Model peptide for anti-sigma factor domain HHCC zinc fingers: high reactivity toward $^{1}$O₂ leads to domain unfolding†

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All organisms have to cope with the deleterious effects of reactive oxygen species. Some of them are able to mount a transcriptional response to various oxidative stresses, which involves sensor proteins capable of assessing the redox status of the cell or to detect reactive oxygen species. In this article, we describe the design, synthesis and characterization of Zn₄L₅(HHCC), a model for the Zn(Cys)₂(His)₂ zinc finger site of ChrR, a sensor protein involved in the bacterial defence against singlet oxygen that belongs to the family of zinc-binding anti-sigma factors possessing a characteristic H/C–X₂₄/2₅–H–X₃–C–X₂–C motif. The 46-amino acid model peptide L₅(HHCC) was synthesized by solid phase peptide synthesis and its Zn²⁺-binding properties were investigated using electronic absorption, circular dichroism and NMR. L₅(HHCC) forms a 1 : 1 complex with Zn²⁺, namely Zn₄L₅(HHCC), that adopts a well-defined conformation with the Zn²⁺ ion capping a 3-helix core that reproduces almost perfectly the fold of the ChrR in the vicinity of its zinc site. H₂O₂ reacts with Zn₄L₅(HHCC) to yield a disulfide with a second order rate constant of 0.030 ± 0.002 M⁻¹ s⁻¹. Zn₄L₅(HHCC) reacts rapidly with singlet oxygen to yield sulfinites and sulfonates. A lower limit of the chemical reaction rate constant between Zn₄L₅(HHCC) and $^{1}$O₂ was determined to be 3.9 × 10⁶ M⁻¹ s⁻¹. Therefore, the Zn(Cys)₂(His)₂ site of Zn₄L₅(HHCC) appears to be at least 5 times more reactive toward these two oxidants than that of a classical ββσ zinc finger. Consequences for the activation mechanism of ChrR are discussed.

Introduction

Organisms living in an aerobic environment have to cope with the deleterious effects of oxidative stress, which is caused by a family of molecules produced from dioxygen in the living cell, the so-called Reactive Oxygen Species (ROS).¹ ROS are classified into two categories according to their production pathways: Type I for those arising from electron(s) addition to O₂ (e.g.: superoxide anion O₂⁻, hydrogen peroxide H₂O₂, hypochlorous acid HOCl, hydroxyl radical HO’…) and Type II for electronically excited states of dioxygen arising from a physical activation of the ground (triplet) state ($^{3}$O₂).²–⁴ The lowest energy excited state of O₂, commonly called singlet oxygen ($^{1}$O₂), is produced mainly in photosynthetic organisms. These organisms, as well as others like mammalians, have been shown to elicit a specific response to $^{1}$O₂, demonstrating that sensing pathways exist for this ROS.⁵–⁷ One of the best characterized is that of Rhodobacter sphaeroides. It involves the protein σ⁵, a group IV sigma factor up-regulating the expression of the response against $^{1}$O₂ stress.⁸–¹¹ σ⁵ is negatively regulated by its cognate anti-sigma factor, ChrR. In normal conditions, the anti-sigma factor sequesters its cognate sigma factor (Fig. 1A). Under $^{1}$O₂ stress, the anti-sigma factor undergoes a structural change and releases its sigma factor, which can then bind RNA polymerase, up-regulating the expression of specific genes involved in the response to $^{1}$O₂ stress. The anti-sigma factor interacts with the sigma factor mainly via its N-terminal domain, named anti-sigma factor domain (ASD).¹⁰ It is estimated that 33% of the group IV sigma factors are regulated by an anti-sigma factor containing an anti-sigma factor domain. Among the predicted cytoplasmic group IV anti-sigma factors, 92% contain a conserved zinc binding motif H–X₄–C–X₂–C–C, classifying them in the zinc-binding anti-sigma factor (ZAS) subfamily.⁸

In 2011, Andreini et al. classified 93% of the 15 763 known structures of protein zinc sites into a minimum set of structural motifs.¹² The two existing structures of ChrR⁹ could not enter in any of the families defined by Andreini, which demonstrates the singularity of the zinc finger site of their anti-sigma factor domain. ChrR contains an anti-sigma factor domain featuring
question, we developed a 46-amino acid peptide modelling the anti-sigma factor domain zinc finger site of ChrR and we studied its reactivity towards $H_2O_2$ and $1O_2$. We show that its oxidation leads to $Zn^{2+}$ release and peptide unfolding. Remarkably, the cysteines of this uncommon Zn(Cys)$_3$(His)$_2$ site found in ZAS are rapidly oxidized by $1O_2$ compared to classical $\beta\beta\beta$ zinc fingers, in agreement with their putative involvement in $1O_2$ detection.

