

Chapter 2

Purification of O₂-Sensitive Metalloproteins

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Abstract

The most dependable factor to perform successful biochemical experiments in an O₂-free environment is the experience required to set up an efficient laboratory, to properly manipulate samples, to anticipate potential O₂-related problems, and to maintain the complex laboratory setup operative. There is a long list of O₂-related issues that may ruin your experiments. We provide here a guide to minimize these risks.

Key words Schlenk line, Glove box, Anaerobic protein purification, Metalloprotein, Metal clusters, O₂-free device, Sodium dithionite (DTH), Liquid nitrogen (LN₂), Liquid oxygen (LO₂), Radical-SAM enzyme

1 Introduction

It is estimated that 30 % of all proteins in a given organism contain associated metal(s) [1]. Metalloproteins are central to prokaryotic metabolic diversity, being essential to the global biogeochemical cycles of N, C, O, S, and H [2]. Arguably, the most important metalloenzymes in this context are the following: the [Ni-Fe] and [Fe-Fe] hydrogenases, involved in hydrogen production and consumption [3]; the molybdenum (Mo), vanadium (V), and iron-only (Fe) nitrogenases that fix atmospheric N₂ into NH₃ [4]; the [Ni-Fe] carbon monoxide dehydrogenase (CODH) carrying out a biological water-shift reaction [5, 6]; and more generally, [Fe-S] cluster-containing proteins involved in other fundamental biochemical processes such as respiratory and photosynthetic electron transport chains [7, 8].

Metal centers of proteins may be involved in catalysis, electron transfer, metal trafficking, sensing environmental conditions, or may simply have structural roles [9]. The catalytic activities of many metal-containing proteins, including all examples listed above, are sensitive to O₂ [10]. In some cases, O₂ reversibly inactivates the enzyme, which might be able to recover activity after its

removal (e.g., [Ni–Fe] hydrogenase [11]). In some other cases, O₂ irreversibly destroys the enzyme metal center(s), as it occurs in the [Fe–Fe] hydrogenase [12] and nitrogenase [13]. Other well-studied O₂ sensitive metal clusters are those of the iron–sulfur proteins aconitase and Fumarate Nitrate Reductase regulatory protein (FNR, *see* Chapter 4). Active aconitase carries a [4Fe–4S] cluster that is partially degraded into an inactive [3Fe–4S] form when exposed to O₂. Interconversion of both types of clusters occurs and is dependent on redox potential and Fe availability [14]. FNR [4Fe–4S] to [2Fe–2S] cluster conversion, caused by oxidation, inactivates FNR as transcriptional regulator and is used to regulate gene expression in response to cellular O₂ levels [15] (*see* Chapter 4). Finally, another broad example of metalloprotein sensitivity to O₂ can be found in the Radical-SAM protein superfamily of which more than 48,000 member proteins have been identified in databases [16–18]. Radical-SAM enzymes are involved in many different thermodynamically unfavorable reactions and require redox-active [4Fe–4S]-SAM conserved cluster for activity.

Specific laboratory setup and special techniques are required to work with O₂ sensitive proteins. We detail here material and methods used in anaerobic protein purification experiments, taking into account that not all laboratories have access to O₂-free glove boxes. A description of Schlenk line assembly and operation, O₂-free buffer preparation, sample manipulation, anaerobic purification tricks, protein concentration and storage methods will guide the reader through the virtual setup of an anaerobic biochemistry laboratory.

2 Materials

2.1 Carrier Gases

Ultrahigh purity gases with O₂ content lower than 3 ppm are required. High-pressure cylinders (usually 50 l at 200 atm) are probably the most popular solution for gas delivery. The cylinders have to be attached to a proper pressure reductor, and they are mobile and cheap, but laborious to move, may occupy essential laboratory space, and present safety concerns (*see* **Note 1**). Therefore, it is recommended that gas cylinders should be stored in a dedicated space on the outside of the research building (Fig. 1g). In this external space, the cylinders should be secured against a wall and connected to pressure reducers from where stainless steel or copper tubing should conduct gases to the required labs, but at a much lower pressure (12 atm or lower). In these laboratories, fine-pressure reducers (0–10 atm) should be located in the pipeline-end, in which attachment of an O₂-trapping catalyst is recommended (*see* **Note 2**).

