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Inorganica Chimica Acta 361 (2008) 941–946
Inorganica Chimica Acta
www.elsevier.com/locate/ica

Studies of the Mo(V) center of the Y343F mutant of human sulfite oxidase by variable frequency pulsed EPR spectroscopy

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Received 6 May 2007; accepted 15 May 2007
Available online 26 May 2007

Dedicated to Professor Edward I. Solomon on the occasion of his 60th birthday.

Abstract

The Mo(V) forms of the Tyr343Phe (Y343F) mutant of human sulfite oxidase (SO) have been investigated by continuous wave (CW) and variable frequency pulsed EPR spectroscopies as a function of pH. The CW EPR spectrum recorded at low-pH (~6.9) has *g*-values similar to those known for the low-pH form of the native vertebrate SO (*original lpH* form); however, unlike the spectrum of *original lpH* SO, it does not show any hyperfine splittings from a nearby exchangeable proton. The detailed electron spin echo (ESE) envelope modulation (ESEEM) and pulsed electron-nuclear double resonance (ENDOR) experiments also did not reveal any nearby protons that could belong to an exchangeable ligand at the molybdenum center. These results suggest that under low-pH conditions the active site of Y343F SO is in the "blocked" form, with the Mo(V) center coordinated by sulfate. With increasing pH the EPR signal from the "blocked" form decreases, while a signal similar to that of the *original lpH* form appears and becomes the dominant signal at pH >9. In addition, both the CW EPR and ESE-detected field-sweep spectra reveal a considerable contribution from a signal similar to that usually detected for the high-pH form of native vertebrate SO (*original hpH* form). The nearby exchangeable protons in both of the component forms observed at high-pH were studied by the ESEEM spectroscopy. These results indicate that the Y343F mutation increases the apparent pK_a of the transition from the *lpH* to *hpH* forms by ~2 pH units.

Keywords: Molybdenum enzymes; Sulfite oxidase; ESEEM; ENDOR; HYSCORE

1. Introduction

Sulfite oxidase (SO) is a physiologically vital molybdenum enzyme that catalyzes the oxidation of sulfite to sulfate, the final step in sulfur metabolism [1]. The X-ray crystal structures of SO from chicken [2], plant [3] and bacterial [4] sources show essentially identical five-coordinate square pyramidal geometry about the Mo center, with three sulfur donors in the equatorial plane. Two of these sulfur donors are from the enedithiolate function of the pyranopterindithiolate (molybdopterin [5]) unit that is present in all molybdenum enzymes, and one sulfur is from a conserved cysteine that is essential for catalytic activity [6]. Two oxo groups, one axial and one equatorial, complete the coordination about the metal in the fully oxidized Mo(VI) state [7,8]. During the catalytic cycle the enzyme passes through the paramagnetic Mo(V) state [9], which shows characteristic EPR spectra that are affected by the exchangeable equatorial ligand ("L" in Fig. 1), pH, anions in the media, and mutation of nearby amino acid residues [10]. These EPR signals were originally classified into three

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doi:10.1016/j.ica.2007.05.023

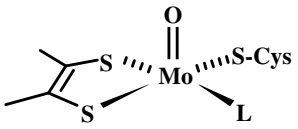

Fig. 1. Coordination geometry of the Mo(V) center of SO enzymes. Examples are known in which L is OH^-, PO_4^{3-}, AsO_4^{3-}, SO_4^{2-}, and possibly H_2O [10,33].

distinct types by Bray in the 1980s from studies of native, wild-type (*wt*) avian SO, namely, low-pH (*lpH*), high-pH (*hpH*), and phosphate inhibited (*Pi*) [11–13].

The continuous wave (CW) EPR spectrum of the *lpH* form of *wt* avian (chicken) SO observed at pH $\leqslant$ 7 is characterized by principal *g*-values of about 2.004, 1.972 and 1.966, and by characteristic splittings at the EPR turning points caused by a strong (~30 MHz) and predominantly isotropic hyperfine interaction (*hfi*) with an exchangeable proton of an equatorial OH ligand. The principal *g*-values of the *hpH* form of avian SO at pH $\geqslant$ 9.5 are significantly different (1.987, 1.964, 1.953), and the isotropic *hfi* of the exchangeable protons is close to zero [14,15]. Recombinant *wt* human SO shows *lpH* and *hpH* EPR spectra that are virtually identical to the native avian enzyme [16]. These two well-known EPR spectral types will be referred to here as *original lpH* and *original hpH* forms of SO.