**Results**

**Peptide design**

To date, two crystal structures of anti-sigma factor domain-containing proteins displaying a zinc finger site (HHCC type) have been elucidated: ChrR$^{10}$ and RslA.$^{13}$ Anti-sigma factor domains display four $\alpha$-helices and among them, three are involved in the constitution of the zinc finger site, which is displayed in Fig. 1B. These three helices (H1, H2 and H3) are held together by the chelation of a $Zn^{2+}$ ion and by closely packed hydrophobic side chains within the heart of the 3-helix core. Helices H2 and H3 (Fig. 1B) form a knuckle bearing the H–X$_2$–C–X$_2$–C conserved motif, coordinating the $Zn^{2+}$ ion. The fourth zinc ligand is a histidine residue located at the N-terminus resulting in an overall H–X$_{24}$–H–X$_3$–C–X$_2$–C $Zn^{2+}$-binding motif (Fig. 2). In order to reproduce the reactivity of a given zinc finger site, it is important to perfectly reproduce the various features of the site that can influence its reactivity. This includes the coordination sphere of the $Zn^{2+}$ ion, the folding of the peptide around and the hydrogen bonds that are established with the $Zn^{2+}$-bound sulfurs.$^{18,20,21}$ It has been shown with RslA that the anti-sigma factor domain can adopt different folds depending whether it is bound to its sigma-factor or not.$^{17}$ Since our aim is to better understand if the reactivity of ChrR’s zinc site could play a role in the sensing of $1O_2$, we aim here at reproducing the fold as complexed with $\sigma^E$. Hydrophobic cores proximal to the zinc finger site can be important to enhance $Zn^{2+}$ affinity to the model peptide and to ensure its proper folding.$^{24}$ For these reasons, we decided to include the three helices in our model.

The design of the model peptides was based on the sequence and X-ray crystallographic structure of ChrR anti-sigma factor domain (Fig. 2),$^{19}$ which was re-engineered as follows. (i) In the HH motif present at the N-terminus, only the second residue is involved in $Zn^{2+}$ coordination as part of the canonical H–X$_{24}$–H–X$_3$–C–X$_2$–C motif. In order to avoid scrambling of $Zn^{2+}$ ligand, the first histidine of the HH motif was changed for a lysine. (ii) Helices H1, H2 and H3 present several hydrophobic side chains

![A and B: Crystallographic structure of the ChrR–$\sigma^E$ complex (pdb 2Z2S)](Image link)

**Fig. 1** (A) Crystallographic structure of the ChrR–$\sigma^E$ complex (pdb 2Z2S) showing the sigma factor $\sigma^E$ in orange and the anti-sigma factor ChrR in blue with anti-sigma factor domain in dark blue and cup-like domain in light blue. The zinc ion is shown in magenta. (B) View of the anti-sigma factor domain zinc finger site embedded within helices H1, H2 and H3, showing $Zn^{2+}$-binding histidines and cysteines of the H–X$_{24}$–H–X$_3$–C–X$_2$–C motif.

We have previously shown that small metallopeptides modelling zinc finger sites are useful tools to gain interesting insights into the reactivity of zinc fingers toward ROS at the molecular level (oxidation products, kinetic rates).$^{16-20}$ In particular, we have shown that a Zn(Cys)$_2$ zinc finger of the treble clef family of zinc fingers could be efficiently oxidized by $O_2$$^{21}$ whereas $1O_2$ oxidation of the Zn(Cys)$_2$(His)$_2$ site of classical $\beta\beta\beta$ zinc fingers is far less efficient.$^{22}$ This questions the role of the Zn(Cys)$_2$(His)$_2$ zinc finger in ChrR. To address this

![Fig. 2](Image link)

