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Kinetics of substrate inhibition of periplasmic nitrate reductase

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ABSTRACT

Periplasmic nitrate reductase catalyzes the reduction of nitrate into nitrite using a mononuclear molybdenum cofactor that has nearly the same structure in all enzymes of the DMSO reductase family. In previous electrochemical investigations, we found that the enzyme exists in several inactive states, some of which may have been previously isolated and mistaken for catalytic intermediates. In particular, the enzyme slowly and reversibly inactivates when exposed to high concentrations of nitrate. Here, we study the kinetics of substrate inhibition and its dependence on electrode potential and substrate concentration to learn about the properties of the active and inactive forms of the enzyme. We conclude that the substrate-inhibited enzyme never significantly accumulates in the EPR-active Mo(+V) state. This conclusion is relevant to spectroscopic investigations where attempts are made to trap a Mo(+V) catalytic intermediate using high concentrations of nitrate.

1. Introduction

Enzymes of the DMSO reductase family use a mononuclear Mo-bis(pyranopterin guanine dinucleotide) cofactor (MoCo) to catalyze a variety of reactions, mostly transfers of oxo groups [1]. The dissimilatory nitrate reductase (NapAB) from *Rhodobacter sphaeroides* (*Rs*) belongs to the DMSO reductase family and catalyzes the reduction of nitrate into nitrite. It is a 108 kDa, periplasmic, heterodimer which houses a [4Fe-4S] cluster in close proximity to the MoCo, and two surface-exposed, *c*-type hemes [2]. The coordination sphere of the Mo ion consists of the four thiolate ligands of two pyranopterins, a sulfur atom from a cysteine that attaches the MoCo to the protein backbone, and a sixth inorganic ligand, proposed to be oxygen or more recently sulfur [3,2,4,5]. Functional information on nitrate reductase has been obtained mostly from EPR spectroscopy, but this technique is not always conclusive because the EPR signature of the MoCo is heterogeneous, and which signals correspond to species that are actually involved in the catalytic cycle is not entirely clear yet [1].

The heterogeneity of the MoCo is also revealed in direct electrochemistry (or "protein film voltammetry", PFV) experiments [6]. In this technique, *Rs* NapAB is adsorbed onto a rotating pyrolitic graphite edge electrode and the enzyme molecules receive electrons directly from the electrode. When substrate is present in solution, the measured current is proportional to turnover rate. The electrode is spun at a high rate to ensure that there the current is not limited by the diffusion of substrate towards the electrode. The changes in turnover rate in response to changes in experimental conditions (electrode potential, substrate concentration) are instantly detected as changes in current, which makes it very easy to monitor changes in activity resulting from activation or inactivation.

Previously, we used site-directed mutagenesis, EPR and PFV to demonstrate that the MoCo in *Rs* periplasmic nitrate reductase (NapAB) is subject to a slow, irreversible reductive activation [7]. The amount of initially inactive form quantitatively correlates with the amount of the so-called "Mo(V) high-*g* resting" EPR signal. More recently, we have proposed that the inactive form features an open, oxidized pyranopterin, which is closed upon reduction [8].

The subject of this paper is a slow, reversible inactivation/reactivation process that occurs at high nitrate concentration [9] and is distinct from that reported in ref mo:nap:jacques14. Indeed, one is reversible and nitrate concentration-dependent, while the other process is irreversible and independent of nitrate concentration. This inactivation/reactivation is most easily detected in cyclic voltammetry experiments [9], where the electrode potential is repeatedly swept upward and downward to monitor the current/potential response. Fig. 1 shows cyclic voltammograms of *Rs* NapAB recorded at pH 6 and two concentrations of nitrate. According to the conventions we use, a reductive current is negative; therefore, the more negative the current, the higher the turnover rate.

At low nitrate concentration (red trace in Fig. 1), the forward and backward current traces are merely offset by the electrode charging current (dotted black trace). This indicates that catalysis is in a steady-state at each value of the electrode potential. The peculiar wave shape, with a maximum of activity under moderately reducing conditions and a decrease at lower potential, has already been observed with NapAB (from *Rs* [10,11] and *Paracoccus pantotrophus* [12]), and other members

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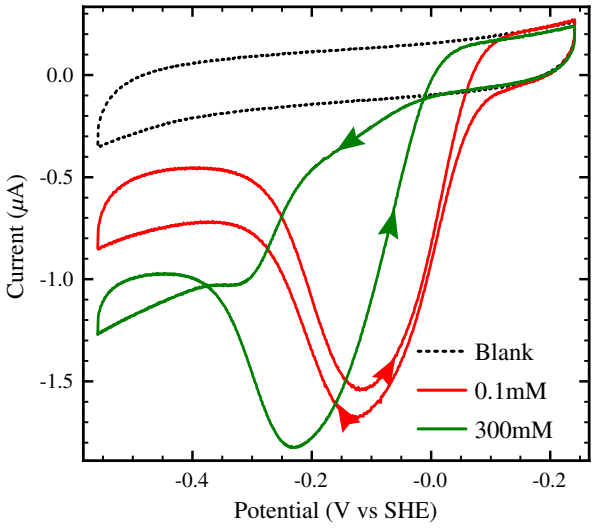

Fig. 1. Cyclic voltammograms of *Rs* NapAB adsorbed onto a rotating PGE electrode. Conditions: pH 6, T = 25 °C, electrode rotation rate $\omega = 5$ krpm, nitrate concentration as indicated. The dotted black line is a blank voltammogram recorded in the absence of enzyme.

of the DMSO reductase family (such as membrane-bound DMSO reductase [13], and respiratory nitrate reductase Nar [14]). Regarding NapAB, a comparison between PFV and solution assays demonstrated that the fact that the enzyme is most active under moderately reducing conditions is not an artifact from the electrochemical experiment [15]. The decrease in activity at higher driving force has been attributed to a change in the reaction pathway under more reducing conditions [10,11].