The most popular inert gases for anaerobic biochemistry are nitrogen (N₂) and argon (Ar). N₂ is normally cheaper and a more

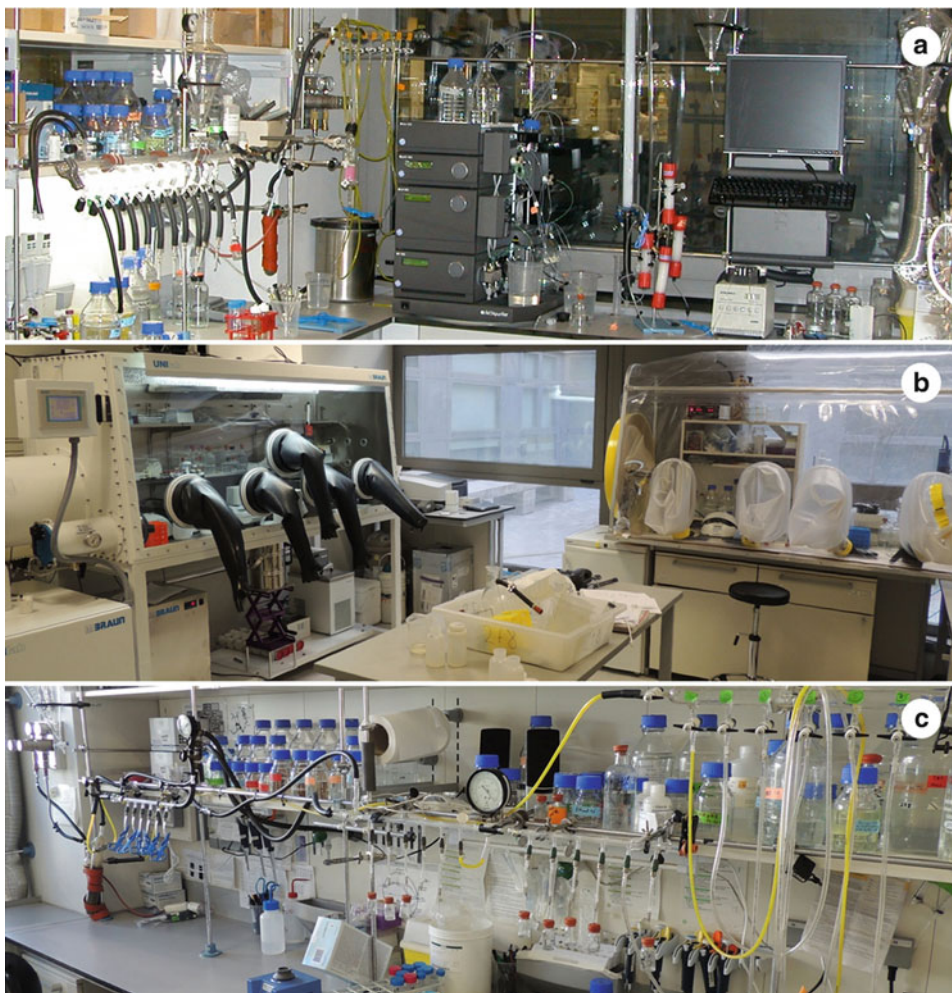


Fig. 1 (a) FPLC system connected to a Schlenk line and to a gas distributing manifold. System is connected to building N_2 and Ar lines through homemade O_2 traps (in red at the center of the picture). The vacuum line contains a water trap between the Schlenk line and the vacuum pump (hidden in the picture). Individual valves allow flowing gas into many flasks and vials independently. A number of chromatography columns are displayed in the center of the picture. (b) MBraun glove box to the left and Coy Labs vinyl glove box to the right. (c) Schlenk line specifically devoted to acetylene reduction assays. It is composed of two attached glass manifolds controlled by a multi-position valve from which it is possible to apply gas (Ar or N_2) or vacuum. Connected “dummy” vials are shown. (d) Co^{2+} affinity purification of His-NifDK performed outside a glove box. (e) NifB-co purification carried out outside a glove box in a jacketed column with double-sealed rubber caps. Note N_2 flow via needle lines on top of the column. (f) N_2 and H_2 gas end lines inside a laboratory. For safety reasons, the H_2 line is properly labeled and built in dual-tubing setup open at the building outside. Two gas mixers are installed: N_2/H_2 (top) and air/ H_2 (bottom). (g) Research facilities should have their highly pressurized gas tanks located outside the building in a dedicated space, with restricted access. Picture includes an N_2 generation system with dual deposit capacity of 3,000 l total (<3 ppm O_2) and ten bar maximum pressure. (h) Schlenk line fed with highly purified N_2 (<2 ppm O_2) from a gas line controlled with valves and a pressure reductor. Homemade trap with a heater to scavenge O_2 traces in N_2 . Vacuum line connected to an Edwards RV3 rotatory pump via a dry-ice water trap. Along the manifold, delivery valves are present, each connected to air-free hermetic vials of known volume through needles puncturing rubber-sealed caps. Additionally, three lines on the right side are dedicated to preparing anaerobic buffers in Büchner flasks. (i) MBraun glove box with FPLC apparatus and anaerobic columns inside