**Fig. 2** Sequence alignment of the N-terminal domain of ChrR (top) and the model peptides presented in this article. $Zn^{2+}$-binding amino acids underlined.
The metal binding properties of LASD(HHCC) were investigated upon addition of Zn$^2+$ in a buffer solution. (i) The C-terminus of the peptide was amidated to remove the carboxylate charge that could destabilize the C-terminus of helix H3. (ii) A random coil peptide with a small content of helical fold. In addition, the CD spectrum of the metal-free peptide was subtracted from each spectrum. (iii) As β-branched amino acids such as threonine or valine may destabilize α-helices, two threonines located in helices H1 and H2, and pointing toward the exterior of the structure were changed for lysines. Similarly, glycines in helix H3, which may destabilize a helical fold, were changed for glutamine and alanines. (iv) The charged amino acids were re-distributed in the sequence for polar amino acids (E, Q or K). (v) Non-essential aspartates and arginines were changed for lysines and to avoid side reactions during solid phase peptide synthesis (SPPS) and maximize synthesis yields. This led to peptide L1, whose sequence is displayed in Fig. 2. As a first try to model the anti-sigma factor domain, zinc binding sites were predicted upon dissolution of the Zn$^2+$ complex, the charged amino acids were re-distributed in the sequence for random disposition onto the 3-helix surface. This led to peptide LASD(HHCC) (Fig. 2), which was synthesized by SPPS. Note that four pseudoproline dipeptides were used to avoid aggregation of the elongating chain during synthesis (see ESI† for details).

**Zinc binding and folding properties**

The metal binding properties of LASD(HHCC) were investigated by combination of UV-Vis absorption, circular dichroism (CD) and NMR. Titrations monitored by UV absorption were used to determine the stoichiometry of the peptide-metal complexes. Upon addition of Zn$^2+$ to a buffered peptide solution, an increasing absorption band is observed at ca. 220 nm, which corresponds to Cys$^-$ → Zn$^2+$ ligand-to-metal charge transfer (LMCT) transitions (Fig. 3A). This signal increases linearly up to one equivalent of zinc and plateaus afterwards (inserts Fig. 3A). This indicates the formation of a 1 : 1 complex only, i.e. Zn$^+$-LASD(HHCC). The intensity of this band is $\Delta \varepsilon = 6600$ M$^{-1}$ cm$^{-1}$, which is in agreement with two zinc-bound cysteinate$^{28,24,29}$. To gain further insight into the coordination sphere of the Zn$^2+$ ion, LASD(HHCC) was titrated with Co$^{2+}$, a Zn$^2+$ ion surrogate commonly used to probe its geometry and coordination sphere. Absorption bands characteristic of Cys$^-$ → Co$^{2+}$ LMCT (in the range 220–400 nm) and d-d (in the range 500–700 nm) transitions appear upon coordination of Co$^{2+}$ by LASD(HHCC) (Fig. 3C). Similarly to the Zn$^2+$ titration, only the 1 : 1 Co-LASD(HHCC) complex is detected. The wavelengths (576, 627 and 670 nm) and intensity (ε = 620 M$^{-1}$ cm$^{-1}$ at 627 nm, i.e. ε > 300 M$^{-1}$ cm$^{-1}$) of the d-d transitions are in agreement with a tetrahedral Co(Cys)$_2$(His)$_2$ site$^{28,24,30,31}$. From these data, we can reasonably infer the formation of a tetrahedral 1 : 1 Zn$^+$-LASD(HHCC) complex with (Cys)$_2$(His)$_2$ coordination. Thereafter, the folding of the peptide was investigated by CD (Fig. 3B). The CD spectrum of the metal-free LASD(HHCC) shows an intense negative signal with minimum at ca. 200 nm and a shoulder at ca. 222 nm. This corresponds to a random coil peptide with a small content of helical fold. In agreement with absorption studies, upon titration of LASD(HHCC) by Zn$^2+$, evolution of the CD spectrum is observed up to 1 eq. Zn$^2+$ with a clean isodichroic point at 205 nm, thereby confirming the formation of a complex with 1 : 1 stoichiometry only. The spectrum of Zn-LASD(HHCC) is characteristic of a peptide with major helix content, as expected for this model. In order to determine the Zn$^2+$ binding constant of LASD(HHCC) at pH 7.0, $K_{\text{Zn,LASD(HHCC)}} = [\text{Zn} \cdot \text{LASD(HHCC)}]/[\text{Zn}^2+] \cdot [\text{LASD(HHCC)}]$, CD titrations were performed in competition with EDTA and TPEN (1 eq.), two high-affinity Zn$^2+$ chelators ($K_{\text{Zn,EDTA}} = 10^{13.1}$ M$^{-1}$ and $K_{\text{Zn,TPEN}} = 10^{14.9}$ M$^{-1}$ at pH 7.0). In the case of EDTA, ca. 95% of Zn$^2+$ is bound to LASD(HHCC) after addition of 1.0 eq. Zn$^2+$ vs. peptide (ESI†), indicating that
$L_{\text{ASD}}(\text{HHCC})$ binds $\text{Zn}^{2+}$ tighter than EDTA but also that EDTA is not suitable for precise determination of the binding constant. In the case of TPEN, we noticed a slow precipitation of the $\text{Zn}^{2+}$-free peptide in presence of this chelator, which precluded any competition experiment. Thus, only a lower estimate of the binding constant can be drawn from the EDTA competition, that is $K_{\text{Zn-L}_{\text{ASD}}(\text{HHCC})} \geq 10^{15.0} \text{ M}^{-1}$.

