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An Efficient Viologen-Based Electron Donor to Nitrogenase

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Supporting Information

ABSTRACT: Nitrogenase catalyzes the reduction of N_2 to NH_3, supporting all biological nitrogen fixation. Electron donors to this enzyme are ferredoxin or flavodoxin (*in vivo*) and sodium dithionite (*in vitro*). Features of these electron donors put a limit on spectrophotometric studies and electrocatalytic applications of nitrogenase. Although it is common to use methyl viologen as an electron donor for many low-potential oxidoreductases, decreased nitrogenase activity is observed with an increasing concentration of methyl viologen, limiting its utility under many circumstances. In this work, we suggest that this concentration-dependent decrease in activity can be explained by the formation of a dimer of the radical cation of methyl viologen $(Me_2V^{\bullet+})_2$ at higher methyl viologen concentrations. In addition, viologens functionalized with positively and negatively charged groups were synthesized and studied using spectroscopy and cyclic voltammetry. A sulfonated viologen derivative, 1,1′-bis(3-sulfonatopropyl)-4,4′-bipyridinium radical $\{[(SPr)_2V^{\bullet}]^-\}$, was found to support full nitrogenase activity up to a mediator concentration of 3 mM, while the positively charged viologen derivative was not an efficient reductant of nitrogenase due to the high standard redox potential. The utility of $[(SPr)_2V^{\bullet}]^-$ as an electron donor for nitrogenase was demonstrated by a simple, sensitive spectrophotometric assay for nitrogenase activity that can provide accurate values for the specific activity and turnover rate constant under argon. Under N_2, the formation of ammonia was confirmed. Because of the observed full activity of nitrogenase and low overpotential, $[(SPr)_2V^{\bullet}]^-$ should also prove to be valuable for nitrogenase electrocatalysis, including bioelectrosynthetic N_2 reduction.

The biological reduction of N_2 to NH_3 is catalyzed by the enzyme nitrogenase. The molybdenum-dependent nitrogenase consists of two component proteins (Figure 1a): the molybdenum−iron protein (MoFeP) and the iron protein (FeP).[1] MoFeP houses two unique cofactors, the electron carrier [8Fe-7S] (P-cluster) and the catalytic [7Fe-9S-1Mo-C-homocitrate] (FeMo-co).[2] FeP contains a single [4Fe-4S] cluster and two MgATP binding sites. During the catalytic cycle, the FeP is reduced by either flavodoxin or ferredoxin (*in vivo*)[3,4] or sodium dithionite or reduced methyl viologen (*in vitro*)[5,6] and binds two MgATP molecules. Then, FeP transiently associates with the MoFeP and transfers an electron, followed by hydrolysis of two MgATP molecules and dissociation from MoFeP.[7] The released oxidized FeP is reduced, and the two MgADP molecules are replaced by two MgATP molecules, making FeP ready for another round of MoFeP reduction. This cycle (called the FeP cycle) is repeated four times to cause the accumulation of four electrons and four protons on FeMo-co as two bridging hydrides and two protons. This four-electron reduced state [called E4(4H)] releases H_2 and binds N_2 with a reduction by two electrons.[8,9] Four more electron/proton delivery cycles must be completed to achieve the reduction of the N_2 to two ammonia molecules (eq 1). In the absence of N_2, hydrides and protons react, and H_2 is evolved (eq 2).

$$N_2 + 16MgATP + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i \quad (1)$$