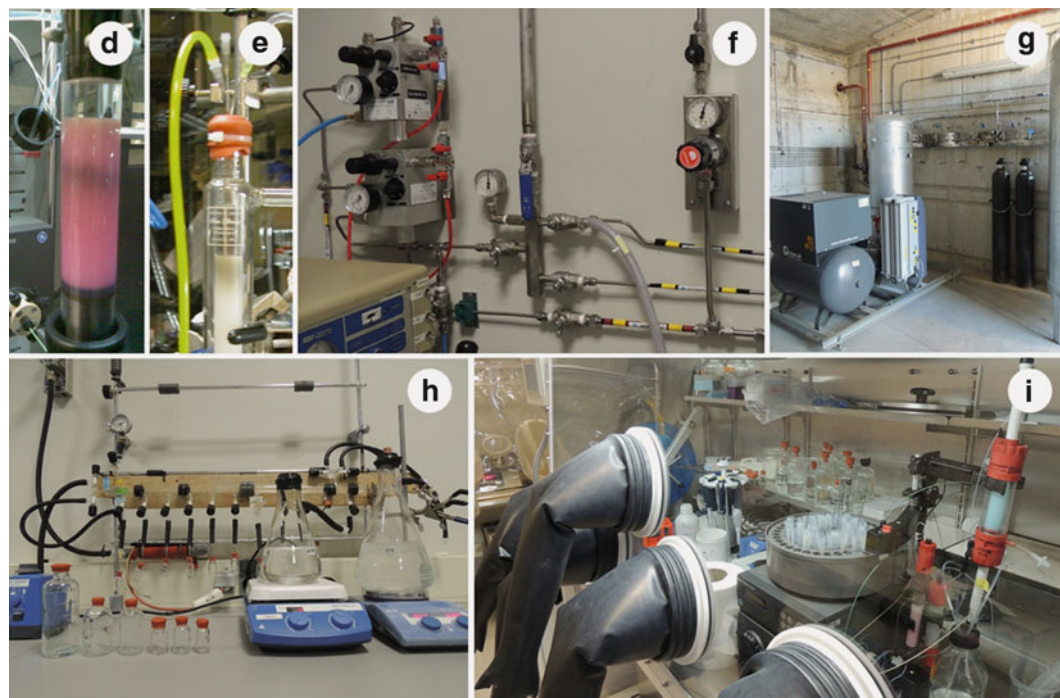


Fig. 1 (continued)

popular alternative. However, the use of Ar gas is required in specific nitrogenase-related experiments when the presence of N_2 might interfere with nitrogenase activity determinations.

In-house N_2 generators are an increasingly popular alternative to gas cylinders because ease of use, N_2 quality output (<5 ppm), and lower running costs. These systems are based on adsorption technology that relies in an adsorbent solid matrix with pores in which H_2O , O_2 , and CO_2 molecules are immobilized whereas N_2 molecules pass through. Because adsorption matrixes eventually saturate, N_2 generators present duplicated fixing vessels and a sensor mechanism that switches regularly between the two, allowing them to run continuously. Thus, while a vessel is operative producing high-quality N_2 , the other vessel is under regeneration by using a controlled-heating process until, eventually, the system switches back again. Importantly, N_2 generators storage gases at lower pressure than cylinders do (normally below 10 atm); therefore, bigger gas reservoirs are required (Fig. 1g). Downie presented a useful guide about industrial gas production and facts [19].

2.2 Gas Mixers

Some experiments require specific gas mixtures of two or more gases. The easiest approach is to order premade mixtures to gas suppliers that check the composition formula accurately. However, this strategy is usually expensive and not recommended when high

volumes are needed. In this case, research facilities can rely in the use of gas mixers and their own gas sensor systems, or other devices, such as gas chromatographers, to calibrate their own gas mixes with high accuracy at a lower cost.

A variety of systems are available in the market: (1) multi-gas flow meters are handy, versatile and cheap to operate; they work at low pressures and flows, but output mix requires external calibration and testing that could be time consuming. However, once the correct parameters are found, these devices provide mixes reliable for routine laboratory work. (2) Mechanical gas mixers allow for higher pressures and flows; they are accurate (± 1 % error in the mix) but are gas specific and more expensive (Fig. 1f). (3) A later generation of gas mixers combine mechanics, sensors, and electronics to deliver accurate gas mixes and flows that are analyzed to control gas quality. However, this sort of high-end devices is costly.

