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Research paper

Is trehalose an effective quenching agent of *Azotobacter vinelandii* Mo-nitrogenase turnover?

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ABSTRACT

H_2-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 H_2-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 N_2ase, which did not change during 1 h 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 (Yan et al., 2011). These results clearly show that 1.5 M trehalose is an effective quench and stabilization agent for Mo-N_2ase studies. Possible applications are discussed.

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-N_2ase) 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 N_2ase [7], it came as a surprise when it was recognized quite recently as a substrate [8]. Its reduction products include CH_4, C_2H_4, C_3H_6, and even C_4 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

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-N_2ase, molybdenum-based nitrogenase; N_2ase, nitrogenase.

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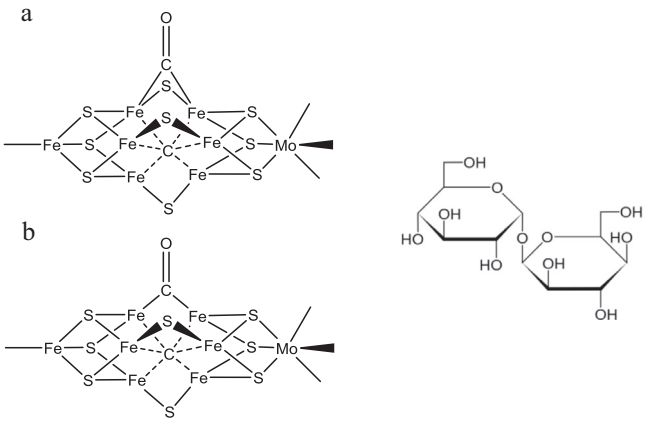

Scheme 1. Left: alternative structures of mono-CO-bound FeMo-cofactor (called 'lo-CO') with: (a) all three 'waist' bridging sulfides (S) in place [3,23]; and (b) with one 'waist' bridging S replaced by CO [16]. Right: structure of trehalose.

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_2ase turnover and capturing intermediates that we were searching for. Here, we show that is indeed the case.

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_2ase 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 1 ppm O_2. The component proteins were concentrated individually using an Amicon microfiltration pressure concentrator before buffer exchange to 25 mM HEPES (pH 7.4), 200 mM NaCl, 10 mM $MgCl_2$ and 2 mM $Na_2S_2O_4$. The specific activity of the Fe and MoFe protein for hydrogen evolution was 2300 and 2100 nmoles H_2 (min^{-1}mg $protein^{-1}$) at 30 °C, respectively. Protein concentrations were determined using the Lowry method [26].

2.2. Trehalose suppression of catalyzed H_2 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 $MgCl_2$, 2.5 µmol of ATP, and 0.125 mg of creatine phosphokinase. Trehalose solutions of varying concentrations were prepared in 25 mM 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 H_2 evolved was measured and the assays then remained at 30 °C for up to 90 min more before total evolved H_2 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% H_2 in N_2 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 M 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 12 K. 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 µm. 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

Fig. 1 shows the effect of adding either EDTA or trehalose as quenching agent on N_2ase 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_2ase 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

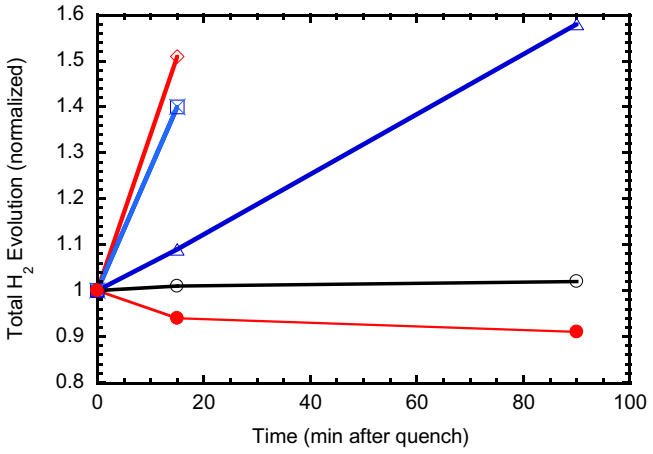

Fig. 1. Plot of total H_2 evolved (normalized to amount present at time zero) versus time (min after adding quenching agent); ● = 0.15 M EDTA, × = 0.2 M trehalose, □ = 0.4 M trehalose, ◇ = 0.5 M trehalose, △ = 1.0 M trehalose, ○ = 1.5 M trehalose.

total H_2 evolved are normalized in Fig. 1 to accommodate data from more than a single experiment.

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

Fig. 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 h (77%). After 1.5 h, 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-N_2ase 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 (Fig. 2, top). The negative (downward) photolysis feature at 1973 cm^{-1} (Fig. 2, top left) is due to loss of a terminally bound CO ligand, whereas that at 1681 cm^{-1} (Fig. 2, top right) reflects the re-arrangement of the second CO in the 'hi-CO' sample. The positive (upward) feature at 1715 cm^{-1} (Fig. 2, top right) is associated with the photolysis product and correlates with the formation of the 'lo-CO' species [27].