More recent EPR studies of *wt* recombinant SO from plant (*Arabidopsis thaliana* (At-SO)) [17] and bacterial (*Starkeya novella* (SDH)) [18] sources show that the classification scheme for vertebrate SOs is not sufficient to describe all of the Mo(V) EPR signals observed for these organisms. For At-SO, the nature of the low pH signal depends upon the mode of reduction [17]. One-electron reduction by Ti(III) citrate at pH 6 gives an EPR spectrum similar to that observed for the *original lpH* form of vertebrates. However, reduction with sulfite at low-pH produces a new form of the active center that is characterized by similar *g*-values, but lacks an exchangeable proton. It was hypothesized that the active site in this form is in the closed conformation that blocks water access to the molybdenum center (therefore, this form was referred to as the *blocked* form), and as a result, the molybdenum center retains the sulfate ligand. Indeed, the presence of coordinated sulfate was recently confirmed by electron spin echo (ESE) envelope modulation (ESEEM) studies using ^{33}S-labeled sulfite as the substrate [19]. Thus, At-SO exhibits either the *blocked low-pH* form or the *original lpH* form, depending upon the mode of reduction.

For *wt* SDH from *S. novella*, only one EPR form, which closely resembles the *original hpH* form of vertebrates, is observed at all pH values [20]. Pulsed EPR studies show the presence of exchangeable protons in the vicinity of the Mo(V) center of bacterial SDH, even though no hyperfine splittings can be detected by CW EPR [18].

These more recent EPR studies of the sulfite oxidizing enzymes from plant and bacterial sources demonstrate that the relationships among the observed EPR spectra, and external conditions (pH, anions), and the structure surrounding the Mo(V) center [10], are more complicated than was originally proposed [13]. In order to understand details of these relationships it is necessary to judiciously and systematically vary the nearby environment of the Mo center of Fig. 1 by site directed mutagenesis. Particularly interesting are nearby amino acid residues that are conserved across species and which have been implicated in the overall catalytic reaction.

One such important residue is Tyr322 in chicken SO (Tyr343 in human SO) that is located close to the Mo active site and is hydrogen bonded to the sulfate anion that crystallizes in the binding pocket [2]. This tyrosine is conserved in the plant and bacterial enzymes and occupies a similar position relative to the molybdenum in the active site [3,4]. Studies of intramolecular electron transfer (IET) kinetics for chicken SO as a function of pH and concentration of anions led to the proposal that this tyrosine residue plays an important intermediary role in the coupled electron–proton chemistry of SO, especially when an anion blocks direct access of H_2O or OH^- to the equatorial Mo^V–OH group [21]. Competing H-bonding interactions of the Mo–OH moiety with Tyr322 (chicken numbering) and with the anion occupying the active site were also proposed to be important in the equilibrium between *original lpH* and *original hpH* forms of *wt* SO [21]. Subsequent laser flash photolysis studies showed that the IET rate constant of Y343F at pH 6.0 is only about one-tenth that of the *wt* enzyme, suggesting that the OH group of Tyr343 is important for efficient IET in SO [22]. Moreover, the pH dependences of the IET rate constants in the *wt* and Y343F forms of human SO are consistent with the previously proposed coupled electron–proton transfer mechanism [21].

The importance of this conserved tyrosine has also been demonstrated for bacterial SDH from *S. novella*, where the analogous mutant (Y236F) is unable to carry out IET and exhibits a CW EPR spectrum that is distinctly different from the *wt* enzyme and closely resembles the *original lpH* form of *wt* vertebrate SO [23]. X-ray crystallography shows only minor differences between the *wt* and Y236F enzymes from *S. novella*, and it has been suggested that changes in the intervening water structure between the molybdenum and heme domains may be responsible for the large differences observed in the kinetics and the EPR spectra for *wt* and Y236F proteins of *S. novella* [23].

In recent years EPR spectroscopy, especially variable frequency pulsed EPR spectroscopy, has proven to be extremely informative and indispensable tool for characterizing the nearby environment of the Mo(V) active centers of SO enzymes (Fig. 1) in order to gain unique insight concerning structure and reaction mechanism [10,24]. In this work, we use these techniques to address the structural changes at the molybdenum center of human SO enzyme that are induced by the Y343F site mutation, which substitutes the conserved active site tyrosine by the hydrophobic phenylalanine residue.