2.3 *O₂ Traps and Sensor Alarms*

The need for the purest N₂ for experimentation may require control sensor systems and additional O₂-free measures beyond the quality of supplied gas [20]. It is recommended that each gas workstation should have an O₂ trapping system attached after the pressure regulator. Although there are commercial solutions from a variety of companies, including Alltech Inc, PerkinElmer, Agilent, Sigma-Aldrich, it is also feasible to build an O₂ trap yourself (Fig. 1a). Most O₂ traps rely on molecular copper-oxide based adsorbents that act as O₂ scavengers. These systems can reduce O₂ presence to ppb values. BASF produces a variety of adsorbents dedicated to eliminate O₂ traces in pure gases. Importantly, it is possible to regenerate these catalysts periodically by applying a stream of H₂ and/or controlled heat.

Monitoring O₂ traces in gas lines is strongly recommended as a control measure. Currently, there are three main O₂ sensor technologies present in the market: (1) zirconia based sensors, (2) electrochemical sensors, and (3) paramagnetic sensors. The zirconium and electrochemical sensors are the most prevalent and both can detect O₂ in ppb values. Zirconium sensors are more robust and expensive, whereas the electrochemical sensors are relatively inexpensive but have a short-life, usually lasting no longer than a year.

Once the basic setup to deliver pure inert gas into a laboratory has been completed, attention must focus on how gas should be delivered to perform experiments in O₂-free atmosphere.

2.4 *Schlenk Line and Rubber Septa Containers*

A Schlenk line (or vacuum gas manifold) is an air-free system that connects a line of inert gas and a vacuum line by a controlling valve. In addition, Schlenk lines have multiple valve-controlled ports to connect hermetic containers thus allowing the manipulation, under O₂-free conditions, of several samples simultaneously (Fig. 1c, h).

In a nitrogenase research laboratory, independent N₂ or Ar lines exiting O₂-traps are connected to Schlenk manifolds.

A rotary vane RV3 Edwards pump connected to another manifold-end provides the necessary vacuum. If the Schlenk line is used for anaerobic buffer production, a water-trap is placed in between the manifold and the pump to prevent water vapor from reaching the pump oil reservoir and corroding the internal mechanism. Simple water-traps are based on an external jacket surrounding an internal deposit filled with ethanol and dry ice. Water vapor present in the flowing gas freezes in the deposit outer-jacket.

Because vacuum gas manifolds are usually made out of glass and because their design is inherently delicate, careful attention must be placed on the pressure and vacuum conditions applied. Installing accurate pressure and vacuum gauge(s) as well as an overpressure valve system is recommended as control measures. Glass manifolds should not be manipulated at pressures higher than 0.5 bars (50 kPa) or at vacuum conditions below -0.9 bar. It is very important that glass connectors and lines be properly sealed using vacuum grease and Teflon tape. Keeping positive pressure in any container or manifold, while not under vacuum, helps avoiding O_2 contamination.

2.5 Anaerobic Jars

Also named as McIntosh and Filde's anaerobic systems, these are hermetic anaerobic containers in which a modified atmosphere can be applied using a Schlenk line. Anaerobic jars are mostly used for culturing microbes under air-free conditions in agar-based media, but may also be used to culture microbes in liquid media, or to set up biochemical reactions under a specific gas composition. However, anaerobic jars are very limiting because no air-free manipulation is possible once the experiment is set. To monitor gas transfer to and from the jar, these systems usually present a vacuum/pressure gauge. It is also possible to eliminate O_2 inside the jar by inserting an anaerobic generation bag, which minimizes the need for a Schlenk line (*see Note 3*). The use of anaerobic indicator strips to confirm O_2 -free atmosphere within the jar is recommended (*see Note 4*).

2.6 Glove Box

Glove boxes allow manipulating objects and processes in a closed chamber with controlled gas composition, typically an O_2 -free N_2 atmosphere. Basic anaerobic glove boxes are designed with a set of systems to ensure O_2 -free operation, including (1) a hermetic chamber, (2) butyl gloves for manipulation, (3) chemical catalyzer device to trap O_2 traces, (4) O_2 sensor, and (5) a transfer box or airlock. Most of these systems work in a 0–5 ppm O_2 range.

