Supplementary Information

The radical-SAM enzyme Viperin catalyzes reductive addition of a 5´-deoxyadenosyl radical to UDP-glucose *in vitro*

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Methods

Chemicals. All chemicals were reagent grade. Uridine diphosphate glucose (UDP-glucose) sodium salt (≥ 98%), S-adenosylmethionine (SAM) (≥ 75%), uridine diphosphate N-acetylglucoseamine (UDP-GluNAc) sodium salt (≥ 98%), Guanosine diphosphate mannose (GDP-Man) sodium salt (≥ 97%), and farnesyl pyrophosphate (FPP) ammonium salt (≥ 95%) were purchased from Sigma Aldrich.

Protein overexpression. The gene encoding *Thielavia terrestris* Viperin (THITE_118376), which was codon optimized for overexpression in *E. coli*, was obtained from Life Technologies (Invitrogen, The Netherlands). A Kpn-I restriction site was introduced after the predicted N-terminal membrane-anchoring domain (Supplementary Figure 1) without introducing any mutation. To introduce the Kpn-I site, the codons AAA (K25) and GTT (V26) were replaced by their synonymous codons AAG and GTC. An EcoR-I restriction site was introduced after the last amino acid of the C-terminus. This gene was then cloned into pBAD/His-C (Invitrogen) using Kpn-I and EcoR-I restriction sites. The final insert lacked the N-terminal membrane-anchoring domain and its sequence was confirmed (Baseclear, the Netherlands). This vector was used to transfect chemically competent NEB 10-beta competent *E. coli* cells (New England Biolabs). For overexpression of the protein cells were grown overnight using 25 ml of LB medium containing ampicillin (100 μM) in a shaker (200 rpm) at 37 °C. This pre-culture was then inoculated into 500 ml of TB-medium. Cells were grown at 30 °C with 145 rpm shaking speed. After two hours, when the optical density reached approximately 0.5-0.6, expression of protein was induced by addition of 0.04% L-(+)-Arabinose (final concentration). One hour after addition of L-(+)-Arabinose 1 ml of 100 mM $FeSO_4(NH_4)_2$ solution was added. The $FeSO_4(NH_4)_2$ solution was prepared in acidic Milli-Q water, pH 2.0. Addition of $FeSO_4(NH_4)_2$ was repeated at 1 hour time intervals for a further seven hours. After 8 hours growth, cells were harvested by centrifugation and were stored at -20 °C. All steps were performed under aerobic conditions.

Protein purification. Cells were broken using a cell disruptor (constant system) operating at 20 kbar and were subjected to ultracentrifugation, 25,000 rpm, for 45 minutes. The buffer was 100 mM Tris, 300 mM NaCl, 10 mM DTT, plus DNase and protease inhibitors. The cell-free extract, which contained soluble proteins, was collected. The solution was kept on ice and subsequent steps of protein purification were performed at 4 ºC. The protein solution was loaded into a his-trap column (5 ml) (GE Healthcare Life Sciences) using an AKTA chromatography system (GE Healthcare Life Sciences). For the column two elusion buffers were used: Buffer A was Tris-HCl 20 mM, NaCl 350 mM, pH 7.2, Imidazol 60 mM, Triton 100X 0.02 (W/v), and 5 mM DTT; Buffer B was Tris-HCl 20 mM, NaCl 350 mM, pH 7.2, Imidazol 750 mM, Triton 100X 0.02 (W/v), and 5 mM DTT. A linear gradient of 0% to 100% B with length of 16.00 (CV) was used. The purified protein was further concentrated using an Amicon Ultra centrifugal filter (cut-off of 10 kDa). The concentrated protein was subjected to buffer exchange using a desalting column (PD-10) (GE Healthcare Life Sciences). The final buffer was 100 mM Tris, 300 mM NaCl, 5 mM DTT, pH 7.5. The resulting purified protein was frozen and kept in liquid nitrogen. Typically more than 50 mg of protein per litter of culture was obtained. For each experiment the required aliquot of protein was transferred to an anaerobic glove box (Belle, O_2 < 5.0 ppm). Concentration of protein was determined using Bradford assay.

