Spectroscopic characterization of the acid–alkaline transition of a thermophilic cytochrome P450

Shohei Hayakawa, Hirotoshi Matsumura, Nobuhumi Nakamura, Masafumi Yohda, Hiroyuki Ohno

Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan
Division of Environmental and Biomolecular Systems, Institute of Environmental Health, Oregon Health and Science University, Beaverton, OR 97006, USA

Article info
Article history:
Received 19 October 2012
Revised 15 November 2012
Accepted 15 November 2012
Available online 27 November 2012

Abstract

The spectroscopic properties of thermophilic cytochrome P450 from the thermoacidophilic crenarchaeon Sulfolobus tokodaii strain 7 (P450st) were investigated in acidic and basic solutions. The resting form of ferric-P450st in weakly-acidic and neutral solutions contained a thiolate/H2O coordinated low-spin heme. Below pH 1.5, P450st underwent cleavage of the Fe–S bond and was converted into apo-P450st. Above pH 8, the acid–alkaline transition due to the deprotonation of the water ligand was observed. The produced thiolate/OH–coordinated ferric-P450st was stable at room temperature. The pK_a value of 8.7 for the transition reflects the protonation properties of the distal side of the heme.

1. Introduction

Cytochrome P450s (P450s) [1] are a ubiquitous family of monooxygenases that are able to activate dioxygen for insertion into inactivated hydrogen–carbon bonds. P450 has a protoporphyrin IX (heme) group with cysteinate as the fifth ligand, and in many cases, one of several exogenous molecules (e.g., water, dioxygen, or peroxides) as the sixth ligand. The coordination state and the electrostatic environment around the heme have been investigated thoroughly using various spectroscopic techniques (e.g., optical absorption spectroscopy, resonance Raman (RR) spectroscopy, electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR), and Mössbauer spectroscopy) because these properties are key factors affecting the enzymatic functions of P450s. The previous spectroscopic studies were, however, performed only under mild conditions (in neutral aqueous solution at room temperature and pressure) because most P450s are easily inactivated and denatured under harsher condition. Thus, the spectroscopic properties of P450s under extreme conditions, such as high temperature, high pressure, and acidic or basic solutions, are currently poorly understood.

Thermophilic P450s, which are derived from a variety of thermophilic bacteria, are enzymatically active even under extreme conditions. Several thermophilic P450s have been isolated and characterized [2–6]. For example, cytochrome P450 from the acidothermophilic archaeon Sulfolobus solfataricus (CYP119A1) exhibits a high melting temperature (T_m) of 91 °C, whereas the melting temperature for the mesophilic cytochrome P450cam from Pseudomonas putida is 54 °C [7]. CYP119A1 has sufficient hyperbaric stability to maintain its native form at pressures up to 200 MPa [7]. Thermophilic P450s are especially useful for scientific investigations and industrial applications because their high structural stability makes it possible to greatly extend the usable conditions.

The cytochrome P450 from the thermoacidophilic crenarchaeon Sulfolobus tokodaii strain 7 (P450st) is a soluble thermophilic P450. P450st has a sequence identity of 64% with respect to CYP119A1 and has sufficient structural stability to stay enzymatically active even at 80 °C [4]. This high structural stability allows the use of P450st as a model enzyme when analyzing the behavior of P450s under extreme conditions. In this study, the effects of pH on the spectroscopic properties of P450 were investigated using P450st. Three forms of P450st were observed when varying the pH, and the coordination states of each P450st are discussed.
2. Materials and methods

P450st was expressed in *Escherichia coli* BL21 (DE3) and purified as reported previously [4]. Optical absorption spectroscopy of ferric-P450st was performed at 25 °C in 40 mM glycine–HCl (pH 1–3), acetate (pH 4, 5), potassium phosphate (pH 6–8), and glycine–NaOH (pH 9–11) buffers. The pKₐ values were determined by the absorbance change of the Soret region with fitting to the Henderson–Hasselbalch equation. The far-UV circular dichroism (CD) spectra of ferric-P450st and optical absorption spectra of the Fe⁺–CO complex of P450st were measured in 40 mM buffer (potassium phosphate, pH 7; glycine–NaOH, pH 10) at 25 °C. The Fe⁺–CO complex of P450st was prepared by addition of sodium dithionite with bubbling the carbon monoxide. The EPR studies of ferric-P450st were carried out in 40 mM buffer (potassium phosphate, pH 7; glycine–NaOH, pH 10) at 77 K.