A good example of traditional anaerobic system would be the Coy Labs vinyl polymer series (Grass Lake, Michigan, USA) (Fig. 1b). Coy Labs glove boxes are affordable, highly reliable, and easy to service. The use of vinyl allows for a clear visual access while an external aluminum structure holds the bag. A catalyzer device based on a palladium matrix circulates the internal atmosphere gas while trapping contaminating traces of O_2 . This catalyzer device

works optimally at 30 °C and in the presence of 3–6 % H₂ in the gas phase. If O₂ contamination occurs the catalyst rapidly fixes it generating H₂O. The O₂ sensor unit reads O₂ contamination in ppm values (0–2,000) and also determines H₂ presence in % values. Delivery of gas (N₂/H₂ mix) or objects inside the glove box is performed through the airlock using a manual or automatic vacuum–N₂–vacuum–N₂–vacuum–N₂/H₂ mix cycle process. Analog and electronic airlocks exist that have door alarm systems to minimize risk of contaminating the glove box with external O₂.

More demanding experimental needs have further refined the glove box concept. Manufacturers such as Jacomex (Dagneux, France), MBraun (Garching, Germany), and GloveBox Systems (Massachusetts, USA) build metal anaerobic glove boxes units with front (and back) panels made of crystal clear polymer (Fig. 1b). Importantly, these systems may work at positive and negative pressures and have dedicated sealed ports for connecting devices out and inside the glove box. Several glove box systems can be interconnected allowing larger working space. In these glove boxes the O₂-trapping systems are placed on the outside, saving valuable working space, and operate by circulating the atmosphere through the catalyst and back into the box in coordination with O₂ and H₂O sensors. Safety is also improved because H₂ is not used routinely during glove box operation. Electronics play a major role integrating all sensor data, i.e., temperature, O₂ (in ppb), and humidity (*see Note 5*). Most of these glove boxes present large airlocks that can be manipulated in automatic mode, and also present very popular manually managed small airlocks. These systems can incorporate external cooling systems to work at temperatures 10–15 °C below room temperature, a very important asset when conducting lengthy protein purifications (Fig. 1i). In summary, these glove boxes are versatile and can be customized to researcher needs, but at a higher price tag.

3 Methods

3.1 Operation of Schlenk Lines

Transfer of vacuum or O₂-free N₂ or Ar gas from the manifold to the experimental vials is carried out through airtight Schlenk lines (*see Note 6*).

1. Make the Schlenk line anaerobic by performing three cycles of vacuum/gas with dummy vials connected.
2. Seal the sample vials hermetically with rubber stoppers and connect them to Schlenk line ports via tubing and needles (Fig. 1h).
3. Start by applying –0.9 bar of vacuum for 30 s.

4. Switch Schlenk line main valve from vacuum to N₂ flow-in position to fill the vials with N₂
5. Repeat the vacuum/N₂ cycle at least three more times.
6. After the last cycle, the vials must be kept under slight positive pressure to minimize O₂ contamination through the stopper. Remove the vials from the manifold.
7. At the end of the day, make sure that the manifold has at least one port open to atmospheric pressure before switching off vacuum pump.

3.2 Preparation of Anaerobic Buffers

Total procedure takes 1–2 h per liter of buffer.

1. Connect a hermetically sealed Büchner flask containing aerobic buffer solution to a Schlenk line while flowing out N₂. Switch to vacuum mode (9.48 kPa or –0.9 bar) with gentle magnetic stirring. Vacuum mode forces dissolved gases out of the liquid phase.
2. Switch the Schlenk line main valve at least three times from the vacuum position to the N₂ flow-in position to refill the flask with gas to accelerate air removal.
3. Fill the Büchner flask with excess N₂ after the final cycle to keep positive gas pressure on the inside.
4. Büchner flasks can be stored in the laboratory bench or inside a glove box.

3.3 Addition of Sodium Dithionite as Reductant and O₂ Scavenger

Sodium dithionite (Na₂S₂O₄), abbreviated DTH, is an important component of buffers used for anaerobic protein purification. DTH stock solutions must be freshly prepared (*see* **Note 7**).

1. Store solid DTH stocks away from the light inside anaerobic jars with desiccant sieve at room temperature.
2. Weigh DTH and degas it at the Schlenk line within serum vials hermetically closed with rubber stoppers.
3. Add anaerobic 50 mM Tris–HCl buffer, pH 8, to make a 100 mM DTH stock solution.
4. Use this stock solution to make 1–2 mM DTH-containing anaerobic buffers.
5. Check buffer reduction with methyl viologen-soaked paper strips (*see* **Note 8**).