In order to confirm that the N-terminal histidine, which is remote from the core H–X$_3$–C–X$_2$–C binding motif in the sequence, is bound to the $\text{Zn}^{2+}$ ion in our $\text{Zn-L}_{\text{ASD}}(\text{HHCC})$ model, a peptide variant with the N-terminal histidine replaced by an alanine, namely $L_{\text{ASD}}(\text{AHCC})$, was synthesized (ESI†). Upon $\text{Zn}^{2+}$ titration, the formation of a 1 : 1 complex only is evidenced by both UV-Vis absorption and CD spectroscopies (Fig. 3A and B). The spectral features are very similar for both peptides, including LMCT absorption and CD spectra, either in their Zn-free and Zn-loaded forms. The intensity of the LMCT band ($\Delta = 8400 \text{ M}^{-1} \text{ cm}^{-1}$) is compatible with two zinc-bound cysteinates. Noteworthy, the CD spectrum of Zn–L$_{\text{ASD}}(\text{HHCC})$ is very similar to that of Zn–L$_{\text{ASD}}(\text{HHCC})$, indicating a similar helix content in the Zn-loaded form. Regarding Co$^{2+}$ binding, analysis of the UV-Vis Co$^{3+}$ titration of L$_{\text{ASD}}(\text{AHCC})$ shows a completely different d–d transition pattern compared to L$_{\text{ASD}}(\text{HHCC})$ with a two-step growing phase (the bands at 660 and 695 nm have the same intensity at the beginning of the titration (<0.5 eq.), then the band at 661 nm becomes the most intense, Fig. 3C), indicating the formation of both 2 : 1 and 1 : 1 Co/peptide species during the titration, with tetrahedral geometry as attested by the $\epsilon$ values above 300 M$^{-1}$ cm$^{-1}$ for the d–d transitions. Additionally, no plateau is observed after 1.0 eq., indicating a less stable 1 : 1 complex compared to Co–L$_{\text{ASD}}(\text{HHCC})$. Finally, the binding affinity of L$_{\text{ASD}}(\text{AHCC})$ for $\text{Zn}^{2+}$ was assessed by competition with EDTA monitored by CD. When a 1 : 1 : 1 L$_{\text{ASD}}(\text{AHCC})$/EDTA/Zn$^{2+}$ mixture is prepared, ca. 25% of $\text{Zn}^{2+}$ is bound to L$_{\text{ASD}}(\text{AHCC})$ versus 95% for L$_{\text{ASD}}(\text{HHCC})$ in the same conditions (ESI†). This corresponds to a value of ca. 10$^{12}$ for $K_{\text{Zn-L}_{\text{ASD}}(\text{AHCC})}$, indicating that the replacement of the N-terminal histidine by an alanine lowers the Zn$^{2+}$ affinity by at least 3 orders of magnitude.

