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Applicability of fluorescence-based sensors to the determination of kinetic parameters for O₂ in oxygenases

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Abstract

Optical methods for O₂ determination based on dynamic fluorescence quenching have been applied to measure oxygen uptake rates in cell culture, and to determine intracellular oxygen levels. Here we demonstrate the applicability of fluorescence-based probes in determining kinetic parameters for O₂ using as an example catalysis by a cofactor-independent oxygenase (DpgC). Fluorescence-based sensors provide a direct assessment of enzyme-catalyzed O₂ consumption using commercially available, low-cost instrumentation that is easily customizable and thus constitute a convenient alternative to the widely-used Clark-type electrode, especially in cases where chemical interference is expected to be problematic.

Keywords

oxygenases; enzyme assays; fluorescence quenching; oxygen; enzyme kinetics

Optical methods for O₂ determination are mainly based on dynamic fluorescence quenching, first described by Kautsky in 1939. [1] Recent advances in light-emitting diodes enabled the development of portable, low-cost, commercially available instrumentation. [2–4] This constitutes an alternative to the widely used Clark-type electrode. Although the latter is relatively inexpensive, easily calibrated, and has a low response time, it has the disadvantages of consuming O₂ during the measurement, being influenced by stirring speed, and being easily poisoned by H₂S, CO₂, functional groups present on proteins, and various organic compounds. [2,5]

One of the main advantages of modern fluorescence-based O₂ probes over the Clark-type electrode is their inherent insensibility to some of the previously mentioned interferents, and the possibility of further limiting chemical interference through the use of protective inert matrices. [5,6] Optical O₂ sensors typically consist of fluorophores (organic dyes or, more commonly, ruthenium and platinum metal complexes) immobilized in solvent impermeable-

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gas permeable polymeric films or organically modified silicate sol-gel matrices. [2,5] These sensors feature response times, limits of detection and sensitivities similar to Clark-type electrodes, and have a predictable temperature dependence. [7] Fluorescence-based probes have been applied to measure oxygen uptake rates in cell culture [6], and to determine intracellular oxygen levels [8–10], but to the best of our knowledge, they have not been applied in kinetic studies of enzyme-catalyzed O_2 consumption.

O_2 features prominently in many crucial enzyme-catalyzed processes including aerobic respiration, degradation of toxic compounds and the biosynthesis of many primary and secondary metabolites. [11] Many of these involve oxygenase enzymes, which are capable of transferring at least one atom from O_2 to an organic substrate. Most oxygenases rely on metals or organic cofactors to bind and activate O_2 , but cofactor-independent oxygenases require neither. [12]. Among these is DpgC, an enzyme that catalyzes a key step in the biosynthetic pathway of last-resort antibiotics vancomycin and teicoplanin. [13,14] The fundamental question of how O_2 reacts enzymatically in the absence of cofactors makes DpgC an interesting target for mechanistic studies. The ability to measure enzyme kinetic parameters for O_2 provides valuable information about O_2 binding and activation. [15,16] In the field, the Clark electrode is the common analytical tool used to monitor O_2 levels, [14,15] although other methods that rely on mass spectrometry are available. [17] We previously determined the kinetic parameters for O_2 in wild-type DpgC and a number of mutants using a traditional Clark-type O_2 electrode and a gas proportioner to generate buffers with different O_2 concentrations. [14] Here we demonstrate the facile applicability of fluorescence-based probes to determine kinetic parameters for O_2 in reasonable agreement with those previously determined.

Figure 1 depicts the setup used in all the kinetic assays reported in this work. The reaction vessel used is a 1 cm path length quartz cuvette containing a 8 mm oxygen-sensing patch (RE-FOX-8, Ocean Optics, FL, USA) placed in a temperature-controlled cuvette holder (qpod 2e, Quantum Northwest, WA, USA). The modified cuvette cover includes three injection ports, which can be used to inject reactants and to bubble different gases. At the cuvette holder's attachment site, we connected a bifurcated optical probe (RE-BIFBORO-2, Ocean Optics, FL, USA) to a blue LED and a phase fluorometer (NEOFOX, Ocean Optics, FL, USA). A magnetic stirring bar provided continuous 1000 rpm stirring.

Using the NeoFox Viewer Software (Ocean Optics, FL, USA) we recorded the solution oxygen level as a percentage every 0.1 s, with 100% corresponding to O_2 saturated water (~ 1.2 mM O_2). [18] This software includes a two point Stern-Volmer algorithm to calibrate the fluorometer, providing a quantitative relationship between the fluorescence lifetime and the partial pressure of O_2 in the solution. One calibration point corresponds to 100% O_2 , obtained by bubbling O_2 directly into water inside the cuvette, and the other one was adjusted so that air-equilibrated water corresponds to 21 % O_2 . Since we performed all experiments at a constant temperature of 25 °C, temperature compensation was not required in the calibration. We confirmed the linearity of the response and the validity of the calibration in the range of interest by recording the O_2 level detected for different dilutions of O_2 -saturated buffer with air-equilibrated buffer (Figure S1).

To assess the applicability of fluorescence-based sensors in determining the kinetic parameters for O₂ in cofactor-independent oxygenases, we measured DpgC catalyzed O₂ consumption using the setup shown in Figure 1. At time 0 s, we initiated the reaction by addition of enzyme (1.67 μM) to reaction buffers containing 83 μM DPA-CoA (3,5-dihydroxyphenylacetyl-CoA, synthesized according to published procedures [19]) and different concentrations of O₂ in the 21-100% range.