3.4 Anaerobic Manipulation of Samples from Liquid N₂ into Vials

Liquid nitrogen (LN₂) is useful in sample manipulation outside a glove box because its low boiling temperature point (–196 °C) combined with its low O₂ content prevents samples from oxidation (*see* **Note 9**). O₂-sensitive protein samples are stored as droplets in cryogenic dewars under LN₂ to prevent oxidation. To thaw protein samples proceed as follows:

1. Place an empty glass vial into a beaker and fill both with LN₂.
2. Add frozen droplets to the vial using tweezers. Calculate the volume of sample needed and remove from the dewar the exact number of droplets (estimate 30–50 µl per droplet).
3. Approach the Schlenk line. Decant excess LN₂ from the vial while applying a flow of pure N₂ from the manifold and into the vial.
4. Seal the vial with a rubber stopper, connect it to the Schlenk line via a needle and switch immediately to vacuum for 1 min, or until LN₂ completely evaporates. Switch back to the N₂ position.
5. Repeat vacuum/N₂ gas cycle three more times.
6. Remove vial from manifold and place it on ice until protein is thawed.

3.5 How to Set Up an Anaerobic Protein Purification Method Without a Glove Box

It is possible to perform anaerobic protein purifications without a glove box if procedures described in this chapter are carefully applied. By ensuring that running buffer is anaerobic, and that tubing connections, peristaltic pump, chromatography column, UV/Vis detector, and sample collecting vials are airtight, there is a good chance that anaerobic protein purification will succeed.

3.5.1 Preparation of Chromatography Columns

1. Buffers must be O₂ free.
2. If possible, add 1–2 mM DTH to the buffers to scavenge O₂ traces and keep reducing conditions (*see Note 10*).
3. Peristaltic pump tubing and connectors to the sample flask and to the column must be airtight. Wash all tubing with anaerobic buffer before handling the sample. When disconnecting tubes, use clamps to stop buffer flow. Reassemble as fast as possible avoiding bubbles.
4. Apply 10–20 column volumes (CV) of anaerobic buffer to the column to turn stationary phase anaerobic. For instance, an FPLC system attached to a column with 50 ml of Q-sepharose resin may need around 0.6–0.8 l of DTH-containing anaerobic buffer for the methyl viologen strip test to indicate a reduced condition (blue) at the column exit (*see Note 11*).
5. Connect peristaltic pump tubing to flask containing protein sample using clamps to stop flow.
6. Load sample onto the column and run chromatography (Fig. 1d).

3.5.2 Collecting Fractions from a Chromatography Column

Sample collection has to be performed manually. Degassed vials with dual-sealed rubber caps are used (Subheading 3.1). Column outlet must be connected to tubing ending in a needle. To collect samples, simply insert needle into a degassed vial. Two additional needles must be in place in the vial rubber cap before inserting the collection needle: an entry N₂ line and a needle to release overpressure (Fig. 1e).

3.5.3 Concentrating Proteins in an Amicon Cell

It is important to carefully render the membrane and the Amicon cell anaerobic before applying the protein sample.

1. Assemble the Amicon cell and membrane on the bench and connect the former to the tubing that will deliver N₂ to pressurize the device.
2. In addition, apply a constant low flow of N₂, by using tubing and a bended needle, into the upper opening of the Amicon cell for 3–5 min. This gas should be supplied anytime the pressure valve is not in position.
3. Fill one half of the Amicon cell with DTH-reduced anaerobic buffer. Wash the cell walls by gentle twirling. Remove upper gassing needle and close pressure valve.
4. Place the Amicon cell on slow magnetic stirring and let all the buffer solution flow through the ultrafiltration membrane to reduce it. Check buffer reduction status with methyl viologen strips.
5. Once reduced, open the pressure valve and load the protein sample by using an anaerobic syringe and needle. Close the valve and place the cell back on magnetic stirrer until desired volume is left in the cell.
6. Remove sample with anaerobic syringe and needle (protected with soft rubber to avoid puncturing the ultrafiltration membrane), and transfer it to an anaerobic vial.

3.5.4 Storing Proteins Under Liquid N₂

O₂-sensitive proteins are routinely stored under LN₂ as droplets (*see* **Note 12**).

1. Place an empty polypropylene vial, with tiny holes in it, inside a beaker and fill both with LN₂. Use safety glasses.
2. Use anaerobic syringe and needle to slowly drop protein solution into LN₂. Do not place the needle too close to the LN₂ because it will freeze, thereby stopping the flow.

4 Notes

1. Safety and maintenance tips. Gas leaks are not just a menace to experimental work, but also expensive and potentially dangerous due to the nature of some gas mixes used for experimentation. Proper maintenance of gas systems and tubing must be periodically carried out. Maintenance of high-pressure systems attached to gas cylinders should be outsourced to professionals. Special attention should be paid to pressure regulators, valves, anti-return valves, tubing connections, and cylinder manifolds. Importantly, only certified professionals should perform modifications to these installations.

