BBA - General Subjects 1862 (2018) 451–459

Contents lists available at ScienceDirect

BBA - General Subjects

journal homepage: www.elsevier.com/locate/bbagen

Unveiling the water-associated conformational mobility in the active site of ascorbate peroxidase

Wei-Chih Chao[a,b,c], Li-Ju Lin[b,c], Jyh-Feng Lu[b,*], Jinn-Shyan Wang[b,*], Tzu-Chieh Lin[c], Yi-Han Chen[c], Yi-Ting Chen[c], Hsiao-Ching Yang[a], Pi-Tai Chou[c]

[a] Department of Chemistry, Fu Jen Catholic University, New Taipei City, Taiwan
[b] School of Medicine, Fu Jen Catholic University, New Taipei City, Taiwan
[c] Department of Chemistry and Center for Emerging Material and Advanced Devices, National Taiwan University, Taipei, Taiwan

ARTICLE INFO

Keywords:
Ascorbate peroxidase (APX)
Conformational mobility
Tryptophan analogues
Fluorescence probe

ABSTRACT

We carried out comprehensive spectroscopic studies of wild type and mutants of ascorbate peroxidase (APX) to gain understanding of the conformational mobility of the active site. In this approach, three unnatural tryptophans were applied to replace the distal tryptophan (W41) in an aim to probe polarity/water environment near the edge of the heme-containing active site. 7-azatryptophan ((7-aza)Trp) is sensitive to environment polarity, while 2,7-azatryptophan ((2,7-aza)Trp) and 2,6-diazatryptophan ((2,6-aza)Trp) undergo excited-state water-catalyzed double and triple proton transfer, respectively, and are sensitive to the water network. The combination of their absorption, emission bands and the associated relaxation dynamics of these fluorescence probes, together with the Soret-band difference absorption and resonance Raman spectroscopy, lead us to unveil the water associated conformational mobility in the active site of APX. The results are suggestive of the existence of equilibrium between two different environments surrounding W41 in APX, i.e., the water-rich and water-scant forms with distinct fluorescence relaxation. Our results thus demonstrate for the first time the power of integrating multiple sensors (7-aza)Trp, (2,7-aza)Trp and (2,6-aza)Trp in probing the water environment of a specifically targeted Trp in proteins.

1. Introduction

Oxidative stress resulting from excessive production of reactive oxygen species (ROS), such as superoxide radical ($\cdot O_2^-$), hydroxyl radical ($\cdot$OH) and hydrogen peroxide (H_2O_2), damage cells and tissues in all aerobic organisms [1]. Cellular damages by ROS have been implicated in a number of neurological and cardiovascular diseases, and biological aging in humans [2–5]. Antioxidants provide protection against ROS by donating electrons to counteract oxidizing effect of ROS. Ascorbate is a common and efficient antioxidant in both animals and plants. In plants, during ascorbate metabolism, an increase in ascorbate concentration accompanied by an increase in multiple isoforms of enzymes has been demonstrated during evolution [6]. In particular, ascorbate peroxidase (APX) is a key enzyme of the plant ascorbate anti-oxidation system responsible for ROS scavenging in maintaining cellular homeostasis [7].

Ascorbate peroxidases (APXs) are heme-containing enzymes that are known to dismutate H_2O_2 to water and molecular oxygen using ascorbate as an electron source [8]. The crystal structure of APX reveals that the overall fold feature of the active site (Fig. 1) is very similar to that of cytochrome *c* peroxidase (CcP); both belong to Class I superfamily of heme peroxidase [9]. APX contains only two tryptophans (W41 and W179) and is of special interest to assess the functional role of these two key Trps nearby the heme center. Although proximal site W179 in APX corresponds to W191 in CcP, a porphyrin π-cation radical was observed during APX catalysis [10–12] rather than a protein-based Trp radical as seen in typical CcP catalysis. The other Trp residue, W41, is present on the distal heme side of APX corresponding to W51 in the CcP. Mutation of W41 to alanine increased conformational mobility of the distal H42 and in its oxidized form coordinates to the heme iron [13]. H42 is critical for porphyrin π-cation radical formation in APX as demonstrated by kinetic studies of H42A and H42E variants, which support the existence of a transient enzyme intermediate prior to compound I formation [14]. X-ray structure of W41A confirms that the ligation of H42 to iron is the consequence of removal of the bulky W41 residue, suggesting that the functional role for W41 is to keep the heme in a 5-coordinate state for more efficient catalysis. In addition, APX with the W41A mutation retains about 10% of enzymatic activity,