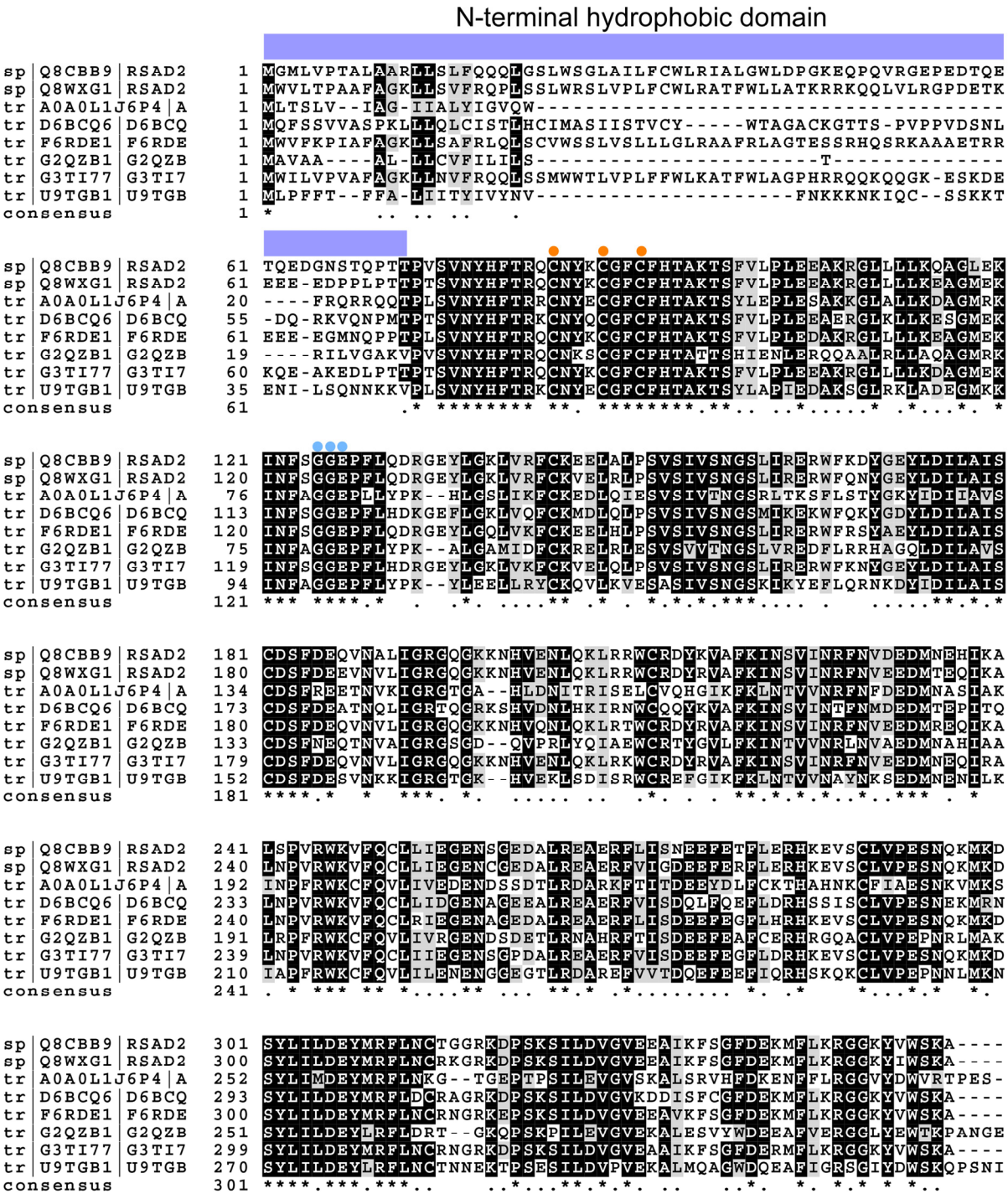
Activation of enzyme. The purified protein contained iron-sulfur cluster. To fully form the active [4Fe-4S] cluster, 1 ml of as-isolated protein was transferred into anaerobic glovebox (Belle, O_2 < 5.0 ppm). Subsequently, 20 μl ferricyanide (100 mM) was added to the protein and the solution was incubated overnight at least for 18 hours. Ferricyanide was removed and the protein was buffer-exchanged to 100 mM Tris, 300 mM NaCl, pH 7.5 using a PD-10 desalting column. Formation of the active [4Fe-4S] cluster was confirmed using electron paramagnetic resonance (EPR) spectroscopy to observe the $[4Fe\text{-}4S]^{1+}$ cluster and by mass spectrometry to observe cleavage of SAM and formation of 5′-dAdoH. Spin quantification (see below) showed that the ratio of the concentration of $[4Fe\text{-}4S]^{1+}$ cluster to that of protein was approximately 0.7-0.9 depending on the protein preparation. Therefore, approximately 70-90% of Viperin monomers contained one [4Fe-4S] cluster.

