Is Trehalose an Effective Quenching Agent of *Azotobacter vinelandii* Mo-Nitrogenase Turnover?

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Abstract

H₂-evolution assays, plus EPR and FTIR spectroscopies, using CO-inhibited *Azotobacter vinelandii* Mo-nitrogenase have shown that the disaccharide trehalose is an effective quenching agent of enzymatic turnover and also stabilizes the reaction intermediates formed. Complete inhibition of H₂-evolution activity was achieved at 1.5 M trehalose, which compares favorably to the requirement for 10 M ethylene glycol to achieve similar inhibition. Reaction-intermediate stabilization was demonstrated by monitoring the EPR spectrum of the 'hi-CO' form of CO-inhibited N₂ase, which did not change during 1 hr after trehalose quenching. Similarly, in situ photolysis with FTIR monitoring of 'hi-CO' resulted in the same 1973 and 1681 cm⁻¹ signals as observed previously in ethylene glycol-quenched systems.[a] These results clearly show that 1.5 M trehalose is an effective quench and stabilization agent for Mo-N₂ase studies. Possible applications are discussed.

[a.] "Photolysis of Hi-CO Nitrogenase - Observation of a Plethora of Distinct CO Species Using Infrared Spectroscopy", Yan, L.; Dapper, C. H.; George, S. J.; Wang, H.; Mitra, D.; Dong, W.; Newton, W. E.; Cramer, S. P., *Eur J Inorg Chem*, **2011**, 2064-2074.

Keywords: Mo-nitrogenase, trehalose, EPR, FTIR, photolysis, carbon monoxide

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1. Introduction

Polyols have long been used in studies with biological materials. Their major uses include protection from denaturation either on freezing (cryo-protection) or during relatively severe purification techniques that could damage the often fragile structure of polypeptide or prosthetic group or both. More recently, polyols have been used to probe catalytic questions by stabilizing reactive intermediates and so allowing studies that would otherwise be impossible or impracticable. In the nitrogenase field, glycerol and ethylene glycol have been the polyols of choice with the latter being used very effectively both to quench Mo-nitrogenase (Mo-N₂ase) activity and to stabilize three EPR-observable CO-bound intermediates[1-4] and one EPR-silent CO-bound intermediate. [5]

These experiments have proved their considerable worth by providing direct insight into substrate binding[3,5,6] because, although CO has long been known as a potent inhibitor of N₂ase[7], it came as a surprise when it was recognized quite recently as a substrate.[8] Its reduction products include CH₄, C₂H₄, C₃H₆, and even C₄ species.[9-11] This resemblance to Fischer-Tropsch catalysis[12,13] has reinvigorated interest in the mechanism of biological nitrogen fixation, which is responsible for providing about half of the fixed nitrogen available globally.[14,15] But even more startling is the very recent crystallographic result, where the structure of a CO-bound form of Mo-N₂ase showed that CO binding to the FeMo-cofactor (Scheme 1, left) was at the expense of a bridging 'waist' sulfide, which somehow regained its place, along with full enzymatic activity, when CO was removed.[16]

However, ethylene glycol and glycerol have their drawbacks; one being the difficulty in removing them from aqueous solutions when, for example, a very concentrated solution or a film of the substance of interest is required. Our search for a more tractable alternative led to a very different polyol, trehalose. Trehalose is a non-reducing homodisaccharide formed by linking two glucose molecules via a α -1,1-glycosidic bond (Scheme 1, right), but has similar quenching and stabilizing properties.[17,18] It is found in microorganisms, plants and animals, often produced in response to environmental stress.[19] Trehalose treatment has been used previously to suppress protein relaxation between different CO-

myoglobin conformations, even at room temperature and, at different concentrations, can even affect the proportions of CO species captured by myoglobin. [20,21] In addition, a trehalose-based glass has allowed capture of photo-intermediates of rhodopsin at room temperature that are ordinarily seen only at low temperatures or on short-time scales.[22] These observations suggested that trehalose treatment might be the alternative polyol for quenching N_2 ase turnover and capturing intermediates that we were searching for. Here, we show that is indeed the case.

Scheme 1. Left: alternative structures of mono-CO-bound FeMo-cofactor (called 'lo-CO') with: (a) all three 'waist' bridging sulfides (S) in place [3,23]; and (b) with one 'waist' bridging S replaced by CO.[16] Right: structure of trehalose.