* Corresponding author.
E-mail addresses: 049696@mail.fju.edu.tw (J.-F. Lu), 034407@mail.fju.edu.tw (J.-S. Wang).

https://doi.org/10.1016/j.bbagen.2017.10.022
Received 2 June 2017; Received in revised form 27 October 2017; Accepted 30 October 2017

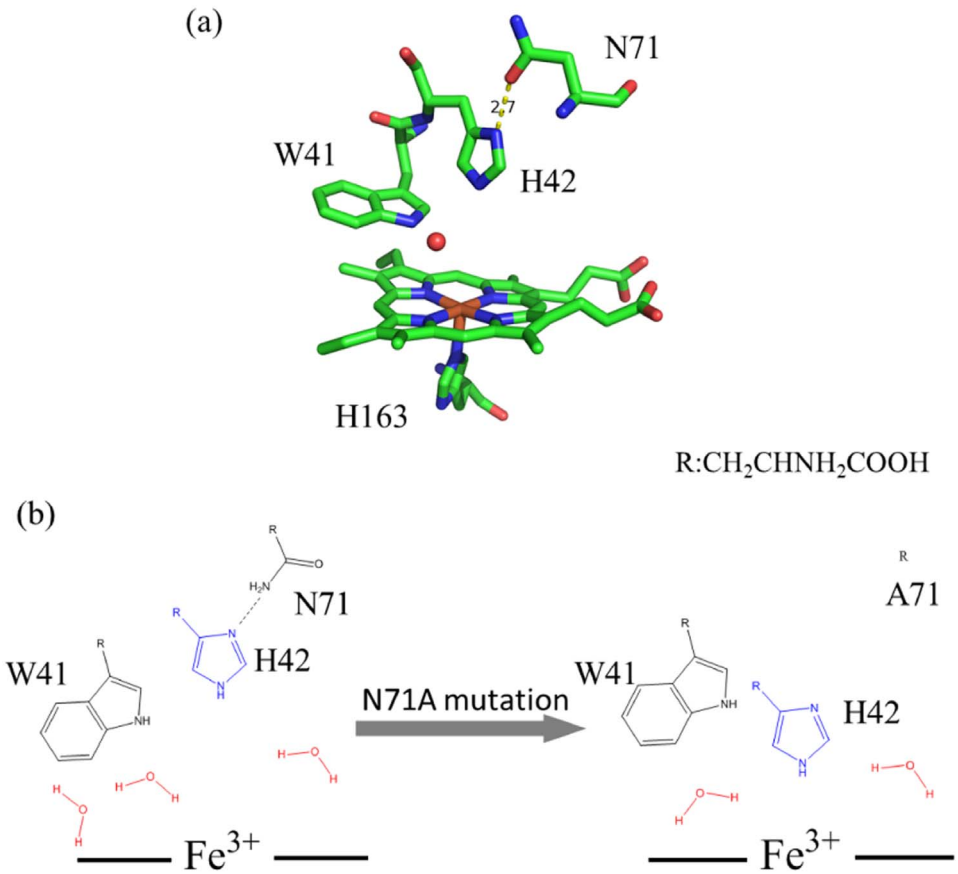

Fig. 1. (a) Illustration showing the active site of APX (PDB: 1APX). The key residues are labeled, in which water oxygen atoms are shown as red spheres. Hydrogen bonds are indicated by yellow dotted lines. (b) Schematic depicting the conformational mobility of distal H42 induced by N71A mutation.

suggesting the ability of H42 to swing "on" and "off" the heme iron to allow residual catalysis. Furthermore, N71 has been suggested to tether H42 via a hydrogen bond and control the mobility of this distal histidine (His) in the active site pocket [15]. Mutation of N71A in APX resulted in dominant low spin species resembling the active site structure of cytochrome *b*, in which the heme iron is ligated to two His ligands [13,16].

