Mechanistic studies of the copper-dependent quercetin 2,3-dioxygenase from A. japonicus

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Dioxygenases are enzymes that catalyse reactions in which both atoms of molecular oxygen (O_2) are incorporated into organic substrates. These enzymes play a key role in the degradation of numerous aromatic and heteroaromatic molecules. With a very few exceptions dioxygenases are metalloproteins. The function of the metallic prosthetic groups is that of relaxing the spin-forbiddeness of the direct reaction of the triplet ground state O_2 with singlet ground state organic substrates to form singlet ground state products. The metal generally found in dioxygenases is iron in either heme or non-heme form.

Quercetin 2,3-dioxygenase is the only dioxygenase unambiguously known to rely on copper for activity and it catalyses the dioxygenation of flavonols. These important polyphenolic compounds are converted into depsides (phenolic carboxylic acid esters) with concomitant evolution of carbon monoxide (Figure S.1). The crystal structure of *A. japonicus* quercetin 2,3-dioxygenase has been elucidated in our group at 1.6 Å resolution providing an excellent platform for functional studies.

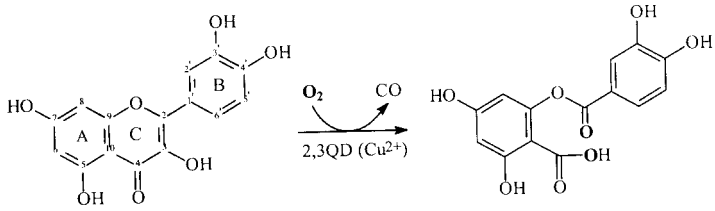

Figure S.1. Scheme of quercetin 2,3-dioxygenase-mediated cleavage of the flavonol substrate quercetin. Flavonol ring and atom nomenclature is given in the left-hand part of the figure. In the reaction the two oxygen atoms deriving from the dioxygen molecule are inserted in the C-ring. C3 and its bound oxygen atom leave as carbon monoxide.

The work described in this thesis is focused on the catalytic mechanism of *A. japonicus* 2,3-dioxygenase. A multidisciplinary approach has been employed this investigation. X-ray crystallography, electron paramagnetic resonance (EPR) and X-ray absorption (XAS) spectroscopy studies together with density functional theory (DFT) calculations have been performed with the aim of providing different perspectives of the system under study. This thesis is articulated in six chapters. In **Chapter 1**, a general introduction on the oxygenation process, the problems related to its accomplishment, and the enzymes involved in its catalysis are presented in order to offer a background for subsequent discussions. Quercetin 2,3-dioxygenase is then introduced and the structure of the enzyme from *A. japonicus* (2,3QD) is described. 2,3QD belongs to the cupin superfamily and is organised in homodimers. This quaternary arrangement seems shared by *A. flavus* 2,3QD but not by *A. niger* DSM 821. Each 2,3QD monomer is folded into a two-domain structure. A mononuclear type 2 copper centre is hosted in a shallow cavity located in the N-terminal domain. The cavity is solvent exposed owing to the disorder of an amino acidic stretch located in front of it. The copper centre interestingly displays two distinct geometries: a main

distorted tetrahedral coordination, formed by His66, His68 and His112 and a water molecule, and a minor distorted trigonal bipyramidal environment, where the copper is additionally ligated by Glu73.

To understand how ligand-binding affects the heterogeneous metal environment of native 2,3QD and to get insight into the enzymes' functional coordination we have elucidated the crystal structures of 2,3QD in complex with the inhibitors diethyldithiocarbamate (DDC) and kojic acid (KOJ) at 1.70 and 2.15 Å resolution, respectively. These structures are discussed in **Chapter 2**. Both inhibitors are shown to asymmetrically chelate the metal centre displacing the disordered bound solvent molecule. They assume a common orientation in the active site cleft, which is expected to be generally adopted by small-sized inhibitors but not by flavonols. The geometry of the Glu73 side chain is profoundly affected by the binding of the inhibitors. In particular, when KOJ is bound, Glu73 fully ligates the Cu ion through its Oε1 atom with a monodentate geometry. Compared to the native coordinating conformation, this conformation is approximately 90° rotated about the χ_3 angle. In virtue of the structural similarity between KOJ and the central flavonol ring, which is expected to bind to the metal, it is anticipated that in the enzyme·flavonol complex the enzyme is penta-coordinated with Glu73 as a ligand.