$$4MgATP + 2H^+ + 2e^- \rightarrow H_2 + 4MgADP + 4P_i \quad (2)$$

Elucidation of aspects of the mechanism of nitrogenase and the application of this enzyme in biofuel and biosynthetic cells are of high interest for chemical conversion and energy-related efforts. This requires an efficient reductant for nitrogenase that does not affect the enzyme activity. In addition, it would be valuable if the reductant showed a large change in its extinction coefficient between its reduced and oxidized forms so that it could be used for spectroscopic studies, and it showed reversible electrochemical behavior at the electrode surface with a low overpotential to support nitrogenase electrocatalysis (i.e., the standard potential difference between the mediator and [4Fe-4S] cluster of FeP of nitrogenase). Most *in vitro* studies of nitrogenase over decades have utilized the reductant sodium dithionite. While convenient for fixed-time product measurement, sodium dithionite is of limited value in spectroscopic investigations due to its low extinction coefficient and in electrocatalysis due to its irreversible electrochemical reaction at the electrode surface. Alkyl viologens, for example, methyl viologen, represent a sound alternative to sodium dithionite and have been applied as stoichiometric reductants and as electrochemical mediators for enzymes such as hydrogenase,[10−12] formate dehydrogenase,[13,14] and CO dehydrogenase.[15,16]

Our recent studies[17] revealed that methyl viologen is an efficient reductant for nitrogenase (Figure 1b,c), supporting substrate reduction activity similar to the activity measured

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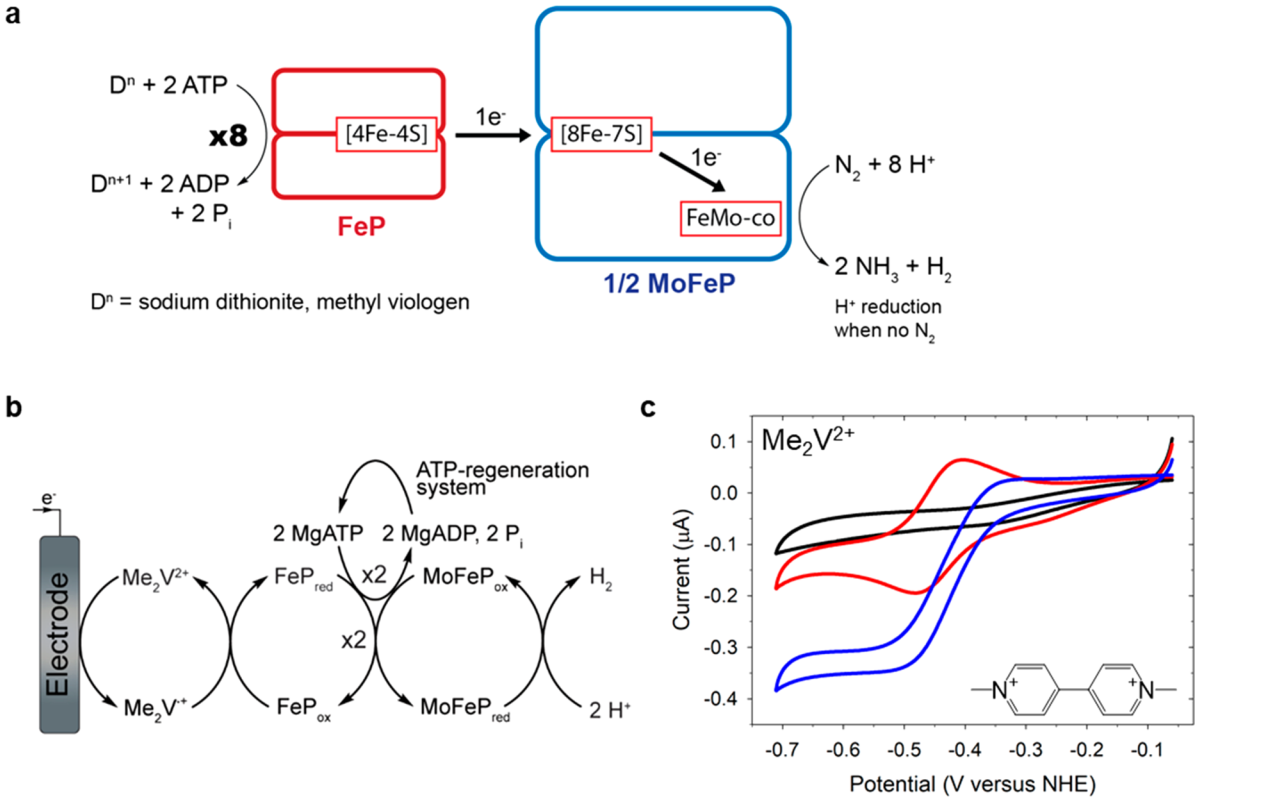