Laboratory personnel must be trained to manage the low-pressure gas lines connected to the different instruments. Importantly, proper tools should be available in any anaerobic biochemistry laboratory. A regular wrench, a grip adjustable wrench, a ratchet set, a drive socket set, screwdrivers, pliers and locking plier sets, a metric tape, and hammer tools should be available. Thread seal tape and pipe thread tube compound are essential to ensure that gas line components are properly sealed.

2. Screw thread, fittings and tubing. The two main screw thread options found in gas lines and laboratory equipment are the ISO Metric screw thread and the British Standard Pipe (BSP) thread, which is based on the pioneer British Standard Whitworth. Additionally, some USA-made pieces of equipment use National Pipe Thread Taper (NPT) standard. It is necessary to emphasize that, although these standard systems have—on occasions—quite similar thread patterns (for example, ISO Metric and NPT use an identical 60° angle), these should not be mixed (for example, the British Standard Whitworth uses a 55° angle).

Problems emerge when a laboratory has pieces of equipment from different geographic origins, because manufactures usually rely in their home country thread standards. As a result, interconnecting equipment and gas lines may become a practical nightmare. Traditionally, gas lines used to be installed in stainless steel or copper tubing, which guarantee robustness at the cost of lacking flexibility, further complicating the compatibility issue. The proliferation of new fast-lock and semi-fast-lock systems, complemented with the use of better plastic tubing materials (polyamide, polyurethane, and PTFE), has dramatically simplified this problem.

3. Fluka Analytical provides one fast and convenient solution to limit O_2 concentration to $<1\%$ in anaerobic jars (up to a volume of 3.5 l). This technology is based on anaerobic atmosphere generation bags (Oxoid AnaeroGen developed by Mitsubishi Gas Company Inc.). Interestingly, this solution does not rely in the use of H_2 and a catalyst to produce water but in the use of ascorbic acid as an active component yielding an atmosphere with 9–13 % CO_2 content [21].
4. Fluka Analytical provides a simple solution to test anaerobiosis in enclosed compartments based on test strips saturated with resazurin solution. When the strips change color from pink to white anaerobic conditions have been achieved.
5. Most modern glove boxes are designed for processes that require a $<0.1\%$ relative humidity (i.e., atomic applications, chemical synthesis, batteries, laser welding, etc.). Therefore, these systems are equipped with reliable zirconium sensors. However, biochemical anaerobic work normally requires some

humidity inside the glove box to avoid rapid desiccation of samples. Importantly, zirconium sensors are not recommended in those humid conditions and need to be replaced by electrochemical sensors.

6. An easy method to spot gas leaks is applying soapy water to the suspected leak area. If positive, bubbles immediately form. There are spray cans specifically manufactured for this task. However, these solutions only work well for significant leaks and are not effective to spot small leaks that can be as damaging to the experimental work. It is recommended to have one gas leak detector system in the laboratory to test gas lines and glove boxes when a leak is suspected. Ultrasonic detectors, infrared point sensors, and electrochemical gas detectors are commercially available. Ultrasonic detectors are probably the most prevalent because they are not gas specific and pinpoint the leak relying in the fact that most gas leaks are audible in the 25 kHz to 10 MHz range.
7. Although added to the buffers to prevent protein oxidative damage, DTH is, itself, susceptible to oxidation breaking down into sulfur compounds that lower buffer pH and might damage protein [Fe-S] clusters. Therefore, DTH must be added to the buffers only after they have been made anaerobic and just before their use. It is strongly recommended not to use DTH-containing buffers older than 1 day. However, there are some drawbacks when using DTH. For example, it over-reduces Ni and is not recommended in Ni^{2+} affinity chromatography protocols.
8. Methyl viologen detecting paper strips are used to test buffer anaerobiosis. Whatman filter paper is soaked in 250 mM Tris-HCl with 1 % (w/v) methyl viologen and then dried in the oven at 80 °C. The dried paper is cut into small strips of $8 \times 5 \text{ mm}^2$ surface. When exposed to a DTH-reduced buffer solution, white paper strips turn blue.
9. LN_2 is generated after purifying N_2 from air in a size exclusion process followed by compression, and is stored in cryogenic pressurized reservoirs. The resulting LN_2 presents O_2 concentrations lower than 0.05 %. Commercial LN_2 with O_2 contents of 5 ppm or lower can also be obtained. A potential oxidation risk for metalloproteins immersed in LN_2 dewars occurs when O_2 from air condensates overtime to form liquid O_2 (LO_2 , boiling point $-183 \text{ }^\circ\text{C}$). LN_2 contaminated with LO_2 turns pale blue. This situation should be avoided at all costs.
10. The anaerobic buffer container must be pressurized to avoid O_2 contamination. For this purpose, we use gas lines with needles to puncture Büchner flask caps. Slow N_2 or Ar flow is needed, but also a release needle valve of smaller diameter has to be in place. This system must be replicated in the collecting sealed tubes.