Further insights into the structure of Zn–L$_{\text{ASD}}(\text{HHCC})$ and Zn–L$_{\text{ASD}}(\text{AHCC})$ were obtained by $^1$H NMR. The 1D $^1$H NMR spectra of metal-free L$_{\text{ASD}}(\text{HHCC})$ and L$_{\text{ASD}}(\text{AHCC})$ in H$_2$O/D$_2$O 9 : 1 display broad peaks with amide NH in the range 7.2–8.5 ppm indicating that these peptides are mostly random coil, in agreement with CD. Regarding $\text{Zn}^{2+}$ complexes, the $^1$H NMR spectrum of Zn–L$_{\text{ASD}}(\text{HHCC})$ displays sharp amide NH resonances spread over a wider range from 6.9 to 9.3 ppm (Fig. 4A, top, and ESI†). Many of them present $J_{HN,HN}$ $< 6$ Hz indicative of helical folding (Table S2 and Fig. S4 of ESI†). The 2D NOESY spectrum shows numerous correlation peaks corresponding to non-sequential NOEs that are characteristic of helical folding (Fig. S4 of ESI†). Additionally, several long-range NOEs between hydrophobic amino acid remote in the sequence indicate the formation of a hydrophobic core. This suggests the formation of a $\text{Zn}^{2+}$ complex with a well-defined stable conformation. On the contrary, the $^1$H NMR spectrum of Zn–L$_{\text{ASD}}(\text{AHCC})$ (Fig. 4A, bottom) shows very broad resonances in the amide NH region although this complex displays a similar helix content as Zn–L$_{\text{ASD}}(\text{HHCC})$ as judged from its CD spectrum. This suggests a conformational equilibrium for Zn–L$_{\text{ASD}}(\text{AHCC})$. Indeed, the coordination of the N-terminal histidine to the $\text{Zn}^{2+}$ ion is not necessary to fold the three helices, as indicated by CD but it plays a major role in freezing the conformation of the peptide in HHCC variant. Finally, the structure of Zn–L$_{\text{ASD}}(\text{HHCC})$ was calculated using X-PLOR with 466 H–H distance constraints (142 intraresidue, 138 sequential and 186 medium- and long-range) extracted from the NOESY spectrum and 35 $\phi$ dihedral constraints derived from $J_{HN,HN}$ values. The superimposition of the ten lowest energy structures, which is depicted on Fig. 4B shows that Zn–L$_{\text{ASD}}(\text{HHCC})$ adopts a well-defined conformation with three helices (the backbone root mean square deviation over the ten structures is 0.83 Å). The zinc finger site caps this three-helix domain (Fig. 4C). The superimposition of the lowest energy structure of Zn–L$_{\text{ASD}}(\text{HHCC})$ with the zinc finger site taken from the crystallographic structure of ChrR anti-sigma factor domain (Fig. 4D) shows that the model reproduces almost perfectly the fold of the native protein, including helices H1, H2 and H3 as well as the loops between the helices. To summarize, L$_{\text{ASD}}(\text{HHCC})$ is able to bind only one $\text{Zn}^{2+}$ ion to form a tetrahedral Zn(Cys)$_2$(His)$_2$ site that folds the peptide into a unique 3-helix conformation reproducing almost perfectly that of ChrR anti-sigma factor domain.

**Oxidation of Zn–L$_{\text{ASD}}(\text{HHCC})$ by H$_2$O$_2$**

The reactivity of Zn–L$_{\text{ASD}}(\text{HHCC})$ toward H$_2$O$_2$ and $^1$O$_2$ was investigated in order to assess the propensity of the anti-sigma factor domain zinc finger site to be oxidized by these two...
oxidants in comparison with other zinc fingers. The reaction of Zn-LASD(HHCC) (20 μM) with H2O2 (1 mM) in phosphate buffer (10 mM, pH 7.0) was monitored by CD (Fig. S3C of ESI†). This reaction is slow and after 15 h, the CD spectrum resembles that of zinc-free LASD(HHCC), indicating that the peptide unfolds upon reaction with H2O2. The product of the reaction was identified by ESI/MS as a disulfide (loss of two mass units). A similar CD spectrum was obtained by reacting Zn-LASD(HHCC) with 2.5 eq. HOCl, a more efficient oxidant for zinc-bound thiols, known to form disulfides.3,4 The kinetics of the reaction of Zn-LASD(HHCC) with H2O2 at 298 K was determined using previously described procedures,5,9 monitoring either the loss of the LMCT absorption in the UV or zinc release by using 4-(2-pyridylazo)resorcinol (PAR), which forms the Zn(PAR)2 complex with intense absorption in the visible when Zn2+ is released (10 mM, pH 7.0) was monitored by CD (Fig. S3C of ESI†), indicating that the peptide unfolds upon reaction with H2O2. The reaction of Zn-LASD(HHCC) by 1O2 was investigated as previously described for other Zn(Cys)4 and Zn(Cys)2(His)2 zinc fingers.19,20 Indeed, the rate-determining step corresponds to the nucleophilic attack of H2O2 by the zinc-bound thiolate and its rate is given by [H2O2][Zn-LASD(HHCC)] with k = 0.030 ± 0.002 M−1 s−1 at 297 K.