4. Discussion

The high electron-flux hydrogen-evolution assays clearly showed that 1.5 M trehalose (final concentration) totally inhibits N_2ase activity. No H_2 production beyond that quench could be

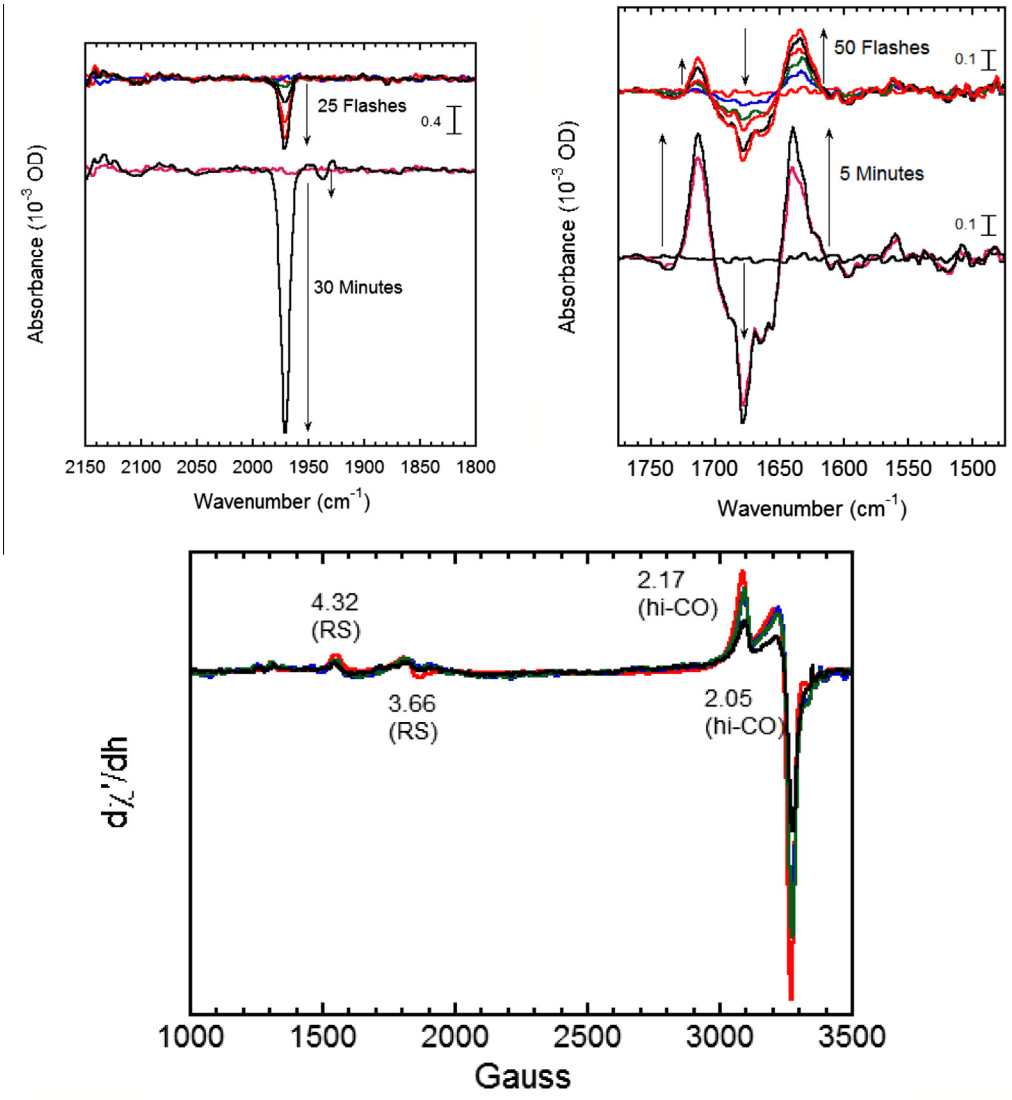

Fig. 2. FTIR-monitored cryophotolysis and EPR Spectroscopy of the 'hi-CO' form of trehalose-quenched N_2ase. Top: FTIR difference spectra for the high-energy (left) and low-energy (right) regions (see Section 3.3 for band assignments). Bottom: Stacked EPR spectra for 'hi-CO' N_2ase: prior to trehalose quench (red); immediately after trehalose quench (blue); 1 h 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 H_2, 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 N_2ase 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-N_2ase, 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 h. 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 Fig. 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 N_2ase [5,27].

These experiments clearly show that trehalose is an effective quench for Mo-N_2ase 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 N_2ase 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 1.5–2.2 M solutions cause formation of large, recursively associating aggregates [29]. The critical concentration for formation of these aggregates coincides with the concentration that results in N_2ase 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 N_2ase-based research.

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