Figure 1. (a) Simplified catalytic scheme for *in vitro* nitrogenase catalysis. (b) Mediated electrocatalytic system utilizing nitrogenase showcased under argon. (c) Cyclic voltammograms recorded with solutions of activity buffer (black), upon addition of 50 μM methyl viologen (red), and then upon addition of the nitrogenase complex (MoFeP:FeP, 1:15, blue). The activity buffer contains 100 mM MOPS (pH 7.0), 6.7 mM $MgCl_2$, 5 mM ATP, and an ATP regeneration system (30 mM phosphocreatine, 0.2 mg/mL phosphocreatine kinase, and 1.3 mg/mL bovine serum albumin).

with sodium dithionite. However, the catalytic performance of nitrogenase is observed to be dependent on the methyl viologen concentration. At millimolar concentrations, methyl viologen causes significant inhibition of nitrogenase activity as a reductant in spectrophotometric activity assays[5] and electrolysis cells.[18] This effect is not well understood. As a result, most nitrogenase kinetic studies still rely on dithionite. In this Communication, we studied the inhibitory effect of methyl viologen and further report a viologen derivative that can be efficiently used in the millimolar concentration range that enables a high-utility spectroscopic activity assay to quantify the flow of electrons through nitrogenase. In addition, the viologen derivative is useful for electrocatalysis studies with nitrogenase.

The effect of methyl viologen concentration on nitrogenase activity was demonstrated via a spectroscopic study of nitrogenase (0.4 μM MoFeP and 6 μM FeP) and a radical cation form of methyl viologen ($Me_2V^{\bullet+}$) in the activity assay buffer containing 100 mM MOPS (pH 7.0), 6.7 mM $MgCl_2$, 5 mM ATP, and an ATP regeneration system (30 mM phosphocreatine, 0.2 mg/mL phosphocreatine kinase, and 1.3 mg/mL bovine serum albumin). The mixture led to rapid oxidation of $Me_2V^{\bullet+}$ to Me_2V^{2+} followed at 606 nm, reflecting the nitrogenase catalytic reduction of protons to H_2 under argon (Figure 2a). A typical time curve is shown in Figure 2b. The rate constant (k_{obs}) obtained with different $Me_2V^{\bullet+}$ concentrations reveals that increasing concentrations of $Me_2V^{\bullet+}$ result in decreasing rate constants (k_{obs}) as shown in Figure 2c. The highest value of k_{obs} (11 s^{-1}) was observed at 250 μM methyl viologen. No significant background process took place in the absence of nitrogenase components.

We sought to understand the origin of the inhibitory effect at higher concentrations of $Me_2V^{\bullet+}$. It is known that persistent π-radicals such as $Me_2V^{\bullet+}$ can take part in radical−radical interactions[19] that result in the formation of a dimer $(Me_2V^{\bullet+})_2$ (Figure 2d). The reported association constant of $Me_2V^{\bullet+}$ is 1300 M^{-1}.[19] Figure 2c (red) shows the development of the dimer as a function of the total $Me_2V^{\bullet+}$ concentration, which is consistent with the decrease in k_{obs} for nitrogenase catalysis.

The dimerization of methyl viologen can increase the formal potential (see the Supporting Information for more details), thereby decreasing the driving force between this mediator and nitrogenase. Our theoretical analysis of the methyl viologen redox couple based on the Nernst equation showed a maximum positive shift of +30 mV as a result of increasing methyl viologen concentration in the range of 0.05−3 mM (Figure S3). Similar results were obtained experimentally by using cyclic voltammetry (Figure S3). This shift should not have a major effect on nitrogenase catalysis. This suggested that dimerization inhibits the activity of nitrogenase by another mechanism.