3. Results

The optical absorption spectra of substrate-free ferric-P450st were measured at various pH values (Fig. 1). In weakly-acidic and neutral solutions (pH 2–7), P450st exhibited a Soret maximum at 415 nm with distinct α- and β-bands at 567 and 535 nm, respectively. These spectral features are in agreement with those of typical ferric-P450s, indicating that P450st contains a thiolate/H₂O-coordinated heme, as revealed by a previous resonance Raman spectral study [8].

Below pH 1.5, P450st underwent a remarkable spectral transition. P450st exhibited a broad Soret band at 370 nm and an additional band at approximately 650 nm, and the α- and β-bands disappeared. This spectral transition proceeded suddenly as the pH changed and was irreversible at room temperature. No aggregates or precipitates were observed, even at pH 1. It has been reported that various heme proteins release their heme at low pH (pH < 2) as the result of a change in the conformation around the heme [9,10]. The spectral features of the acidic P450st observed in this study are consistent with those of metmyoglobin (metMb) [11] at low pH and with those of P450cam [12] in which the proximal Fe–S bond is broken. Thus, the spectral changes for P450st in acidic solutions are most likely due to the cleavage of the Fe–S bond. This hypothesis is also supported by the fact that the stable apo-P450st was prepared by the addition of 2-butanol after this spectral changes.

When raising pH above 8, P450st underwent a spectral transition, which is known as “the acid–alkaline transition” in heme proteins. During this transition, the absorption maximum of the Soret region shifted from 415 nm to 422 nm and decreased in intensity. Along with this change, there appeared a distinct peak at 363 nm. The intensity of α-band decreased and became lower than that of the β-band. Well-defined isosbestic points were observed at 378, 424, and 512 nm, demonstrating that only two states of P450st are present during the transition (Fig. S1). The aggregation and the precipitation were not observed at all. The transition occurred reversibly (Fig. S2). The data were fitted to a single pKₐ equation, giving the value of 8.7 (Fig. 1, inset).

Spectroscopic characterizations of the acid–alkaline transitions of various heme proteins have been reported previously (e.g., cytochrome c [13–15], hemoglobin [16], myoglobin [17,18], horseradish peroxidase [17,19], and chloroperoxidase [20]). In many cases, the acid–alkaline transitions of these heme proteins are due to changes in the heme coordination state, which are often due to the deprotonation of the water ligands or the substitution of the ligands as the result of pH-induced changes to the protonation states and/or to the local structure around the heme. In the case of P450st, the spectral features of the acid–alkaline transition are similar to those of the type II spectral change, which is induced by nitrogen coordination with the heme [21]. The spectral features are also similar to those of pressure-inactivated P450cam (P420cam), which are produced by the protonation of the proximal thiolate ligand or the elongation of the Fe–S bond [22]. The acid–alkaline transition of P450st is therefore considered to be induced by (i) the binding of an anionic molecule as the sixth ligand of the heme or (ii) the conversion of the protein into the inactive P420 form. To determine the cause of the acid–alkaline transition of P450st, additional spectroscopic analysis of the alkaline-form of P450st was performed.

The far-UV CD spectra of ferric-P450st and the optical absorption spectra of the Fe⁺–CO complex of P450st in neutral and weakly-basic solutions are shown in Fig. 2. Despite the higher pH, no spectral changes were observed during these investigations. These results indicate that the alkaline-P450st keeps both its secondary structure and the local conformation around the proximal side of the heme group, and thus maintaining its activity. Hence, the acid–alkaline transition of P450st is not caused by the formation of the P420 form, but by the binding of an anionic ligand.