The main challenge that must be addressed to apply the setup in Figure 1 to determine the kinetic parameters for O₂ is correctly accounting for non-enzymatic changes in solution O₂ levels. The solution generated by injecting O₂-saturated buffer into the reaction vessel containing air-equilibrated buffer and DPA-CoA is not in equilibrium with the air in the cuvette headspace, and as a consequence the oxygen levels in the reaction buffer decrease with time. In addition, we must take into account that the enzyme solution is air-equilibrated and will affect the oxygen levels in the cuvette when it is injected to start the reaction. Injecting very small volumes could minimize this issue, but smaller injections would imply a loss of precision in the injected volume. In order to account for the non-catalytic decrease in O₂ levels, we performed blank trials in which instead of injecting enzyme we injected the same volume of air-equilibrated buffer (Figure 2A, top). The resulting data can be used to correct the assay data and obtain a curve showing the progress of the enzyme-catalyzed reaction as a function of time (Figure 2A, bottom).

The linear range of the progress curves at each O₂ concentration can be used to determine the initial velocities of the enzyme-catalyzed reaction. Plotting these initial velocities (calculated based on 50 data points) against the corresponding initial O₂ concentrations results in linear graphs for WT (wild-type) DpgC and two mutants (Figure 2, B). The K_M value observed for DpgC is larger than the O₂ concentration in a saturated solution, making it impossible to obtain data points above K_M. Low O₂ binding affinities are common among O₂-using enzymes as a strategy to control O₂ reactivity. [16,20] As a consequence, we can only determine the kinetic parameters with low precision and instead of a full Michaelis-Menten equation fit, it is more convenient to work under the assumption that [O₂] << K_M and fit the data to a linear equation with slope V_{max}/K_M. For WT DpgC, k_{cat}/K_M = 2.2 ± 0.1 mM⁻¹ s⁻¹. This value is in reasonable agreement with the one determined using a Clark-type electrode, 1.2 mM⁻¹ s⁻¹, [14] especially considering the low precision in the determination of the kinetic parameters that results from K_M being larger than the maximum O₂ concentration in solution (see SI). For the L316A mutant, the k_{cat}/K_M value of 2.1 ± 0.2 mM⁻¹ s⁻¹ is statistically equal to the one for WT DpgC, and is consistent with a mutation where O₂ access is not affected. In contrast, for F432W k_{cat}/K_M is significantly lower (1.35 ± 0.05 mM⁻¹ s⁻¹) because this mutation hinders O₂ access, increasing K_M. The setup presented here effectively distinguished between the two cases and is applicable to assess the effect of mutations on O₂ access in oxygenase enzymes.

In summary, we established the applicability of fluorescence-based sensors to the measurement of kinetic parameters for O₂ in a cofactor-independent oxygenase and its mutants. To the best of our knowledge, these sensors had not been previously used to study O₂-dependent enzyme kinetics. Using this method to measure DpgC-catalyzed O₂ consumption, we obtained k_{cat}/K_M in reasonable agreement with those previously reported

using a Clark-type electrode. [14] The use of optical methods is especially convenient in cases where chemical interference is expected for Clark-type electrodes, which can be poisoned by proteins and various organic compounds. [2,5] These issues may be partially responsible for the differences in the values of k_{cat}/K_M reported with the two methods.

Our results show that fluorescence-based sensors constitute a portable, low-cost, commercially available alternative to Clark-type electrodes for the measurement of enzyme-catalyzed O_2 consumption. These sensors provide a direct, continuous, sensitive and real-time assay to determine the kinetic parameters for O_2 in oxygenase activity. The assay described in this paper could be easily modified and applied to other O_2 consuming or evolving enzymes and is capable of distinguishing between mutants with different O_2 accessibilities. As fluorescence-based sensors become more widely available, it is likely that their applications in enzyme kinetics will grow, especially for enzymes where reaction products like CO_2 cause errors in the estimation of O_2 levels when a Clark-type electrode is used. [17]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental setup used for kinetic measurements. (1) Quartz cuvette, (2) Oxygen sensing patch, (3) magnetic stirring bar, (4) cuvette cover fitted with an O-ring (not shown), (5) sample/gas injection ports, (6) Screw-on metal piece to create airtight seal, (7) temperature-controlled cuvette holder, (8) attachment site for the bifurcated optical probe. The cuvette is placed so that the oxygen sensing patch is facing the optical probe.

Figure 2.

(A) Top: O_2 concentration as a function of time for the reaction catalyzed by DpgC (black), and corresponding blank trial (gray). At time 0 s, we initiated the reaction by addition of WT DpgC to a final concentration of $1.67 \mu M$ in a total volume of 1.2 mL, or the same volume of air-equilibrated buffer in the case of the blank trial. Bottom: Progress of the enzyme catalyzed reaction as a function of time, obtained by point-by-point subtraction of the reaction data from the blank data. (B) Initial rate of enzyme-catalyzed O_2 consumption as a function of the initial O_2 concentration for WT DpgC (grey), a mutant where O_2 diffusion is not affected (L361A, green), and a mutant where O_2 diffusion is hindered (F432W, purple). The solid lines are linear fits with slope 3.66, 3.45, and 1.35 and R^2 0.99, 0.98, and 0.99, respectively.