2. Materials and Methods

2.1. Cell growth and protein purification

The *Azotobacter vinelandii* wild-type strain (DJ527) was grown in a 24-L fermenter at 30°C in a modified fixed-nitrogen-free liquid Burk medium.[24] The N_2 ase component proteins were separated and purified as described previously.[25] All subsequent protein manipulation was done anaerobically with either a Schlenk line or within an anaerobic

glove box operating at less than 1ppm O₂. The component proteins were concentrated individually using an Amicon microfiltration pressure concentrator before buffer exchange to 25 mM HEPES (pH 7.4), 200mM NaCl, 10mM MgCl₂ and 2mM Na₂S₂O₄. The specific activity of the Fe and MoFe protein for hydrogen evolution was 2300 and 2100 nmoles H₂ (min⁻¹mg protein⁻¹) at 30°C, respectively. Protein concentrations were determined using the Lowry method.[26]

2.2. Trehalose suppression of catalyzed H₂ evolution

All wild-type MoFe-protein activities were measured using a high electron-flux assay that contained a 20-fold molar ratio of wild-type Fe protein under an atmosphere of argon gas at 30°C. Each assay contained 0.25 mg total nitrogenase proteins, 30 μmol of creatine phosphate, 25 μmol of HEPES buffer (pH 7.4), 20 μmol of sodium dithionite, 5 μmol of MgCl₂, 2.5 μmol of ATP, and 0.125 mg of creatine phosphokinase. Trehalose solutions of varying concentrations were prepared in 25mM HEPES (pH 7.4). Assays were quenched after 8 min by addition of an aliquot of either 0.5 M EDTA to final concentration of 0.15 M (as a control) or one of several trehalose solutions to achieve a final concentration of 0.2 M to 1.5 M trehalose. Total evolved H₂ evolved was measured and the assays then remained at 30°C for up to 90 min more before total evolved H₂ was again measured. Hydrogen was measured by an 8A Shimadzu gas chromatograph using a molecular sieve 5A column and a thermal conductivity detector with calibration by a standard 1% H₂ in N₂ mixture.

2.3. Trehalose stabilization of CO-bound species

The so-called 'hi-CO' complex[2,3] was prepared by enzyme turnover under a 100% CO atmosphere using low electron-flux conditions generated by a 1:4 molar ratio of Fe protein:MoFe protein as described previously.[27] The reaction was then quenched by the addition of trehalose to a final concentration of 1.5 M and the product concentrated in an Amicon microfiltration pressure concentrator under 100% CO gas.

2.4. Electron paramagnetic resonance spectroscopy

EPR spectra were collected at X-band using a Bruker CW Elexsys-II E500 instrument connected to a Bruker SHQE-W resonator at 12K. Cryogenic temperatures were achieved with an Oxford ESR-900 He-flow cryostat.

2.5. Photolysis monitored by FT-infrared spectroscopy

Photolysis was performed using a Sutter Instruments Lambda LS xenon-arc lamp and a Profoto ZoomSpot Flash Lamp essentially as performed previously.[5,27] Samples were held in custom-built CaF_2 -windowed cells with Teflon spacers giving a path length of 50 microns. Infrared spectra were recorded with a Bruker Vertex 70V vacuum FTIR instrument operating at 4-cm^{-1} resolution. The samples were held at liquid helium temperatures inside a modified Oxford helium flow cryostat and photolyzed with 25-50 flashes of white light followed by direct illumination. The data are presented as difference spectra.

3. Results

3.1. Effect of trehalose on enzyme activity

Figure 1 shows the effect of adding either EDTA or trehalose as quenching agent on N_2 ase activity. High electron-flux hydrogen-evolution assays are totally inhibited by addition of 0.15 M EDTA (final concentration), which is used as the control. Similar total inhibition of N_2 ase activity also occurs with trehalose, but only at a final concentration of 1.5 M. Final concentrations up to 0.5 M trehalose are ineffective and 1.0 M only partially inhibitory. Values for total H_2 evolved are normalized in Figure 1 to accommodate data from more than a single experiment.

Figure 1. Plot of total H_2 evolved (normalized to amount present at time zero) versus time (min after adding quenching agent); ● = 0.15 M EDTA, × = 0.2 M trehalose, ◻ = 0.4 M trehalose, ◊ = 0.5 M trehalose, Δ = 1.0 M trehalose, ○ = 1.5 M trehalose.

3.2. Trehalose stabilization of the EPR signal from the 'hi-CO' inhibited form

Figure 2 (bottom) shows the results of a low electron-flux turnover experiment under 100% CO. After 10 min, an aliquot was removed and rapidly frozen *before* trehalose addition; it shows the axial EPR spectrum ($g=2.17, 2.05$) of the expected 'hi-CO' form of CO-inhibited N_2ase . [2-4] This form has one terminally bound CO and a second CO-based ligand, possibly protonated. [27] Very low concentrations of both the 'lo-CO' form (visible as small baseline disturbances centered at ~3650 gauss) and the resting-state form (labeled as RS with $g=4.3, 3.7$) are also present. Immediately following the trehalose quench (to 1.5 M final concentration), the integrated intensity of the $g=2.17$ 'hi-CO' signal had decreased to

83% of the unquenched value, but was then stable for 1 hr (77%). After 1.5 hr, about 55% of its integrated intensity remained.