Preparation of samples for liquid chromatography-mass spectrometry (LC-MS) analysis. Samples were prepared under strict anaerobic conditions (O_2 < 5.0 ppm). For LC-MS analysis concentration of protein was typically 130-150 μM unless otherwise stated. For accurate mass analysis

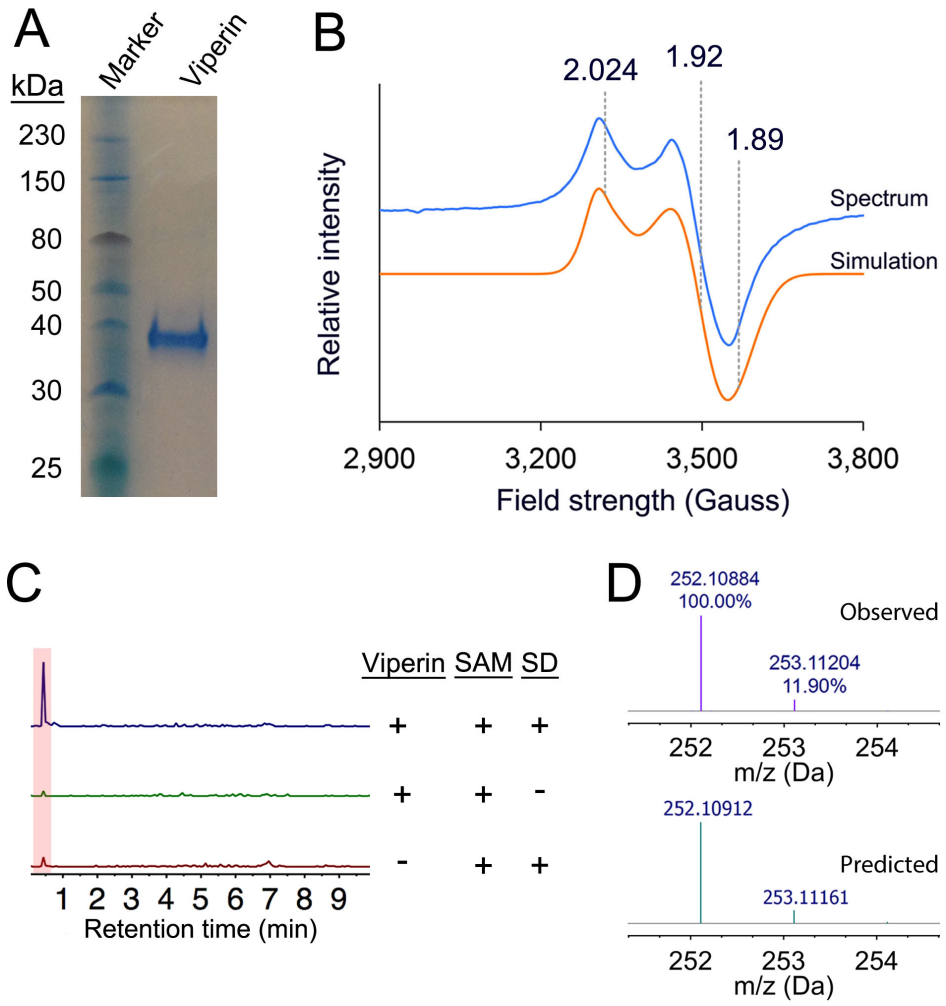
concentration of protein was approximately 1.3 mM. For accurate mass analysis of the reactions in D_2O buffer, the protein and all reagents were prepared in D_2O buffer. For LC-MS analysis a sample containing all components of the reaction, i.e. Viperin, SAM, UDP-glucose, and sodium dithionite was prepared. In parallel three different control samples were prepared to each of which Viperin, UDP-glucose, or sodium dithionite was not added. In a separate control sample UDP-GluNAc was added instead of UDP-glucose. Protein was in 100 mM Tris, 300 mM NaCl, pH 7.5. For preparation of the control samples when a component of the reaction (Viperin, SAM, UDP, or sodium dithionite) was not added, an aliquot of the buffer was added to have exactly the same final volume for all the samples. Stock solution of SAM and UDP-glucose had typically a concentration of 20-40 mM. A small aliquot of these stock solutions (10-20 µl) was added to reach the desired final concentration. To determine the time course of product formation and UDP-glucose consumption, 800 µl of Viperin (120 µM) was used to which 80 µl SAM (20 mM) and 80 µl UDP-glucose (20 mM) was added. 100 µl of each sample was removed at t= 0 minute, which is before the addition of sodium dithionite to start the reaction, and at 10, 20, 40, 80, 160, and 420 minutes after addition of 20 µl (100 mM) sodium dithionite. At each time point 100 µl of reaction mixture was removed and was mixed with 100 µl of ethanol (100%) to denature the protein and quench the reaction. The solutions were then subjected to centrifugation at 13,000 rpm for 8 minutes to remove the denatured protein. Each solution was transferred to a clean microcentrifuge tube (Eppendorf) and kept on ice for immediate analysis by LC-MS. Alternatively to quench the reaction each solution was passed through an Amicon ultra centrifugal filter (3 kDa cut-off). The temperature inside the glove box was 20 ºC.