**Oxidation of Zn-LASD(HHCC) by 1O2**

In previous studies,21–22 we have shown that the reaction of Zn(Cys)4 and Zn(Cys)2(His)2 zinc finger models with 1O2 yields sulfinate species as major products, and disulfides in a lesser extent. Additionally, in a classical ββ Zn(Cys)4(His)2 zinc finger, Zn2+ coordination inhibits photooxidation of histidines. Oxidation of Zn-LASD(HHCC) by 1O2 was investigated as previously described for other Zn(Cys)4 and Zn(Cys)2(His)2 zinc finger models:21–22 the oxidation products were identified by combination of HPLC and ESI-MS analyses and the reaction rate was assessed in competition experiments with a reference compound. Rose bengal or methylene blue were used as photosensitizers to produce 1O2 in this study. Zn-LASD(HHCC) was photooxidized in D2O buffered with phosphate or ammonium acetate. HPLC analyses were performed with acidic eluent (0.1% TFA) so that Zn2+ is removed from the peptide during analysis. The HPLC chromatogram of a solution of Zn-LASD(HHCC) containing the photosensitizer but maintained in the dark showed a single peak at tR = 20.3 min corresponding to the reduced peptide LASD(HHCC) (Fig. 5B). Upon irradiation, a second peak appears at a shorter retention time (tR = 18.1 min). Prolonged irradiation shows an increase of the peak at 18.1 min at the expense of the one at 20.3 min. The main product detected by ESI-MS analysis of the crude corresponds to the addition of two oxygen atoms to the peptide, suggesting the formation of sulfinates. Other peaks corresponding to the addition of three and four oxygen atoms are also observed. The two HPLC peaks were collected, digested with glutamate carboxypeptidase (GluC, a peptidase that cleaves peptides at carboxylic side of glutamates or aspartates), and digestion mixtures were analysed by ESI-MS (Fig. 5A and Table S1†). GluC digestion of LASD(HHCC) can give four fragments: Ac-KHVSKQLKAYAE (F1), GTLSE (F2), AYSKKVAKHLSC (E) (F3) and C14KAKAQQLKAKAA-NH2 (F4). Digestion of both HPLC fractions gave unaltered N-terminal fragments F1 and F2, meaning that neither His2 nor Tyr11 are photooxidized. However, different patterns were observed for the two cysteine-containing fragments F3 and F4 (Table S1 of ESI†). For both HPLC fractions, no mass peak corresponding to the possible F3–F4 disulfide was detected. For the fraction eluting at 20.3 min, fragment F3 is unaltered but fragment F4 was detected in three different chemical forms: unaltered and with increase of 32 and 48 mass units. The product with a 32 mass unit increase was ascribed to the oxidation of Cys34 into a sulfinate by comparison to previous studies.21,22 This was confirmed by the loss of 66 mass units corresponding to H2SO4 upon MS/MS fragmentation. The formation of a sulfinate upon photooxidation of metal-bound thiolate has already been reported in many instances for Pt, Ni, Pd, Co and Cd complexes thereby supporting the formation of a sulfinate for Zn-bound cysteines in Zn-LASD(HHCC) but the product with a 48-mass unit increase is more intriguing. As F4 does not contain any amino acid prone to oxygen incorporation upon photooxidation other than Cys34 (i.e. His, Tyr, Trp or Met), this product could only be ascribed to the formation of a sulfonate on Cys34. This hypothesis is supported by the formation of glutathione sulfonate in the case of photooxidation of glutathione.41 Nevertheless, the mechanism of the formation of this product, especially the breaking of the O–O bond in the putative RS(O)(O)–O–O intermediate remains unexplained.