In an effort to overcome the inhibitory effect of methyl viologen, we synthesized two viologen derivatives: 1,1′-bis[3-(trimethylammonium)propyl]-4,4′-bipyridinium tetrabromide {$[(NPr)_2V]Br_4$} bearing two additional positively charged ammonium groups[20] and 1,1′-bis(3-sulfonatopropyl)-4,4′-bipyridinium $[(SPr)_2V]$ bearing two additional negatively charged sulfonic groups.[21] These derivatives were first tested

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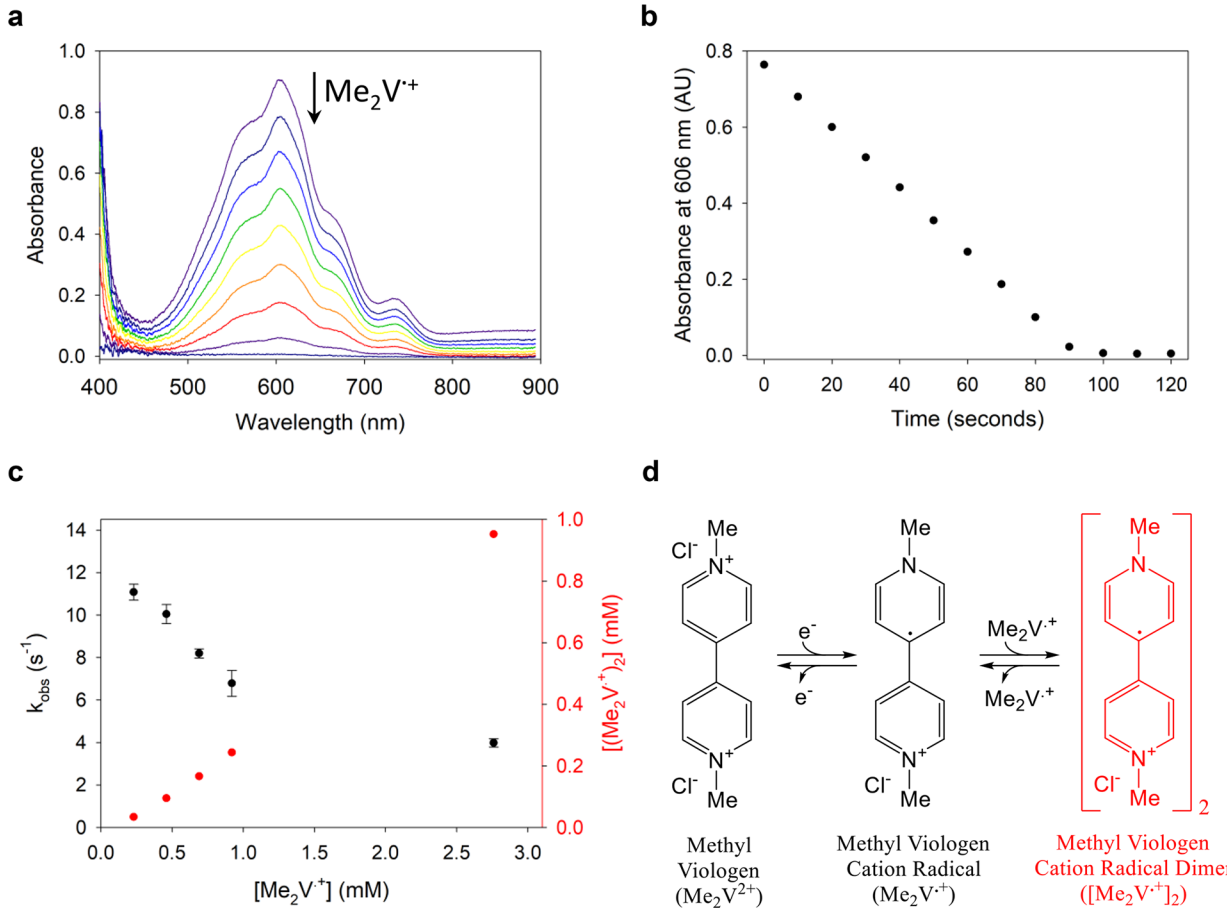