Determining the kinetic parameters of Viperin. To determine the kinetic parameters of Viperin different amounts of UDP-glucose (between 0-10 mM) were added to Viperin (5 mg/ml) in the presence of SAM (2 mM). The reactions were started by addition of sodium dithionite (2.5 mM). Final volume of the reactions was 0.2 ml. After 60 minutes the reactions were stopped by addition of 1:1 V/V ethanol to denature the protein followed by centrifugation at 13,000 rpm to remove the denatured protein. The solutions were immediately used for LC-MS measurements. The concentration of product formed was determined from the intensity of the LC chromatogram of the product (m/z 818). The amount of product formed was determined from the amount of UDP-glucose consumed. 200 µl Viperin (400 µM) was added to a microcentrifuge tube (Eppendorf) followed by addition of 20 µl SAM (10 mM stock), 20 µl UDP-glucose (1 mM stock), and 2 µl sodium dithionite (100 mM stock). The amount of SAM was 10-fold more than UDP-glucose to reach near complete consumption of UDP-glucose. After three hours incubation in the glove box, samples were prepared for LC-MS analysis as explained above. Under these conditions, we assumed that 100 µM product was formed and thus, the observed intensity of the LC chromatogram of the product was assumed to represent formation of approximately 100 µM product. This relationship was then used to determine the concentration of product formed in other experiments from the intensity of the product peak in the LC chromatograms.

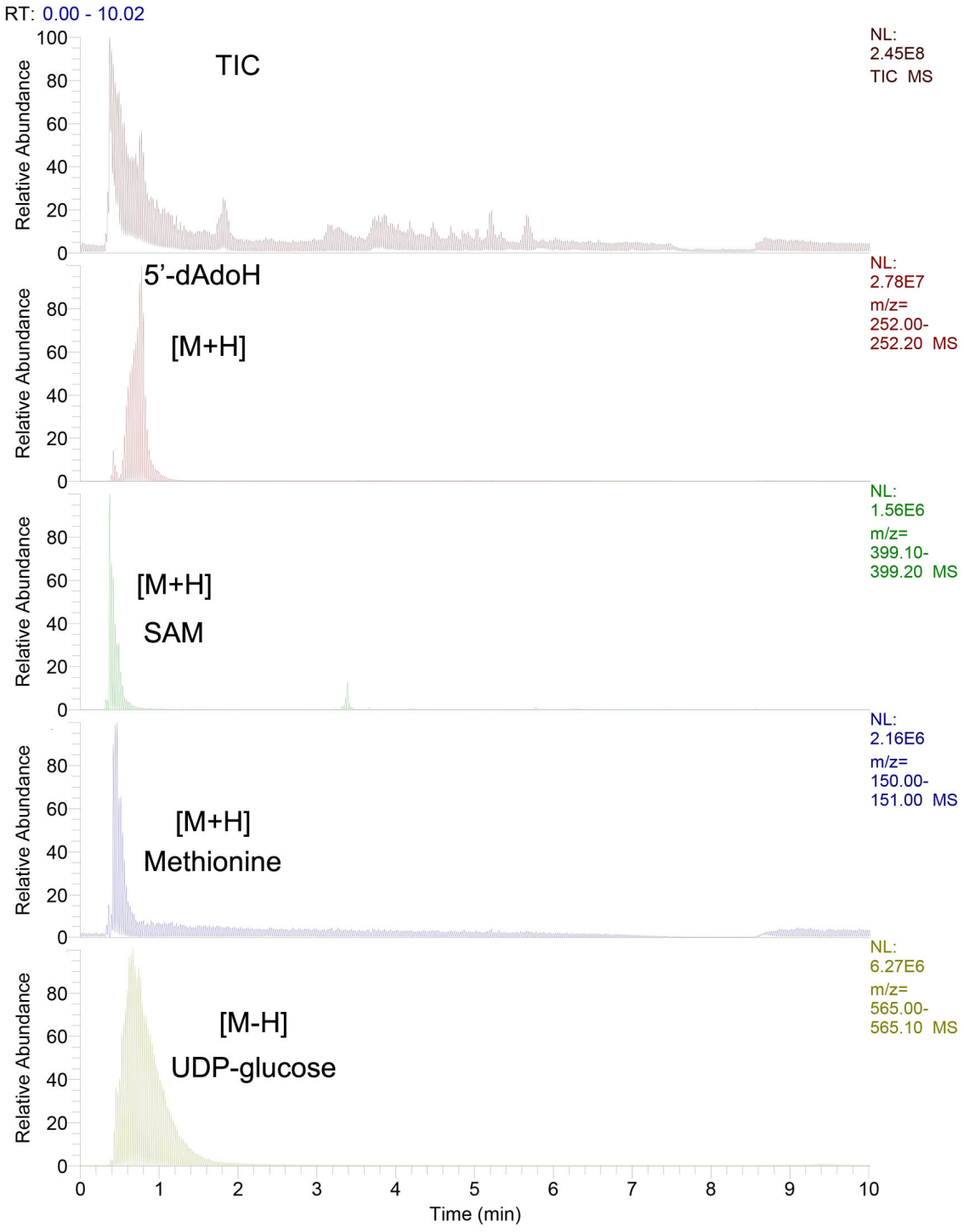
Preparation of samples for NMR spectroscopy and ^{1}H-NMR measurements. All NMR samples were prepared in the glove box (Belle, O_2 < 5.0 ppm). Four samples were prepared. A sample containing all components (Viperin, SAM, UDP-glucose, and sodium dithionite), and three other samples in each of which UDP-glucose, Viperin, or sodium dithionite was not added. The sample containing all components of the reaction was prepared by addition of 30 µl SAM (20 mM stock) and UDP-glucose (30 mM stock) to 300 µl Viperin (400 µM), followed by addition of 15 µl sodium dithionite (100 mM stock). To prepare the samples in which a component was not added, the buffer (200 mM Mops, 300 mM NaCl, pH 7.0) was added to reach exactly the same final volume as the sample containing all the components of reaction. Viperin was in 100 mM Tris, 300 mM NaCl, pH 7.5. Five hours after incubation of the samples in the glove box (temperature around 20 °C), 200 µl D_2O was added to each sample. The solutions were added to 5 mm O. D. NMR tubes. The tubes were tightly closed. The NMR tubes were then transferred outside the glove box and immediately used for measurements.



