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The role of post-translational modification in the photoregulation of Fe-type nitrile hydratase†

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The inactive, nitrosyl bound form of Fe-type nitrile hydratase (NHase) contains two active site cysteine residues that are post-translationally modified to sulfenate (SO^-) and sulfinate (SO_2^-) ligands. DFT and INDO/S calculations support the hypothesis that these unusual modifications play a key role in modulating the electronic absorption spectra and photoreactivity of the Fe(III) centre in the enzyme.

Fe-dependent nitrile hydratase (NHase) catalyzes the hydration of nitriles to yield amides, and is employed industrially for acrylamide production.[1] Several observations have generated interest in NHase. First, the enzyme contains the only known low-spin, mononuclear, non-heme Fe(III) centre in biology,[2] with a coordination sphere of two backbone amide nitrogens, and three cysteine residues. Second, biochemical[3] and X-ray crystallographic[4] studies have shown active NHase requires post-translational oxidation of two of these cysteine residues. Third, NHase is likely regulated *in vivo* by reversible binding of nitric oxide (NO) to the metal center.[5] Thus, incubation of NHase with NO in the dark eliminates activity, but exposure of inactive NO-bound NHase to visible light cleaves the Fe(III)–NO bond[6] in a reaction that is chemically interesting because, to date, only one model Fe(III) complex has been prepared to which NO binds reversibly.[7,8] The cysteinyl sulfenic and sulfinic acid oxygen atoms form a 'claw' that hydrogen-bonds to two conserved, functionally important arginine residues (Fig. 1) in the NO-bound form of NHase.[9] Since Fe–nitrosyl bonds are generally stable to visible light, it was proposed that post-translational oxidation of the active site cysteines may be important to the photochemical reactivity of the NHase/NO complex, as well as to its exceptional stability in the dark.[4] Significant technical difficulties, however, complicate experimental verification of this hypothesis.

Computationally efficient density functional theory[10] permits rigorous theoretical examination of the role played by the modified cysteines in the NHase/NO complex. We have therefore calculated the properties of two models (**1** and **2**) of the Fe(III) centre in the inactive, NO-bound form of NHase (Fig. 1) to characterize the effects of cysteine oxidation upon UV/visible spectroscopy and Fe–NO bonding. Active site model **2** is identical to model **1** except for the removal of the three oxygens marked O2, O3 and O4 (Fig. 1), and therefore corresponds to the inactive NHase prior to post-translational modification. The two, charge-neutral models were optimised *in vacuo*† at the BLYP/6-31G* level using the program TURBOMOLE.[11] In order to model structural constraints on this metal complex imposed by the surrounding protein, the methyl carbons 'capping' the peptide and amino acid fragments were fixed at their crystal coordinates.

Relative to the crystal structure, the RMS deviations in heavy atom positions for the optimised structures of models **1** and **2** were 0.12 and 0.15 Å, respectively. While differences between the Fe–S bond lengths in model **1** and the crystal structure are within the error of DFT calculations,[12] the changes in the cognate lengths in model **2** are significant and consistent with the hypothesis that sulfur oxidation influences Fe–S bonding. Interestingly, the Fe–NO bond angle is not affected by the post-translational modification, although the distance between the NO oxygen (O1) and the third oxygen of the 'claw setting' (O5) lengthens by 29 pm in model **2** relative to its value in model **1**. We also note that a strong hydrogen bonding interaction between O2 and the arginine side chain is present in the BLYP-optimised structure for model **1**.

To determine the importance of post-translational oxidation of the cysteinyl ligands in modulating the photoreactivity of the Fe–NO bond in the NHase/NO complex, we calculated UV/visible spectroscopic transition energies for models **1** and **2** at their optimised geometries, using the semi-empirical INDO/S CIS approximation (Fig. 2).[13,14] The INDO/S calculations for both complexes capture the essential features of the experimentally observed spectrum for the NO-inactivated form of NHase system,[15] namely the near-UV peak at 370 nm and sloping shoulder down to approximately 500 nm. While the agreement between theory and experiment is satisfying, we note that no

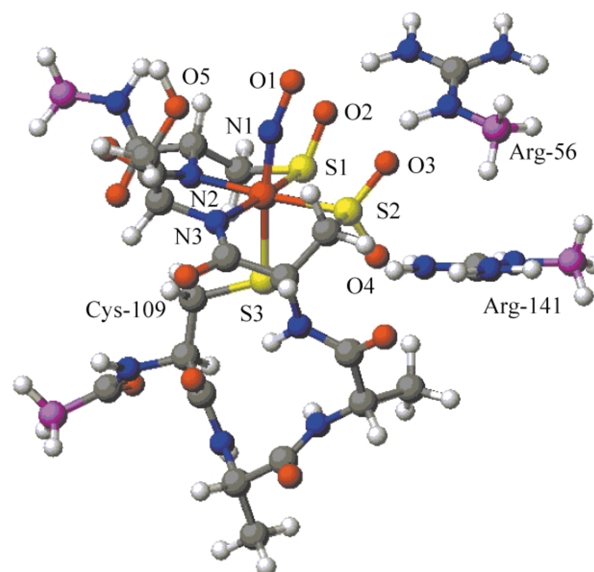

Fig. 1 NHase active site model **1**, derived from the crystallographic coordinates of the NO-complex of *Rhodococcus* R312 NHase refined to 1.7 Å resolution.[4] Conserved arginines (Arg-56, Arg-141) of the ß-subunit were modelled as methyl guanidinium moieties so as to preserve their hydrogen-bonds to the cysteine sulfenic and sulfinic acid oxygen atoms of the Fe(III) ligands. The ends of the peptide chain comprising the Fe-binding site were capped with methyl groups, and intervening residues Ser-110 and Leu-111 were modeled as alanine. Atoms shown in purple were fixed at their crystal coordinates during geometry optimisation. Active site model **2** is identical to model **1** except for the removal of the three oxygens marked O2, O3 and O4, and therefore corresponds to the inactive NHase prior to post-translational modification. [Key: C–grey; H–white; N–blue; O–red; S–yellow; Fe–orange.]