![Image](https://example.com/image.jpg)
unclear. Altogether, the fraction eluting at 20.3 min contains the unoxidized peptide \( \text{L}_{\text{ASD}}(\text{HHCC}) \) as expected but also \( \text{L}_{\text{ASD}}(-\text{HHCCSO}^2) \) and \( \text{L}_{\text{ASD}}(\text{HHCCSO}^3) \) oxidation products in which Cys34 was photooxidized into a sulinate and a sulfonate, respectively (Fig. 5A). For the fraction eluting at 18.1 min, only a 32-mass unit increase was detected for F3, revealing photooxidation of Cys31 into a sulinate (confirmed by loss of 66 mass units upon MS/MS fragmentation). Photooxidation of Cys31 rather than Tyr20 or His27 in F3 was supported by unaltered F1 fragment, which contains also histidine and tyrosine, and was further demonstrated by the loss of 66 Da corresponding to \( \text{H}_2\text{SO}_2 \) upon MS-MS fragmentation. F4 appears within three different forms: unaltered and with +32 and +48 mass units. This indicates the presence of Cys34 in reduced, sulfonate and sulinate forms in the fraction eluting at 18.1 min. Therefore, this fraction contains three peptides: the primary oxidation product \( \text{L}_{\text{ASD}}(\text{HHCCSO}^2) \) and the overoxidation products \( \text{L}_{\text{ASD}}(\text{HHCCSO}^3) \) and \( \text{L}_{\text{ASD}}(\text{HHCCSO}^3\text{-C}^\infty) \) (Fig. 5B). Note worthy, mass analysis always revealed the presence of overoxidation products, even at short irradiation times, indicating that the rate of formation of overoxidation products is at least comparable to the rate of formation of primary oxidation products \( \text{L}_{\text{ASD}}(\text{HHCCSO}^2) \) and \( \text{L}_{\text{ASD}}(\text{HHCCSO}^3) \).

Then, the rate of chemical reaction between Zn-\( \text{L}_{\text{ASD}}(\text{HHCC}) \) and \( \cdot \text{O}_2 \) \( (k_r) \) was assessed by competitive photooxidation with the peptide EGWKG as a competitor \( (k_r(\text{EGWKG}) = (4.6 \pm 0.5) \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \) following a procedure described previously. The method consists in comparing the consumption of the zinc finger peptide and the reference compound by HPLC (ESI). In such a competitive photooxidation experiment, the ratio of the rate constants \( k_{1r} \) and \( k_{2r} \) of two compounds \( C_1 \) and \( C_2 \) is given by eqn (1) where \( [C_{10}] \) and \( [C_{20}] \) are the concentrations of compound \( C_i \) before and after photooxidation, respectively:

\[
k_{\text{1r}} = \frac{\ln([C_{1f}]/[C_{10}])}{\ln([C_{2f}]/[C_{20}])}
\]

Since we were not able to separate unreacted \( \text{L}_{\text{ASD}}(\text{HHCC}) \) and \( \text{L}_{\text{ASD}}(\text{HHCCSO}^3) \) by HPLC and as overoxidation are rapidly formed, this method underestimates the consumption of \( \text{L}_{\text{ASD}}(\text{HHCC}) \) and yields a sizeable underestimation of the limit of 3.9 \( \text{M}^{-1} \text{s}^{-1} \) from the competition experiments. Finally, CD was used to assess the consequences of \( \cdot \text{O}_2 \) oxidation of Zn-\( \text{L}_{\text{ASD}}(\text{HHCC}) \) on the peptide fold. As shown in Fig. 5G, photooxidation causes dramatic loss of helical structure, attesting of peptide unfolding.

**Discussion**

**Design and characterization of the anti-sigma factor domain zinc finger model**

For several years, we have been developing peptidic zinc finger models of small size in order to study the reactivity of zinc finger sites toward reactive oxygen species such as \( \text{H}_2\text{O}_2 \), \( \cdot \text{O}_2 \), \( \text{HClO} \) or \( \cdot \text{O}_2 \) at the molecular level. Our approach involves the use of cyclic and branched peptides, named CPLT (Cyclic Peptides with Linear Tail), to create shortcuts in native zinc finger sequences. This affords models that reproduce almost perfectly the fold of native zinc finger domains around the \( \text{Zn}^{2+} \) ion with a minimal set of amino acids. The small size of these models allowed us to characterize oxidation products and to provide reliable kinetic data to describe the reactivity of zinc fingers. The CPLT design is well suited to zinc finger sites harbouring a CXXC motif in a \( \beta \)-hairpin loop. However, the elaboration of a CPLT model was not possible for the anti-sigma factor domain due to its 3-helix core that supports the four \( \text{Zn}^{2+} \)-binding amino acids. Therefore, we decided to keep this core but to reduce the size of the anti-sigma factor domain sequence as much as possible without altering its fold around \( \text{Zn}^{2+} \). Re-engineering of the amino acid sequence allowed us to get a soluble 46-amino acid protein that folds upon \( \text{Zn}^{2+} \) binding and adopts the same conformation as that found in the sigma factor/anti-sigma factor complex. The design strategy used to elaborate this model revealed some important features: (i) it is necessary to avoid clustering of negatively or positively charged amino acids on the surface to provide sufficient solubility to the model, (ii) once the \( \text{Zn}^{2+} \) ion coordinated to the \( \text{H}(-\text{X})\text{C}(-\text{X})\text{C}(-\text{X})\text{X} \) motif, the constitution of a hydrophobic core within the heart of the 3-helix structure is most probably the driving force for the protein to get the proper folding; and (iii) the N-terminal histidine is absolutely required for conformational stability, providing at least 4 kcal mol\(^{-1}\) stabilization to the system as shown by the comparison between Zn-\( \text{L}_{\text{ASD}}(\text{HHCC}) \) and Zn-\( \text{L}_{\text{ASD}}(\text{HHCC}) \).