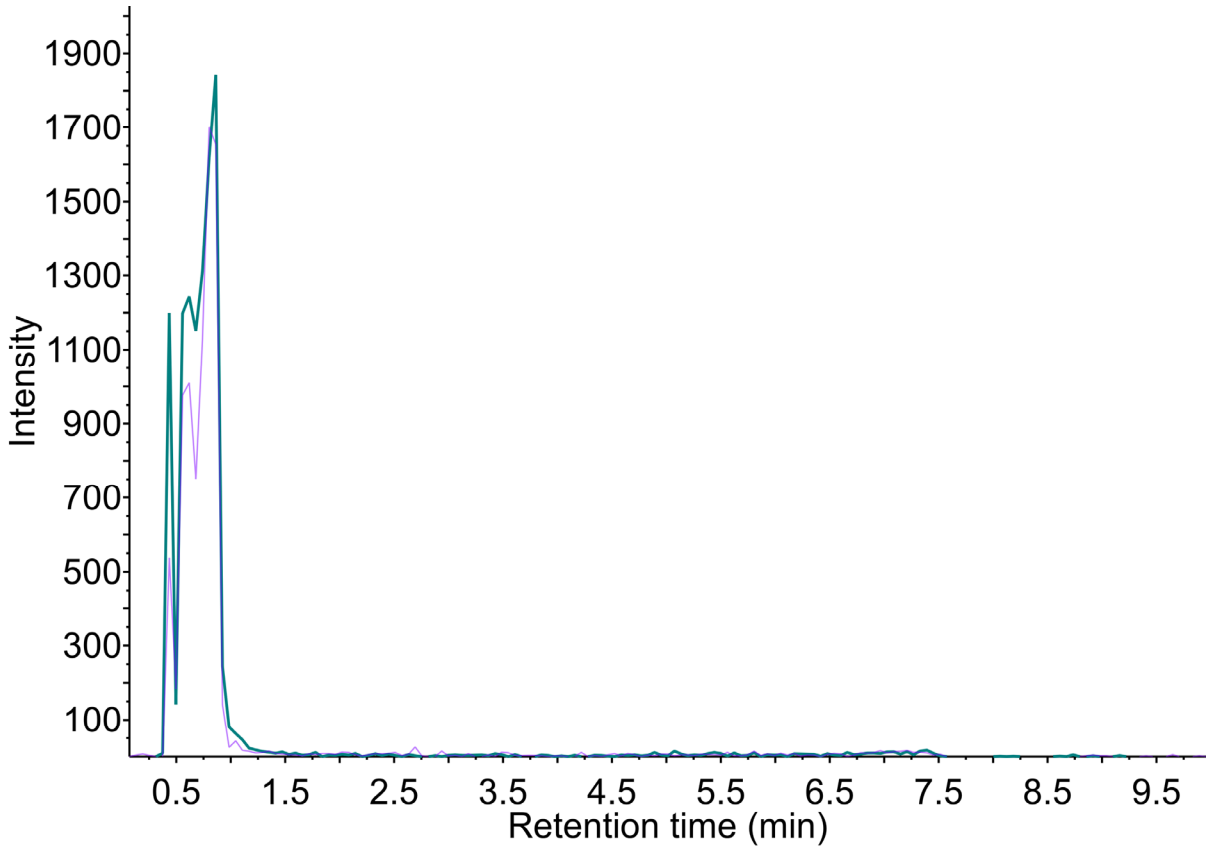
Supplementary Figure 1. Viperin is conserved from fungi to humans. The figure shows multiple amino acid sequence alignment of Viperin from eight different species. Multiple sequence alignment was performed using T-COFFEE online tool, and BoxShade online tool was used to prepare the figure. The amino acid sequences of Viperin from eight species are compared, from top to bottom: *Mus musculus*, human, *Siniperca obscura*, *Loxodonta africana*, *Equus caballus*, *Thielavia terrestris*, *Rhizophagus irregularis*, and *Aspergillus nomius*. The highly variable N-terminal hydrophobic domain is predicted to be a membrane anchor (from analysis using the DAS-TMfilter server). Orange circles mark the highly conserved cysteine residues of the conserved CxxxCxxC motif. Blue circles mark the conserved GGE motif of the SAM binding pocket.