The intrinsic fluorescence of Trp has shown to be sensitive to its local environment, such as the polarity of solvent or amino acids in its proximity [17]. Taking advantages of spatial proximity and the postulated water hydrogen bond network involving W41 and H42 [10], W41 is an ideal in situ probe to investigate intrinsic conformational mobility of H42 in APX. The intrinsic Trp fluorescence has been widely used as an environment sensitive fluorophore. Unfortunately, utilization of Trp is limited due to the complex contribution of both water and charged groups of protein to various ratios to the Trp emission shift. A Trp analogue 7-azatryptophan ((7-aza)Trp) (see Scheme 1(a)) is a powerful sensor to probe the local polarity. Upon electronic excitation, (7-aza)Trp undergoes intramolecular charge transfer from pyrrolic (highest occupied molecular orbital, HOMO) to pyridyl (lowest unoccupied molecular orbital, LUMO) moiety, that is strongly affected by the polarity of the environment, being red shifted from 320 nm in nonpolar solvent to ~400 nm in water [18,19]. Recently, a more direct method of 2,7-diazatryptophan ((2,7-aza)Trp) replacing tryptophan in sensing water molecules has been reported and (2,7-aza)Trp has been evaluated for its functionality in proteins [20–22]. (2,7-aza)Trp shows advantageous property of water catalyzed excited-state proton transfer (ESPT). (2,7-aza)Trp requires forming 1:1 water: (2,7-aza)Trp (in stoichiometry) H-bonded complex to execute water catalyzed ESPT, as shown in Scheme 1(b), which is effective when a water molecule is in proximity of (2,7-aza)Trp. Very recently, 2,6-diazatryptophan ((2,6-aza)Trp) has been synthesized in our lab, in which the increased separation between proton donor and acceptor makes possible the occurrence of water catalyzed ESPT from a 2:1 water: (2,6-aza)Trp H-bonded complex (see Scheme 1(c)) [23]. The requirement of long-range relay for water molecules broadens the horizon of sensing bio-water in proteins.

In this study, we exploited these three unnatural tryptophans, namely (7-aza)Trp, (2,7-aza)Trp and (2,6-aza)Trp, and used them to replace W41 of APX, which then acted as a fluorescence sensor, respectively, for sensing polarity, proximal and relayed water molecules around the active site of APX. Moreover, various mutants of APX were applied as controls to gain understanding on the conformational mobility of the active site. The combination of emission bands and the associated relaxation dynamics, together with the difference in Soret-band absorption spectra and resonance Raman spectroscopy, lead us to unveil the water associated conformational mobility in APX.

2. Materials and methods

2.1. Materials

(2,7-aza)Trp and (2,6-aza)Trp were synthesized according to our previous reports [20,23]. (7-aza)Trp was purchased from Sigma-Aldrich. All the unnatural tryptophans are in racemic forms, however, only *L*-optically active form can be incorporated into the protein.

2.2. Expression and purification of recombinant APX

The recombinant pea APX with 6xHis tag at the N-terminus (MRGSHHHHHHGS) was constructed from pea leaf cDNA via PCR according to a previously described methodology [24], followed by the cloning into a pCW based vector for over expression in *E. coli* driven by the P_{tac} promoter. The expression constructs for the recombinant APX proteins carrying mutation (W179F, N71A/W179F) were generated according to the instruction of the QuickChange® Site-Directed