**\( \text{H}_2\text{O}_2 \) and \( \cdot \text{O}_2 \) oxidation**

In previous studies on the oxidation of model zinc fingers, we have shown that \( \text{H}_2\text{O}_2 \) yields mainly disulfides as products at physiological \( \text{pH} \) conditions whereas \( \cdot \text{O}_2 \) yields mainly sulfinites. Regarding kinetics, we have observed that neutral Zn(Cys)\(_2\)(His)\(_2\) zinc finger sites react slower with \( \text{H}_2\text{O}_2 \) than negatively charged Zn(Cys)\(_3\)(His) and Zn(Cys)\(_3\) zinc finger sites. The reactivity of Zn-\( \text{L}_{\text{ASD}}(\text{HHCC}) \) toward \( \text{H}_2\text{O}_2 \) was studied using the methodology previously described. Reaction with \( \text{H}_2\text{O}_2 \) leads to disulfide formation within the CEEC motif with a second order rate constant of 0.030 ± 0.002 M\(^{-1}\) s\(^{-1}\) at 297 K. This is within the range of second order rate constants determined for other Zn(Cys)\(_2\)(His)\(_2\) zinc finger sites react slower with \( \text{H}_2\text{O}_2 \) than negatively charged Zn(Cys)\(_3\)(His) and Zn(Cys)\(_3\) zinc finger sites. The reactivity of Zn-\( \text{L}_{\text{ASD}}(\text{HHCC}) \) toward \( \cdot \text{O}_2 \) was studied using the methodology previously described. Reaction with \( \cdot \text{O}_2 \) leads to sulfinate formation within the CEEC motif with a second order rate constant of 0.008–0.037 M\(^{-1}\) s\(^{-1}\) but significantly lower than for Zn(Cys)\(_3\)(His) and Zn(Cys)\(_3\) zinc finger sites. Among Zn(Cys)\(_2\)(His)\(_2\) zinc fingers models, Zn-\( \text{L}_{\text{ASD}}(\text{HHCC}) \) reacts faster with \( \text{H}_2\text{O}_2 \) than Zn-CP-1(CCHH) \( (k = 0.008 \text{ M}^{-1} \text{s}^{-1}) \), a classical CCHH zinc finger with well-defined \( \beta \beta \alpha \) fold. Regarding \( \cdot \text{O}_2 \) reaction of Zn-\( \text{L}_{\text{ASD}}(\text{HHCC}) \) is very fast leading to sulfinites but overoxidation products (sulfonate, bis-sulfinate) are also formed very rapidly. No photooxidation of Zn\(^{2+}\) bound histidines was detected, confirming that histidines are protected from \( \cdot \text{O}_2 \) oxidation by Zn\(^{2+}\) coordination, as previously observed in the case of a classical \( \beta \beta \alpha \) zinc finger. Unfortunately, all photooxidation products could not be separated by HPLC precluding precise determination of the chemical reaction rate constant \( k_r \). This was not the case in previous studies. Indeed, the size of the Zn-\( \text{L}_{\text{ASD}}(\text{HHCC}) \) model is large compared to our other
mined for Zn seems that the uncommon topology of the zinc fast reaction with $1O_2$ in order to trigger quickly the cellular nonetheless, to be a sensing unit this zinc 

$\text{NH}_2$ 

"$\text{1MEY}^{+}$" 

$s$ 

$cysteinates$, with a low number of $\text{NH}_2$ hydrogen bonds, the latter being known to 

\[ \text{CPF} (\text{CP-1(CCHH) }), \text{we found that the former has higher} \]

\[ \text{E complex. This would correspond} \]

\[ \text{CBH-EUR-GS (ANR-17-EURE-0003).} \]

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\[ \text{CBH-EUR-GS (ANR-17-EURE-0003).} \]

\[ \text{Notes and references} \]

1. C. C. Winterbourn, in Encyclopedia of Radicals in Chemistry, 