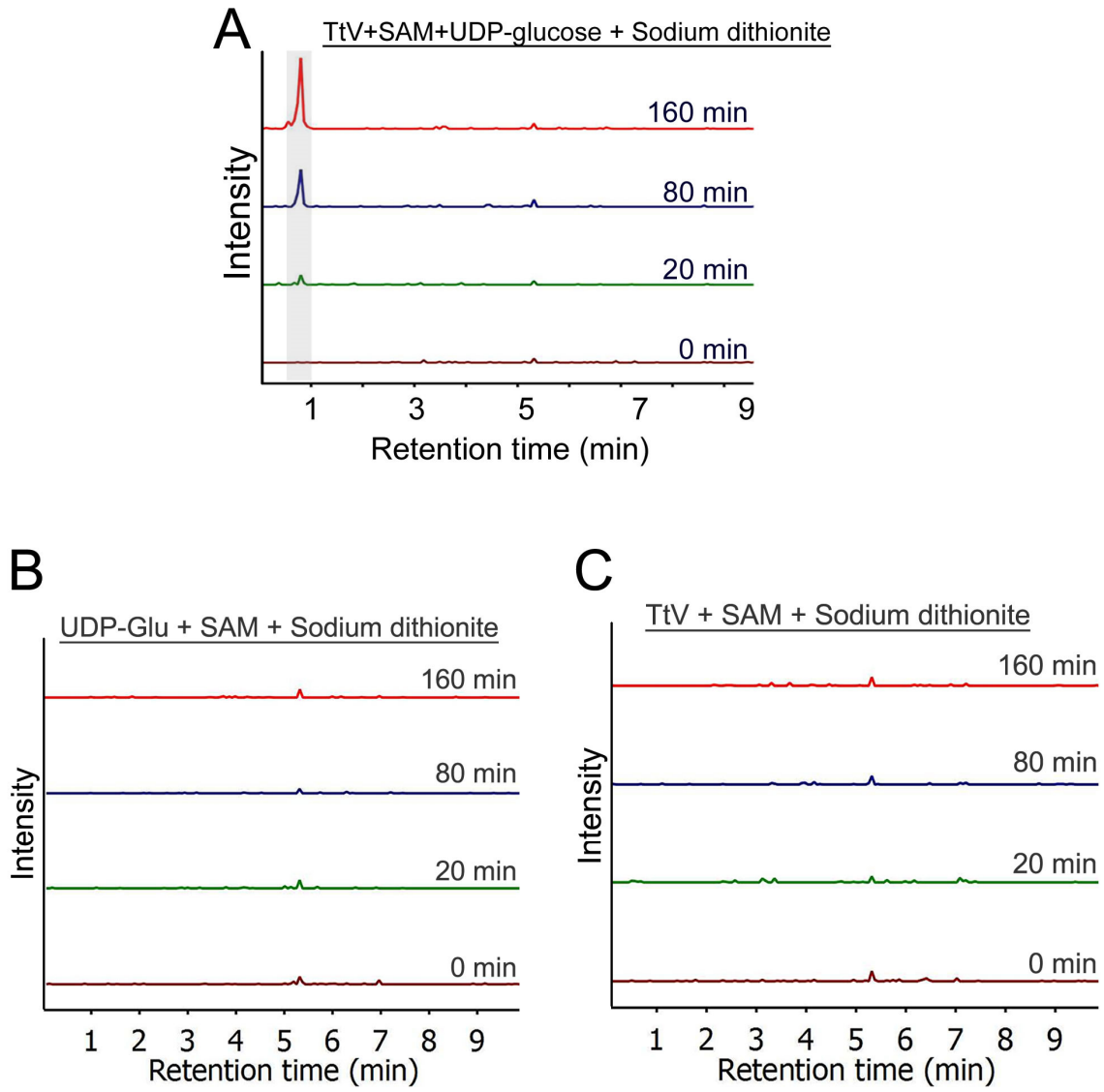
Supplementary Figure 2. After activation the purified Viperin contains a functional [4Fe-4S] cluster. (A) An SDS gel showing the purified fungal Viperin with molecular mass of approximately 37 kDa. (B) After purification the protein was activated (supplementary methods). The EPR spectrum of the $[4Fe\text{-}4S]^{1+}$ cluster in fungal Viperin has the same g values as those of the EPR spectrum of the $[4Fe\text{-}4S]^{1+}$ cluster of human Viperin. (C) The LC chromatogram of formation of a product [M+H] with a m/z of 252.1 upon addition of SAM and sodium dithionite (SD) to Viperin. Samples were incubated for 10 minutes under anaerobic conditions and subsequently were used for LC-MS analysis. (D) Accurate MS analysis of the product of the cleavage of SAM (C) in the absence of substrate confirmed formation of 5'-dAdoH (m/z 252.1096 for [M+H]). The observed MS spectrum of the product in the LC chromatogram (C) is the same as the predicted MS spectrum of the [M+H] for 5'-dAdoH.