The EPR spectra of P450st in weakly-acidic and neutral solutions had three signals at g = 2.42, 2.25, 1.92 (Fig. 3, left), which are consistent with those of ferric-P450s with a hexacoordinated low-spin heme [23]. In weakly-basic solutions (pH 10), P450st exhibited a new EPR signal set (g = 2.49, 2.24, 1.90; Fig. 3, right), which is located in a position similar to that for various anionic ligands bound to ferric-P450cam [21]. Because the structural analysis of P450st has revealed that there are no coordinatable amino acids around the heme’s distal side [9,24], we concluded that the acid–alkaline transition of P450st is due to the formation of a thiolate/OH⁻–coordinated heme due to the deprotonation of the distal water ligand.

4. Discussion

Previously, some researches on pH dependence of spectroscopic properties of P450s have been reported. Sono et al. attempted to characterize the acid–alkaline transition of mesophilic P450cam [21], and they reported that substrate-free ferric-P450cam at pH 10 undergoes type II-like absorption spectral changes at 4 °C and exhibits two additional EPR signal sets, an unstable signal set (g = 2.54, 2.24, 1.86) and a stable signal set (g = 2.47, 2.26, 1.89). They proposed that ferric-P450cam turns into a mixture of the unstable thiolate/OH⁻–coordinated form and the stable P420 form when
investigations revealed that substrate-free ferric-P450st is not characterized by formation of the P420 form [25]. In contrast to these studies, our investigations revealed that substrate-free ferric-P450st is not converted into the P420 form, but converted into a single stable thiolate/OH state of P450st was unaffected by the deprotonation of the water ligand during the acid–alkaline transition and the catalytic step of active oxidants formation [30]. On the other hand, P450st has a hydrophobic active site and there are no proton-acceptor amino acids around the distal side of the heme [7,24], such that a water molecule most likely acts as a proton donor/acceptor for the acid–alkaline transition of P450st. The poor accessibility of solvents toward the heme and the absence of the distal acid–base catalyst probably decrease the pKa value for aquo-P450st.

The pKa value for the deprotonation of the water ligand reflects the Lewis acidity of the heme and proton donors/acceptors around the heme. The Lewis acidity of the heme is a key property affecting P450 oxygenation reactions because this property is thought to explain the protonation states of the active center during the oxygen activation steps and, notably, to determine the reactivity of the active oxidant [31–33]. A previous study in which native myoglobin was reacted with hydrogen peroxide found that the pKa value for the acid–alkaline transition is correlated with the formation of the ferric hydroperoxo species (compound 0), which involves the deprotonation of the H2O2–FeIII complex [30]. Because our previous studies revealed that P450st is able to catalyze hydrogen peroxide–driven monooxygenation reactions [8], a similar argument could be applicable to the reactions catalyzed by P450st. The pKa value of 8.7 for P450st therefore indicates that the compound 0 formation step of P450st might be activated at high pH values. In contrast, the following protonation step in the formation of the oxoferryl porphyrin π-cation radical species (compound I) might be accelerated at low pH values. The pH dependence of the hydrogen peroxide–driven monooxygenation by P450st and the correlation with the pKa value are now under study.

In conclusion, we investigated the pH dependence of the spectroscopic properties of thermophilic P450st and observed two transitions: the cleavage of the Fe–S bond in acidic solutions and the deprotonation of the water ligand of the heme in weakly-basic solutions. To the best of our knowledge, this is the first study to characterize a stable thiolate/OH–coordinated form of P450.

Acknowledgements

H. M. acknowledges the financial support from the Japan Society for the Promotion of Science (Research Fellowship for Young Scientists). This study was supported by a grant from the Japan Society for the Promotion of Science (No. 21605004).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.11.017.
References