Figure 2. Methyl viologen cation radical ($Me_2V^{\bullet+}$) as a reductant in a spectrophotometric activity assay for nitrogenase under argon. (a) Spectrophotometric evidence of the oxidation of 0.45 mM $Me_2V^{\bullet+}$ by nitrogenase and (b) time course at 606 nm measured in a cuvette with a 0.2 cm path length. (c) k_{obs} vs $[Me_2V^{\bullet+}]$ (black) and calculated $Me_2V^{\bullet+}$ dimer concentration (red). (d) Redox transition of methyl viologen to $Me_2V^{\bullet+}$ and the dimerization reaction. Conditions: 0.4 μM MoFeP and 6 μM FeP in activity assay buffer (0.5 mL; n = 3).

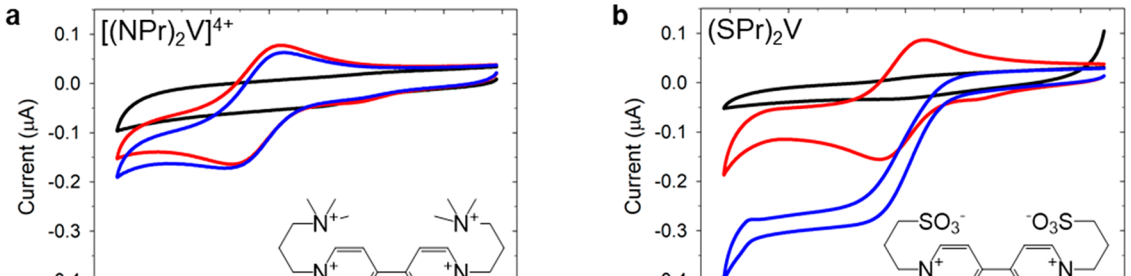

Figure 3. Voltammetric studies of viologen derivatives (a) $(NPr)_2V^{4+}$ and (b) $(SPr)_2V$. Cyclic voltammograms were recorded with solutions of activity assay buffer (black), upon addition of 50 μM viologen derivative (red), and upon addition of the nitrogenase complex (MoFeP:FeP, 1:15, blue).

using the voltammetric approach.[17] Cyclic voltammograms were recorded in the activity assay buffer, after addition of the corresponding viologen at 50 μM and after the addition of 0.4 μM MoFeP and 6 μM FeP (Figure 3). As shown in Figure 3a, in the presence of $[(NPr)_2V]^{4+}$, the current did not change after addition of nitrogenase, reflecting the fact that this derivative does not efficiently reduce nitrogenase FeP (−0.4 V vs NHE, measured at pH 8 in the presence of MgATP),[22,23] likely due to its higher standard reduction potential (−0.32 V vs NHE). The standard potential of $(SPr)_2V$ (−0.40 V vs NHE) was 40 mV more positive than that of methyl viologen, but it was sufficient to reduce nitrogenase (Figure 3b).

The rate constants in the presence of Me_2V^{2+} and $(SPr)_2V$ were 13 and 12 s^{-1}, respectively, with the corresponding viologen at 50 μM. k_{obs} was determined using eq 3 derived for nitrogenase electrocatalysis[17]