In **Chapters 3** and **4** the study of 2,3QD is moved from the crystal state to the solution state. In order to confirm the heterogeneous coordination of native 2,3QD derived from X-ray crystallography and to characterise relevant functional states 2,3QD has been studied by electron paramagnetic resonance (EPR) (**Chapter 3**) and X-ray absorption (XAS) (**Chapter 4**) spectroscopy. Consistent with the mixed coordination observed by X-ray crystallography, native 2,3QD at pH 6.0 shows a mixture of two EPR species. Whereas the major form has parameters typical of type 2 Cu sites ($g_{//}$ = 2.330, $A_{//}$ = 13.7 mT), the minor one appears to have a more distorted geometry ($g_{//}$ = 2.290, $A_{//}$ = 12.5 mT). Anaerobic addition of the substrate quercetin leads to drastic changes in the EPR signal producing a single species spectrum ($g_{//}$ = 2.336, $A_{//}$ = 11.4 mT). Substrate binding brings order in the metal centre without reducing it. After turn-over a new EPR signal is observed. It is ascribed to the carboxylic acid ester product complex. This spectrum has *g*-tensor parameters suggesting a trigonal bipyramidal site. Our EPR analysis indicates that several differences are present between 2,3QD and the enzyme from *A. niger* DSM 821.

XAS spectroscopy also indicates a mixed native environment. The analysis of the extended X-ray absorption fine structure (EXAFS) region of native 2,3QD at functionally relevant pH (pH 6.0) indicates an active site equally well described by either four or five ligands (three N(His) + one/two O) at an average distance of 2.00 Å. Bond valence sum (BVS) analysis confirms that the best model is somewhere in between the two. When, however, 2,3QD is anaerobically complexed with its natural substrate quercetin the copper environment undergoes a transition to a five-coordinated

cage, which is best modelled by a single shell of N/O scatterers at 2.00 Å. This coordination is independently confirmed by the anaerobic complex with myricetin (5'-hydroxy quercetin).

Chapter 5 focuses on the X-ray crystallographic elucidation of the E·S state. Crystal structures are reported for 2,3QD anaerobically complexed with the substrate kaempferol (5,7,4'-trihydroxy flavonol, KMP) and the natural substrate quercetin (5,7,3',4'-tetrahydroxy flavonol, QUE), determined at 1.90 and 1.75 Å resolution, respectively,. The structures show that the copper coordination cage is consistent with the prediction made on the basis of inhibition and XAS studies. The flavonol substrates coordinate to the copper ion in a monodentate way through their 3OH group. Interestingly, they bind in a bent way, in which the C2 flavonol atom is distorted towards a non-planar geometry. The increased local sp^3 character at C2 might stabilise a minor flavonol radical-Cu^+ species activated for attack by the diradical O_2. Such a species allows to circumvent the spin-forbiddeness of the catalytic reaction. Glu73 coordinates the copper through its Oε1 atom. The short distance of about 2.55 Å between its Oε2 atom and the flavonol O3 atom suggests that a hydrogen-bond exists between the two atoms indicating that Glu73 can act as a base in flavonol deprotonation and that it retains the proton. The structure also show that upon substrate binding the flexible loop located in front of the active site becomes partly ordered through a van der Waals contact between Pro164 and the flavonol A-ring. As a result, the catalytic centre is shielded from the bulk solvent when ready for O_2 attack.

In **Chapter 6**, utilising experimentally derived information, a preliminary density functional theory (DFT) investigation of the reaction mechanism of 2,3QD is presented. The calculations support the idea that in the E·S state spin density flows from the flavonolate ligand into the copper. This is therefore consistent with the existence of a flavonol radical-Cu^+ species which represents the state activated for O_2 attack. The binding of molecular oxygen is considered possible on either the C2-centred flavonol radical or the copper ion. The calculations indicate that a 1,2-dioxetane moiety might be formed in the catalytic process. We believe that if such a species is generated it does not lead directly to products. As expected, the endoperoxide species, which represents the best structure in order to rationalise the formation of the enzymatic products is found highly unstable. It is about 21.0 kcal/mole higher in energy than the reactants. Support is obtained for Glu73-dependent flavonol deprotonation in the initial step of the catalytic cycle and Glu73-dependent depside protonation in the last one.