	11 ± 7.8	7.2 ± 8.9	−8.0 ± 22

[a]All reactions started with 10 μM APSK and photolyzed or kept in dark for the indicated time. [b]Sample sparged with argon for 15 min. [c]95% confidence interval. [d]The APSK truncation mutant (APSKΔ96) lacks residues 78−98 of the N-terminal region.

APSK dimer and the photogeneration of **1S** was observed for both reactions starting with 100 and 40 μM of **1**. For 10 μM of **1**, oxidized APSK accumulation was observed; however, **1S** concentration was too low to quantitate. In both plots, the formation of **1S** slowed after 30 min of photolysis. Since **1S** also absorbs at 350 mn, the increase of **1S** in solution reduces the number of photons available for **1**, which results in slowing of the rate of $O(^3P)$ generation. The decreased rate of formation for **1S** after 30 min of photolysis coincided with a slowing of the rate of APSK oxidation. The amount of **1S** generated during the irradiation was 15 and 8.2 μM for the reactions starting with 100 and 40 μM of **1**, respectively. At 45 min, the extent of APSK oxidation was 87% when starting with 100 μM of **1** and 63% when starting with 40 μM of **1**. Since the starting concentration of reduced APSK was approximately 9 μM, this indicates the complete oxidation of Cys86 or Cys119 required a little less than 2 equiv of $O(^3P)$. Of eight total cysteine residues, examination of the structure of APSK reveals two other cysteine residues (Cys191 and Cys245), in addition to Cys86 and Cys119, are partially solvent exposed. Therefore, if $O(^3P)$ selectively oxidizes solvent exposed cysteine residues, 2 equiv of $O(^3P)$ would be required to achieve 100% APSK dimer formation since oxidation of Cys191 or Cys245 does not result in dimeric APSK. However, further work is required to confirm this hypothesis.

Atomic oxygen and molecular oxygen (O_2) react to generate ozone (O_3) with a measured rate constant of 4.1×10^9 M^{-1} s^{-1}.[24] Therefore, O_3 could contribute to oxidation of APSK in the photolysis reaction carried out in ambient conditions. To solely examine the effect of $O(^3P)$ on the redox state of APSK, the same experiments were conducted under anaerobic conditions by sparging with argon for 15 min prior to irradiation. As shown in Table 2 (entry 2), anaerobic photolysis resulted in a 46% oxidation of the available monomeric APSK after 20 min irradiation starting with 40 μM of **1**. Oxidation of APSK in anaerobic conditions was 16% less than in aerobic conditions (62%). However, this change is not statistically significant when considering the 95% confidence interval for these experiments. To probe the role of O_3, similar experiments with the addition of 100 μM of tryptophan, which reacts rapidly with O_3,[25] were performed under both aerobic and anaerobic conditions. Since $O(^3P)$ was not expected to react rapidly with tryptophan, it was not surprising when no significant difference in the extent APSK oxidation for samples with and without tryptophan was observed in anaerobic conditions (SI). However, in aerobic conditions, APSK oxidation decreased from 50% ± 7 to 30% ± 5 in the presence of tryptophan, which suggests O_3 contributes to APSK oxidation in aerobic conditions. The addition of 200 μM benzyl phenyl sulfide, a singlet oxygen trap,[9] showed no effect on the oxidation of APSK (SI).

To confirm that cross-linked dimer formation was the result of the Cys86−Cys119 disulfide, the oxidation of an N-terminal truncation mutant lacking residues 78−96, including Cys86 was examined. Generation of the N-terminal truncation mutant APSKΔ96 used pET-28a-APSKΔ77 as template. Forward and reverse primers harboring NheI and EcoRI restriction sites, respectively, were used to PCR-amplify the new version of the coding region, which was subcloned into pET-28a. APSKΔ96 was purified in an identical manner as wild-type enzyme. For this mutant, oxidation of Cys119 was not expected to generate the dimer since Cys119 has no adjacent thiols that are accessible to form disulfide bonds. Experimental data for APSK and its truncation mutant photolysis are compiled in Table 2 (entries 3−5). No significant accumulation of the dimeric form of APSKΔ96 during photolysis under both aerobic and anaerobic conditions after 30 or 45 min of irradiation was observed.