† Electronic supplementary information (ESI) available: Selected structural parameters of the experimentally observed NHase active site and DFT-optimised models **1** and **2**, and graphical MO representations of the calculated INDO/S spectroscopic transitions for the models discussed. See http://www.rsc.org/suppdata/cc/b2/b207027h/

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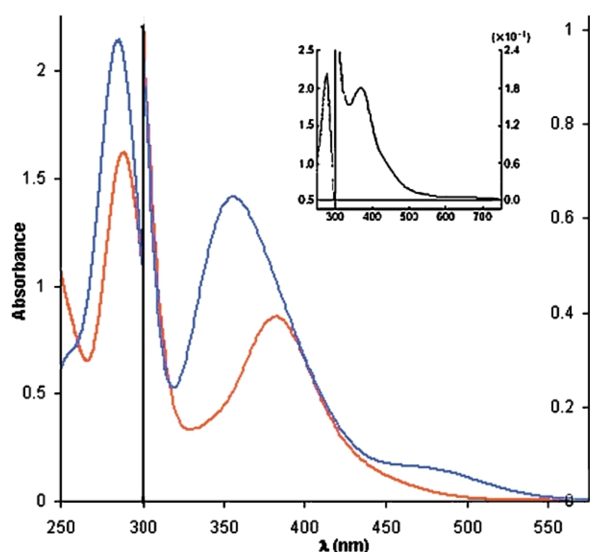

Fig. 2 Simulated UV/visible spectra based on INDO/S CIS calculations on the DFT-optimised structures of NHase active site models **1** (red) and **2** (blue). Bandwidths were taken as 3200 cm^{-1} for all transitions, and absorbance intensities were computed relative to the transition located at approximately 370 nm in each spectrum. The vertical line, at the same location in the theoretical and experimental spectra, indicates that the absorbance scale has been changed so as to simplify data presentation. The experimental spectrum of the inactive NHase/NO complex shown for comparison (inset) is reprinted from *Trends in Biotechnology*, 17, I. Endo, M. Odaka and M. Yohda. An enzyme controlled by light: the molecular mechanism of photoreactivity in nitrile hydratase, pp. 244–248, © (1999) and used with permission from Elsevier Science].

UV/visible spectrum has yet been determined for the complex of NO with the form of NHase in which the metal ligands are unmodified (model **2**). Assignment of the electronic transitions associated with specific absorption peaks for model **1** nevertheless revealed the participation of the oxygen substituents in the sulfinic acid ligand. Thus, for the transition at 380 nm (Fig. 3),† the electron is promoted into the Fe–NO antibonding orbital from an MO associated with the sulfinate ligand. The absence of these two oxygens in model **2** results in a shift of the absorption to 350 nm, which appears to be associated with a transition that does not promote an electron into the Fe–NO antibonding orbital so as to weaken this bond. In fact, this transition in model **2** merely arises from ligand-to-metal charge transfer (LMCT) involving the p-orbitals of N3, N2, and S3 and the Fe d_{z^2} orbital.† The calculated spectra for models **1** and **2** also exhibit LMCT transitions similar to that observed for the enzyme at approximately 280 nm. Detailed analysis reveals, however, that the assignment of these transitions is different in the two models. In the case of **1**, the 288 nm absorption again corresponds to electron transfer from the oxygens of the post-translationally modified cysteinyl ligands into the Fe–NO antibonding orbital.† In contrast, the transition at 285 nm calculated for model **2** is assigned to transfer of the electron from an MO involving S1, S3 and N3 into an unoccupied MO

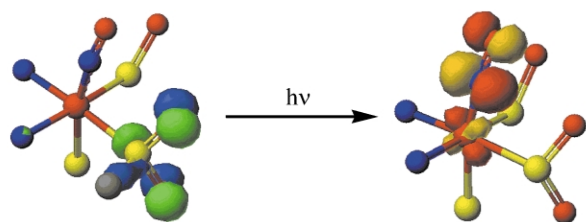

Fig. 3 Graphical representations of the electronic transitions calculated for the 380 nm photoexcitation of NHase active site model **1**. The phases of the initial (left) and final (right) MOs involved in promotion of the electron are shown in blue/green and red/yellow, respectively. Hydrogen atoms have been omitted for clarity.

dominated by the Fe d_{z^2} orbital. These calculations support the hypothesis that the Fe–NO bond is photolabile only if the sulfur ligands are post-translationally oxidized.

The influence of post-translational sulfur modification on Fe–NO bonding is also evident from calculations of the Wiberg indices[16] for the Fe–N1 bond in the ground and excited states of models **1** and **2**. For model **1**, excitation at 380 and 288 nm changes the Fe–N1 bond order from 1.43 (ground state) to 1.11 and 1.21, respectively. In contrast, the Wiberg bond order computed for the Fe–NO bond in model **2** changes from 1.78 in the ground state to 1.44 and 1.39 on absorption at 350 and 285 nm, respectively. Thus, in both the ground and excited states of model **2**, the Fe–NO bond appears stronger than the cognate bond in model **1**.

In summary, these computational studies, which appear to be the first upon the Fe(III) centre in NHase, are consistent with the proposal that post-translational modification of the cysteinyl ligands is an essential element in the photoreactivity of the inactive, NO complex. Their influence upon the mechanism of the enzyme-catalysed reaction remains to be established.

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