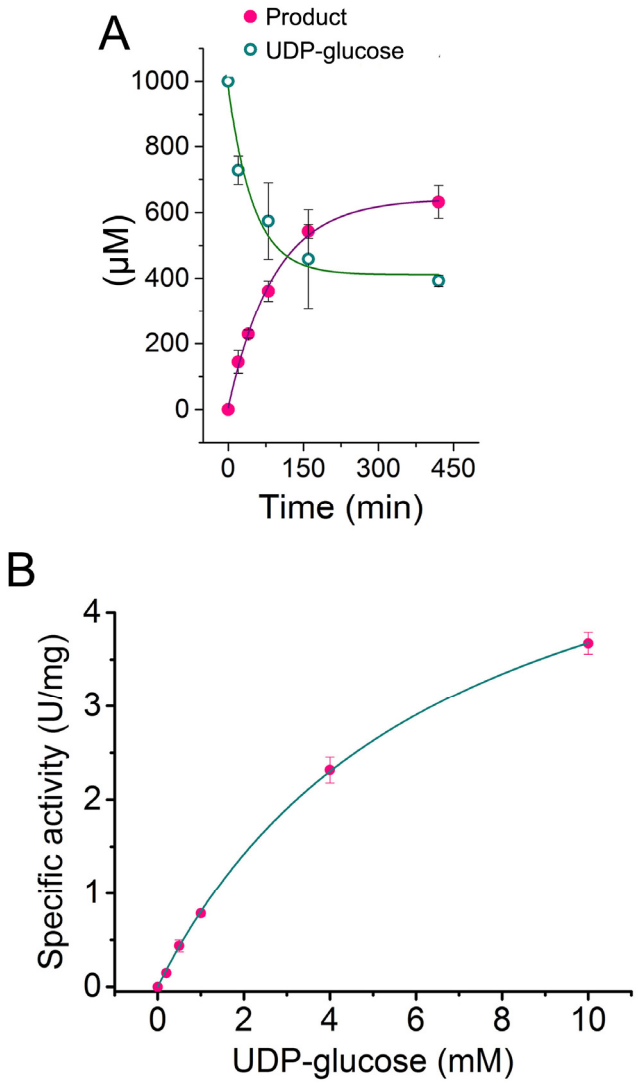
Supplementary Figure 3. Different components of the reaction were detected by LC-MS. From top to bottom: The LC chromatogram for total ion count (TIC), that of [M+H] for 5'-dAdoH with m/z of 252.10, that of [M+H] for SAM with m/z of 399.14, that of [M+H] for methionine with m/z of 150.06, and that of [M-H] for UDP-glucose with m/z of 565.05. Measurements were performed 160 minutes after addition of sodium dithionite to a solution containing SAM, Viperin, and UDP-glucose. All of the components of the reaction were eluted within 2 minutes because of their high polarity.