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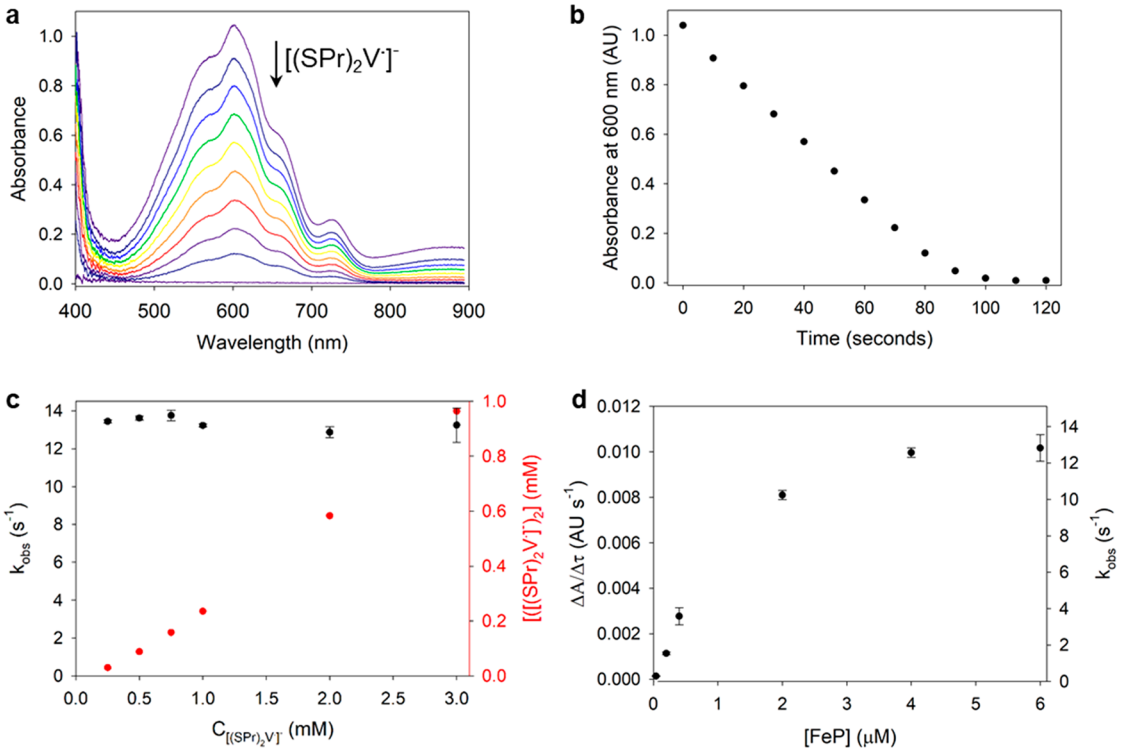

Figure 4. $[(SPr)_2V^{\bullet}]^-$ as an electron donor for the spectrophotometric activity assay for nitrogenase under argon. (a) Spectrophotometric evidence of the oxidation of $[(SPr)_2V^{\bullet}]^-$ by nitrogenase in activity assay buffer and (b) time course at 606 nm. (c) k_{obs} vs $[(SPr)_2V^{\bullet}]^-$ (black) and calculated $[(SPr)_2V^{\bullet}]^-$ dimer concentration (red). (d) Plot of k_{obs} vs [FeP] measured spectrophotometrically at 600 nm in the presence of 0.5 mM $[(SPr)_2V^{\bullet}]^-$. Conditions: 0.4 μM MoFeP, 6 μM FeP in activity assay buffer (0.5 mL; n = 3), and cuvette path length of 0.2 cm.

$$k_{obs} = \left(0.4463\frac{i_{cat}}{i_p}\right)^2 \frac{nFvC^{o}_{Med}}{RTC^{o}_{E} \times 2} \quad (3)$$

where k_{obs} is the observed kinetic constant for substrate reduction, i_{cat} is the current of the catalytic reaction, i_p is the peak current of the mediator in the absence of nitrogenase, n is the number of electrons in the electrochemical reaction (n = 1), F is Faraday's constant, v is the scan rate, C^{o}_{Med} is the mediator concentration, R is the ideal gas constant, T is the reaction temperature, and $C^{o}_{E} = C^{o}_{MoFeP}$ in the presence of an excess of FeP.