11. Because of the large volume required to reduced a resin, it may be convenient to turn the system anaerobic overnight, so that protein purification starts early next morning. Importantly, this procedure has to be repeated anytime a new purification procedure is planned.
12. If a glove box is available, it is possible to use it to store purified protein preparations in aliquots into hermetically sealed cryotubes. In glove boxes with installed cold traps, cryotubes can be frozen inside the glove box. Otherwise, freeze closed cryotubes in LN₂ just after removing them from the airlock.

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References

1. Waldron KJ, Robinson NJ (2009) How do bacterial cells ensure that metalloproteins get the correct metal? *Nat Rev Microbiol* 7:25–35
2. Knoll AH, Canfield DE, Konhauser KO (2012) Fundamentals of geobiology. Wiley-Blackwell, New York
3. Fontecilla-Camps JC, Amara P, Cavazza C, Nicolet Y, Volbeda A (2009) Structure-function relationships of anaerobic gas-processing metalloenzymes. *Nature* 460:814–822
4. Hoffman BM, Lukoyanov D, Dean DR, Seefeldt LC (2013) Nitrogenase: a draft mechanism. *Acc Chem Res* 46:587–595
5. Drennan CL, Heo J, Sintchak MD, Schreiter E, Ludden PW (2001) Life on carbon monoxide: X-ray structure of *Rhodospirillum rubrum* Ni-Fe-S carbon monoxide dehydrogenase. *Proc Natl Acad Sci U S A* 98:11973–11978
6. Lindahl PA (2012) Metal-metal bonds in biology. *J Inorg Biochem* 106:172–178
7. Fontecave M (2006) Iron-sulfur clusters: ever-expanding roles. *Nat Chem Biol* 2:171–174
8. Johnson DC, Dean DR, Smith AD, Johnson MK (2005) Structure, function, and formation of biological iron-sulfur clusters. *Annu Rev Biochem* 74:247–281
9. Dos Santos PC, Dean DR (2008) A newly discovered role for iron-sulfur clusters. *Proc Natl Acad Sci U S A* 105:11589–11590
10. Imlay JA (2006) Iron-sulphur clusters and the problem with oxygen. *Mol Microbiol* 59:1073–1082
11. De Lacey AL, Fernandez VM, Rousset M, Cammack R (2007) Activation and inactivation of hydrogenase function and the catalytic cycle: spectroelectrochemical studies. *Chem Rev* 107:4304–4330
12. Stripp ST, Goldet G, Brandmayr C, Sanganas O, Vincent KA, Haumann M et al (2009) How oxygen attacks [FeFe] hydrogenases from photosynthetic organisms. *Proc Natl Acad Sci U S A* 106:17331–17336
13. Wong PP, Burris RH (1972) Nature of oxygen inhibition of nitrogenase from *Azotobacter vinelandii*. *Proc Natl Acad Sci U S A* 69:672–675
14. Kennedy MC, Emptage MH, Dreyer JL, Beinert H (1983) The role of iron in the activation-inactivation of aconitase. *J Biol Chem* 258:11098–11105
15. Sutton VR, Mettert EL, Beinert H, Kiley PJ (2004) Kinetic analysis of the oxidative conversion of the [4Fe-4S]₂⁺ cluster of FNR to a [2Fe-2S]₂⁺ cluster. *J Bacteriol* 186:8018–8025
16. Sofia HJ, Chen G, Hetzler BG, Reyes-Spindola JF, Miller NE (2001) Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res* 29:1097–1106

17. Marsh EN, Patterson DP, Li L (2010) Adenosyl radical: reagent and catalyst in enzyme reactions. *Chembiochem* 11:604–621
18. University of California-San Francisco Medical Center (2013) Structure-Function Linkage Database <http://sfld.rbvi.ucsf.edu/django/superfamily/29/sequences/all/>
19. Downie NA (2002) Industrial gases. Kluwer Academic, Boston, MA
20. McIlwrick CR, Phillips CS (1973) The removal of oxygen from gas streams: applications in catalysis and gas chromatography. *J Phys E Sci Instrum* 6:1208–1210
21. Beerens H (1998) Bifidobacteria as indicators of faecal contamination in meat and meat products: detection, determination of origin and comparison with *Escherichia coli*. *Int J Food Microbiol* 40:203–207

Metalloproteins

Methods and Protocols

Fontecilla-Camps, J.C.; Nicolet, Y. (Eds.)

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