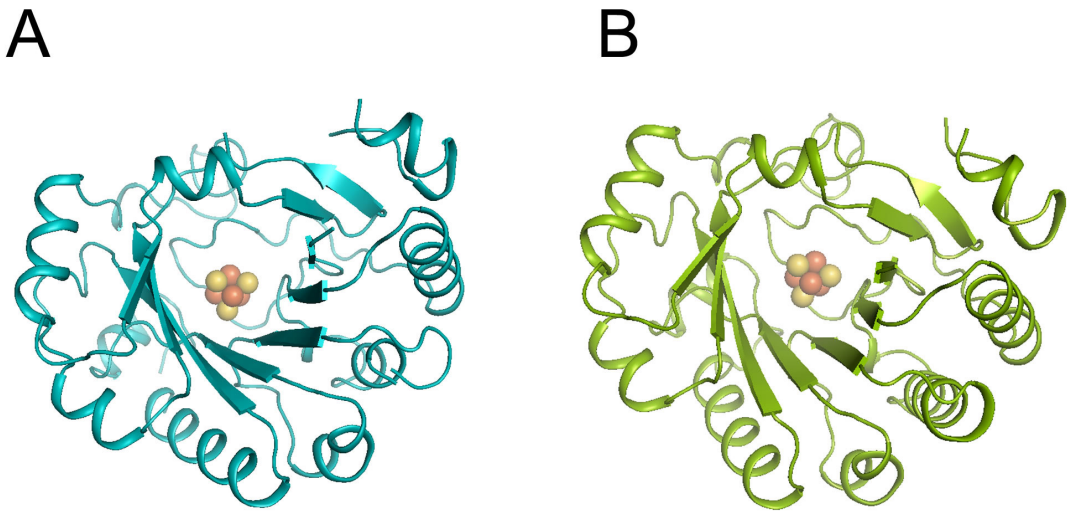
Supplementary Figure 4. The amount of 5′-dAdoH produced in the presence of UDP-glucose was less than that in the absence of UDP-glucose. LC chromatograms of 5′-dAdoH product (M+H) with m/z of 252.10 in positive ion electrospray ionization mode. Chromatograms were recorded for a sample in which SAM and sodium dithionite were added to the fungal Viperin (green trace) and for a sample in which SAM, UDP-glucose, and sodium dithionite were added (purple trace). The results show that when UDP-glucose was added to a mixture of Viperin, SAM, and sodium dithionite (purple) the amount of 5′-dAdoH produced was less than that when UDP nm-glucose was not added to a mixture of Viperin, SAM, and sodium dithionite (green) . UDP-glucose was added in the presence of 2-fold excess SAM (2 mM final concentration) to Viperin (100μM). The reaction was started by addition of sodium dithionite (500 μM final concentration). After 3 hours a sample was prepared (Methods) for LC-MS analysis.



Supplementary Figure 5. The amount of product formed increases as a function of time. (A) The LC chromatograms of the product [M+H] with m/z of 818.1 in positive ion electrospray ionization mode. The reaction was stopped at different time points after addition of sodium dithionite to a solution containing Viperin (137 µM), UDP-glucose (1 mM), SAM (1mM) and sodium dithionite (2 mM). (B-C) The LC chromatograms show the absence of the product [M+H] with m/z of 818 in two control samples: (B) a control sample in which Viperin was absent, and (C) a control sample in which UDP-glucose was absent. Samples were prepared before addition of sodium dithionite (t=0 minute) and at different time points after addition of sodium dithionite.



Supplementary Figure 6. Consumption of UDP-glucose by Viperin follows simple Michaelis-Menten kinetics. (A) The amount of product formed as a function of time is consistent with the amount of UDP-glucose consumed as a function of time. Concentration of UDP-glucose was 1 mM and that of SAM was 2 mM. Sodium dithionite was added to a final concentration of 2 mM. (B) The specific activity of enzyme was plotted as a function of the amount of UDP-glucose added. The amount of product formed was estimated from the intensity of the LC chromatogram of the product [M+H] with m/z of 818, as explained in experimental procedure. The fit to the data is obtained using Michaelis-Menten kinetic model with no cooperativity or substrate/product inhibition. Enzyme activity assays were performed at room temperature. Concentration of enzyme was approximately 5 mg/ml. Concentration of SAM was 2 mM and the reaction was started by addition of sodium dithionite (final concentration of 2.5 mM). Final reaction volume was 0.2 ml. Reactions were incubated at room temperature. Data are average of two measurements ± errors.



Supplementary Figure 7. Comparison of the structure of fungal Viperin obtained by structural homology modeling with the X-ray crystal structure of mouse Viperin. (A) The predicted structure of fungal Viperin. The structure was modelled based on the structure of mouse Viperin (PDB Code: 5VSL). (B) The X-ray crystal structure of mouse Viperin (PDB Code: 5VSM).