It was also considered that formation of the APSK dimer could potentially be the result of other covalent bonds (e.g., such as an ether linkage) rather than a disulfide as expected. To confirm the oxidized APSK subunits were indeed linked through a disulfide bond, the experiments were carried out normally (10 μM APSK, 40 μM **1**, 30 min irradiation, plus the 2 controls); however, prior to loading onto the gel, the solutions for lanes 1−3 were divided in two portions and one portion was incubated with 10 mM DTT for 30 min to reduce any newly formed disulfides. The results of the DTT post-treated samples compared to the untreated samples are shown in Figure 5. In aerobic conditions, the DTT post-treatment sample contained 15% oxidized APSK versus 62% in the same sample with no DTT post-treatment. In anaerobic conditions, only 14% normalized oxidized APSK was detected compared to 48% in the same sample without DTT incubation. To ensure that the oxidized APSK could be completely returned to the reduced state, forcing conditions (100 mM DTT, 60 min incubation) were used for the sample (lane 1). With these forcing conditions, only 2.1% and 3.0% normalized oxidized APSK was detected in the DTT-treated portions from the aerobic and anaerobic photolysis, respectively. DTT-treated portions from the control samples (lanes 2 and 3) showed no significant difference when compared to the untreated portion. The negative normalized oxidized APSK showed in aerobic photolysis were due to double incubation with DTT (before and after irradiation as opposed to lane 4 that was only treated once). The decrease in dimeric APSK upon post-treatment with DTT to its monomeric form is strong evidence that an intersubunit disulfide is generated as a result of $O(^3P)$ production.

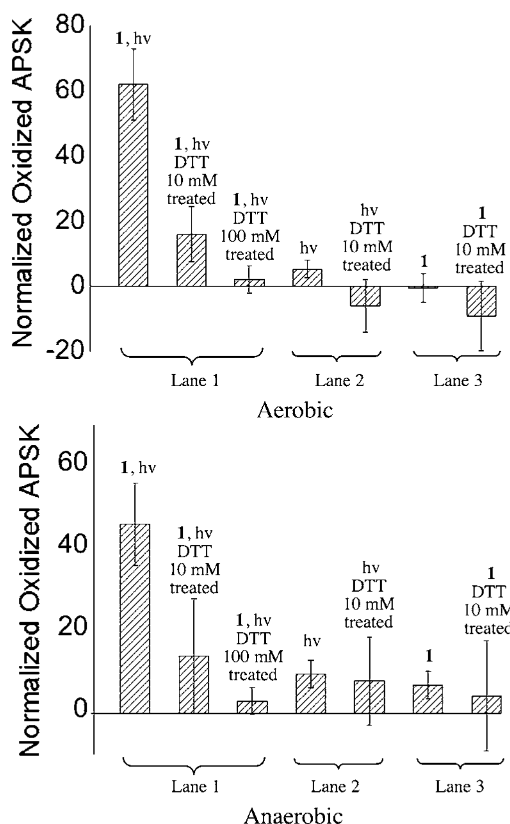

Figure 5. *A. thaliana* APSK oxidation and DTT postphotolysis incubation experiments. Lane 1: sample containing 10 μM APSK, 40 μM **1** was photolyzed for 30 min and then separated into three portions (**1**, hv), untreated; post-treated with 10 mM DTT for 30 min; post-treated with 100 mM DTT for 60 min. Lane 2 (hv): same as Lane 1 except an absence of **1**; lane 3 (**1**), same as lane 1 except the sample was not irradiated. All DTT post-treated lanes are to the right of untreated lane. Anaerobic samples were sparged with argon for 15 min prior to irradiation. Error bars represent the 95% confidence interval.

In summary, generation of O(^{3}P) during the photodeoxygenation of **1** is capable of oxidizing APSK at the regulatory thiol residues. The approach has been demonstrated to offer an efficient means of regulating APSK oxidation, which is expected to provide a convenient method for controlling its activity in future studies. These results also indicate O(^{3}P) can be used to selectively target cysteine residues for oxidation, and provide evidence for the thiophilicity of O(^{3}P). To our knowledge, there are no previous studies of O(^{3}P) reactivity toward proteins in solution.

ASSOCIATED CONTENT

Supporting Information

General materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

rmccull2@slu.edu

Notes

The authors declare no competing financial interest.

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