A spectroscopic study similar to that described above was performed to test the nitrogenase activity at higher concentrations of $[(SPr)_2V^{\bullet}]^-$. Panels a and b of Figure 4 show the spectra and time course at 600 nm in the presence of nitrogenase, respectively. No decrease in activity was observed with an increase in the concentration of $(SPr)_2V$ to 3 mM (Figure 4c). In addition, our studies revealed that $(SPr)_2V$ in its one-electron reduced form is prone to dimerize with an association constant of 841 M^{-1} (Figure S5 and Figure 4c). This finding shows that $[(SPr)_2V^{\bullet}]^-$ achieves high efficiency with nitrogenase, likely due to the additional negatively charged groups preventing interference in the nitrogenase catalytic cycle.

To confirm N_2 reduction in the presence of $[(SPr)_2V^{\bullet}]^-$ as an electron donor, 0.4 μM MoFeP and 6 μM FeP were added to the activity assay buffer containing 1 mM $[(SPr)_2V^{\bullet}]^-$ under N_2 in a sealed vial and left to react until all the reductant was consumed. The formation of 143 μM ammonium was confirmed using a fluorometric assay.[24]

Previously described spectrophotometric activity assays for nitrogenase utilized sodium dithionite[25] or titanium(III) citrate[26] as the electron donor. These methods are limited by the low stability and low extinction coefficients of these electron donors. For these methods, the measurement of nitrogenase activity still relies on the assays requiring the obligatory product quantification: NH_3 by fluorometric assay and H_2 by gas chromatography. Here, we tested $[(SPr)_2V^{\bullet}]^-$ as a stable and easy to prepare reductant for nitrogenase in a spectrophotometric activity assay. The assay was performed in a cuvette with a path length of 0.2 cm in the presence of 0.5 mM $[(SPr)_2V^{\bullet}]^-$. The extinction coefficient was determined to be 9925 M^{-1} cm^{-1} at 600 nm. Figure 4d shows the well-pronounced dependence of the catalytic rate constant on FeP concentration. The dependence of the nitrogenase reaction rate on MoFeP concentration was also studied spectrophotometrically and exhibits a linear relationship between the reaction rate and the concentration of MoFeP (see Figure S6). The lowest concentrations of nitrogenase proteins measured by this assay were 40 nM FeP and 20 nM MoFeP (see Figure S6). The sensitivity of the assay is 0.0074 s^{-1} μM^{-1} for FeP (determined for <0.4 μM FeP, in the presence of 0.4 μM MoFeP) and 0.0266 s^{-1} μM^{-1} for MoFeP (in the presence of 6 μM FeP). It is expected that utilization of $[(SPr)_2V^{\bullet}]^-$ as a reductant for nitrogenase should facilitate many mechanistic and genetic engineering studies because it provides a fast and sensitive determination of nitrogenase activity. The application of $(SPr)_2V$ as an electrochemical mediator in bioelectrosynthetic N_2 reduction is promising because, in contrast to methyl viologen, it shows a lower overpotential and allows the use of higher concentrations of the electrochemical mediator, which

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does not inhibit nitrogenase activity and may result in higher product formation rates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.9b00844.

General considerations, electrolysis of viologens, determination of extinction coefficients, influence of the dimerization of viologens on their formal redox potential, determination of the $[(SPr)_2V^{\bullet}]^-$ dimerization constant, and MoFeP concentration dependence (Figures S1−S6) (PDF)

Accession Codes

MoFeP, UniProtKB P07328 and P07329; FeP, UniProtKB P00459.

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Author Contributions

A.B. conceived the idea of the project and designed and performed all experiments. A.B. led the data interpretation and analysis in consultation with L.C.S. Z.-Y.Y. isolated and purified MoFeP. Z.-Y.Y. and A.B. isolated and purified FeP. J.L., B.H., and M.H. synthesized $(SPr)_2V$ and $[(NPr)_2V]^{4+}$ under the supervision of T.L.L. A.B. and L.C.S. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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