3.3. FTIR-Monitored Photolysis

Upon photolysis with broadband white light at liquid-helium temperatures, a trehalose-quenched CO-bound Mo-N₂ase sample exhibited IR bands typical of the loss or re-arrangement of bound CO ligands.[5,27] These changes are best illustrated in difference spectra as shown (Figure 2, top). The negative (downward) photolysis feature at 1973 cm⁻¹ (Figure 2, top left) is due to loss of a terminally bound CO ligand, whereas that at ~~and~~ 1681 cm⁻¹ (Figure 2, top right) reflects the re-arrangement of the second CO in the 'hi-CO' sample. The-positive (upward) feature at 1715 cm⁻¹ (Figure 2, top right) is associated with the photolysis product and correlates with the formation of the 'lo-CO' species.[27]

Figure 2. FTIR-monitored cryophotolysis and EPR Spectroscopy of the 'hi-CO' form of trehalose-quenched N_2ase . Top: FTIR difference spectra for the high-energy (left) and low-energy (right) regions (see section 3.3 for band assignments). Bottom: Stacked EPR spectra for 'hi-CO' N_2ase : prior to trehalose quench (red); immediately after trehalose quench (blue); 1 hr post quench (green); and 1.5 h post-quench (black). The g-values for the 'hi-CO' species and the small amount of

resting-state (RS) enzyme are labeled. Spectra were corrected for concentration differences caused by dilution with the trehalose solution.

4. Discussion

The high electron-flux hydrogen-evolution assays clearly showed that 1.5 M trehalose (final concentration) totally inhibits N₂ase activity. No H₂ production beyond that quench could be detected for up to 90 min, which was not the case for final concentrations up to 1.0 M trehalose. Three experiments (at 0.4 M, 0.5 M and 1.0 M trehalose) essentially produce the same total amount of H₂, but over quite different time periods, which suggests that a limiting reactant in these assays, most likely dithionite or MgATP, has been exhausted by the time (15 min after the quench was added) of the first post-quench measurement. However, this observation does not negate the overall conclusion that trehalose is an effective inhibitor of N₂ase turnover.

The next question was whether trehalose additionally provides stability to any reaction intermediate trapped by its cessation of turnover. We approached this question by using two techniques with which we are very familiar, in combination with two well-known forms of Mo-N₂ase, namely its CO-inhibited forms called 'hi-CO' and 'lo-CO'. [1-5,27] First, we were encouraged by the results of monitoring a sample of 'hi-CO' using EPR spectroscopy, when found that, after 1.5 M trehalose quenching, the spectrum was stable for up to 1 hr. Then, we applied FTIR-monitored cryophotolysis to 'hi-CO' to determine its response to white-light illumination. To our satisfaction, we found negative (downward) photolysis features at 1973 cm⁻¹ and 1681 cm⁻¹, both of which indicate loss or rearrangement of CO from two different binding sites on the sample plus a positive (upward) feature at 1715 cm⁻¹ (see Figure 2, top right) which is associated with the photolysis product, 'lo-CO'. These results are essentially the same as those previously reported for ethylene glycol-quenched CO-inhibited N₂ase. [5,27]

These experiments clearly show that trehalose is an effective quench for Mo-N₂ase studies at 1.5 M, which compares favorably to the requirement for 10 M ethylene glycol to achieve similar inhibition. They also confirm what we had expected from both earlier

studies on CO-myoglobin[17,18,20,21] and from its recognized role as a stress protectant in many biological systems.[19] Furthermore, there is microbiological evidence for trehalose quenching N₂ase activity. For example, it was found that increased root-nodule trehalose production coincided with decreased catalyzed acetylene-reduction activity in symbiotic systems involving *Rhizobium* during drought-like conditions.[28]

The mechanism by which trehalose inhibits nitrogenase activity and stabilizes reaction intermediates remains unknown. However, previous studies, using all-atom molecular dynamics for trehalose, indicate that 1.5-2.2M solutions cause formation of large, recursively associating aggregates.[29] The critical concentration for formation of these aggregates coincides with the concentration that results in N₂ase inhibition and, therefore, suggests that aggregation may play a role in trehalose's quenching ability.

From a practical standpoint, the goal of this work was to develop a system to produce high concentrations of materials that are currently not easily achieved by simple evaporation or other concentration methods. Toward this goal, we believe we have the beginnings of such a system using trehalose. The properties of trehalose open possibilities for such higher concentrations, which would make spectroscopic work on weaker vibrational, electronic, or nuclear features a real possibility. Likewise, analogous to work on myoglobin, there is the potential to stabilize and trap new forms of intermediates for spectroscopic studies that could help unravel the mysteries of substrate dynamics and/or kinetics that continue to appear in N₂ase-based research.

5. Abbreviations

CW – continuous wave

EPR – electron paramagnetic resonance spectroscopy

FTIR – Fourier-transform infrared spectroscopy

FeMo-cofactor – the iron-molybdenum cofactor of the MoFe protein

Fe protein – the iron protein of Mo-nitrogenase

MoFe protein – the molybdenum-iron protein of Mo-nitrogenase

Mo-N₂ase – molybdenum-based nitrogenase

N₂ase – nitrogenase

Acknowledgements

This work was funded by National Institutes of Health Grant GM-65440, and the National Science Foundation grant CHE 1308384 (S.P.C.)

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We have observed using FTIR, EPR, and activity assays that trehalose quenches the nitrogenase reaction and stabilizes the formation of bound CO species. Trehalose has potential applications in increasing the concentrations of nitrogenase protein while maintaining spectroscopically interesting species.