At higher nitrate concentrations (green trace in Fig. 1), the voltammogram shows a clear hysteresis: at a given potential, the absolute value of the current, and therefore the activity, is much lower on the sweep towards low potential than on the sweep towards high potential (the arrows in Fig. 1 indicate the direction of the sweeps). This hysteresis reveals a potential-dependent process that is slow on the voltammetric time scale (recording the complete voltammogram takes 80 s).

In a previous work, we showed that this hysteresis stems from a slow potential-dependent inhibition by substrate [9]. In this paper, we characterize in detail the kinetics of inhibition. The information we obtain allows us to compute the proportion of substrate-inhibited species as a function of substrate concentration and potential, and the subfraction of inactive species that are paramagnetic. This analysis will prove crucial to determine if a given spectroscopic signature obtained in turnover conditions is likely to be that of an active species.

2. Results

2.1. Thermodynamics of substrate inhibition: the potential dependence of the inhibition constant

Regarding NapAB, we have shown before [9] that the turnover rate tends to zero at high nitrate concentration, as predicted by the following rate law [16,9]:

$$i = \frac{i_{max}}{1 + K_m/s + s/K_i} \quad (1)$$

where i_{max} is the maximum current that would be obtained at infinite substrate concentration if there were no inhibition, K_m is the Michaelis constant and K_i the inhibition constant.

The mechanism that gives substrate inhibition in Nap will be discussed below.

It is possible to determine both K_m and the inhibition constant K_i using protein film voltammetry, in an experiment where the electrode potential is held constant and the substrate concentration is increased in a stepwise fashion by repeatedly adding aliquots of a concentrated solution of nitrate. Fig. 2 shows the result of such an experiment, with in panel A the concentration of nitrate as a function of time, and panel B the resulting current. When we carried out this experiment, we waited long enough between each injection for the current to reach its asymptotic value. This steady-state current is plotted against substrate concentration in Fig. 3.

The result in Fig. 3 is consistent with a steady-state current that asymptotically decreases to zero at infinite nitrate concentration, which demonstrates that the nitrate-inhibited form has no residual activity; Eq. (1) is therefore appropriate to fit the data (dotted line).

We repeated the experiment shown in Fig. 2 for different values of the electrode potential (and at two pH values), analyzed the results with Eq. (1), and plotted the values of K_i against potential in Fig. 4. Consistent with our earlier findings that inactivation occurs at high potentials [9], Fig. 4 shows that K_i decreases as the potential increases. In fact, K_i depends on potential in a way consistent with a reaction involving one electron and two nitrate molecules (that is, one decade of K_i per 120 mV). K_i increases only weakly with pH (less than expected for a reaction that would involve 1 proton and two nitrate molecules), which suggests that inhibition is not coupled to a protonation.

Fig. 2 shows that at high substrate concentration (*i.e.* when substrate inhibition occurs) the current relaxes slowly to its steady state value after each addition of nitrate; in contrast, at low substrate concentration, a steady-state is obtained within the mixing time (<1 s). The slow relaxation at high substrate concentration is consistent with the presence of hysteresis in cyclic voltammograms (Fig. 1), and it reveals a transformation that occurs on the voltammetric time scale ($\approx$100 s). This inactivation process is sufficiently slow that it is possible to measure the rate constants of the interconversion between the active (A) and inactive (I) species:

$$A \underset{k_a(E)}{\overset{k_i(E)}{\rightleftharpoons}} I. \quad (2)$$

The knowledge of the apparent inactivation ($k_i(E)$) and reactivation ($k_a(E)$) rate constants is valuable to understand the chemistry of the process. Indeed, the dependence of the rate constants on potential and substrate concentration can reveal the properties of the active and

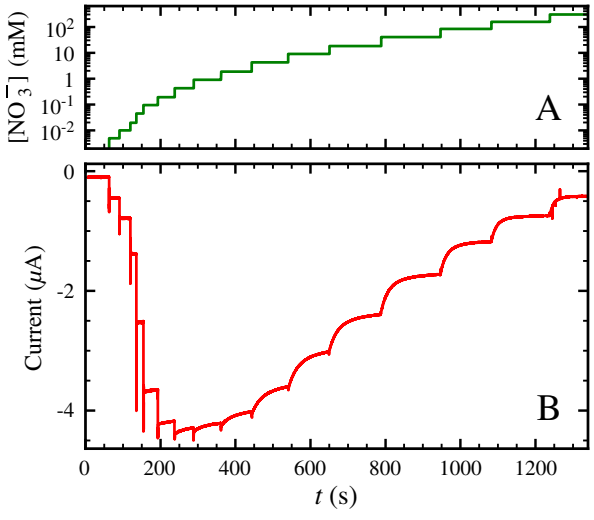

Fig. 2. Chronoamperometric experiment in which an electrode with a film of adsorbed *Rs* NapAB is poised at a constant potential (here −50 mV vs SHE), and various volumes of stock solutions of nitrate are added into the buffer step-by-step. Panel A shows the nitrate concentration and panel B the resulting catalytic current.

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