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Oxidative Stress

Efficient Oxidation and Destabilization of $Zn(Cys)_4$ Zinc Fingers by Singlet Oxygen**

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Abstract: *Singlet oxygen ($^1O_2$) plays an important role in oxidative stress in all types of organisms, most of them being able to mount a defense against this oxidant. Recently, zinc finger proteins have been proposed to be involved in its cellular detection but the molecular basis of this process still remains unknown. We have studied the reactivity of a $Zn(Cys)_4$ zinc finger with $^1O_2$ by combinations of spectroscopic and analytical techniques, focusing on the products formed and the kinetics of the reaction. We report that the cysteines of this zinc finger are oxidized to sulfinates by $^1O_2$. The reaction of the ZnS_4 core with $^1O_2$ is very fast and efficient with almost no physical quenching of $^1O_2$. A drastic (ca. five orders of magnitude) decrease of the Zn^{2+} binding constant was observed upon oxidation. This suggests that the $Zn(Cys)_4$ zinc finger proteins would release their Zn^{2+} ion and unfold upon reaction with $^1O_2$ under cellular conditions and that zinc finger sites are likely targets for $^1O_2$.*

Singlet oxygen ($^1O_2$), the lowest-energy-excited state of molecular oxygen, is an important reactive oxygen species in biology.[1] It is produced in all organisms, whether photosynthetic or not, by several light-dependent or -independent pathways.[2–5] Due to its electronic structure, $^1O_2$ is a strong oxidant that can damage all cell components: DNA, RNA, proteins, and membranes. Various organisms possess a set of defense genes against $^1O_2$ stress, the best characterized defense system being that of *R. sphaeroides*.[2,3] In this organism, $^1O_2$ response genes are regulated by the sigma factor σ^E, which is inactivated by association with its anti-σ factor ChrR, a zinc finger protein.[6] The presence of $^1O_2$ induces the dissociation of the ChrR–σ^E complex and gene expression. The molecular event, which causes the dissociation, is not yet known.

Based on the known reactivity of the building blocks of the cell components (amino acids, nucleic acids, and unsaturated lipids) and their relative abundance, proteins are considered to be the main targets of $^1O_2$.[7] Besides oxidative chemical reactions, $^1O_2$ can interact with molecules by physical quenching to give ground-state triplet oxygen ($^3O_2$) and with regard to isolated amino acids, those with aromatic side chains (tryptophan, tyrosine, and histidine) and with sulfur atoms (cysteine and methionine) are the most reactive. The chemical reaction pathway is prevalent for all these amino acids except tryptophan, for which both pathways are competitive.[7] The reaction rate of cysteine is in the range of $2–9 \times 10^6\ M^{-1} s^{-1}$. Regarding oxidation products, the cysteine of glutathione is oxidized to the corresponding disulfide and thiosulfinate ester (S–S-bond-containing compounds) as major products and sulfinate or sulfonate as minor products.[8] The photooxidation of metal-bound thiolates has been investigated in a few examples with Ni^{II}, Pd^{II}, Pt^{II}, and Co^{III} complexes of organic nonpeptidic ligands[9–13] but never with Zn^{2+}. The products are always metal-bound sulfenates or sulfinates but, in many of these cases, the rigid nature of the ligand is not favorable for disulfide formation. Therefore, these studies may not be relevant to the reactivity of zinc fingers. Concerning the interaction between $^1O_2$ and zinc finger proteins, literature is scarce. It has been demonstrated that $^1O_2$ generated by the decomposition of an endoperoxide can inhibit DNA binding of the nuclear vitamin D receptor (VDR) and retinoid X receptors (RXR) which are transcription factors with treble-clef zinc fingers.[14] However, the interaction was not elucidated at the molecular level.

Herein, we describe the reactivity of a $Zn(Cys)_4$ treble-clef zinc finger with $^1O_2$. We show that the cysteine sulfurs are rapidly and efficiently oxidized into sulfinates, which strongly destabilizes the Zn^{2+} binding. Our observation suggests that $^1O_2$ can target and destroy zinc finger sites of proteins in cells.

We have developed a family of peptides modeling zinc fingers of various folds in order to study their reactivity with oxidants involved in oxidative stress.[15–17] Concerning $^1O_2$, we decided to explore its reactivity with $Zn{\cdot}L_{TC}$, which models one of the most encountered ZF folds, the treble-clef (Figure 1).[18] L_{TC} is a cyclic peptide with a linear tail. Two cysteines (Cys 2 and 5) are located in the PCENCGK^{D}P cycle and the two others (Cys 13 and 16) in the tail that is grafted on the side chain of the glutamate in the cycle.

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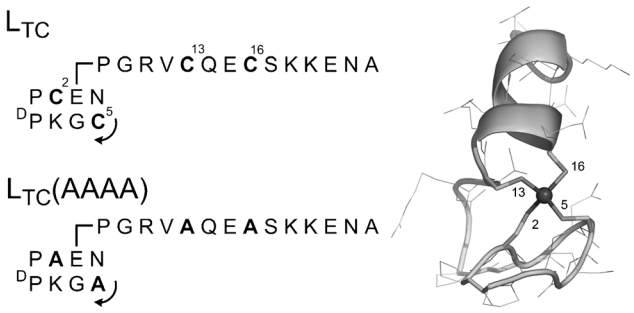


Figure 1. Sequences of the peptides L_{TC} and L_{TC}(AAAA) (left), and the solution structure of Zn·L_{TC} (right).[19] Numbering of the cysteines of L_{TC} is shown.

Zn·L_{TC} is stable to triplet oxygen in phosphate buffer (pH 7.0), but the photooxidation by singlet oxygen, generated in situ by irradiating the Rose Bengal photosensitizer, yields several oxidation products. To focus only on the primary oxidation products, the irradiation time was adjusted so that unreacted Zn·L_{TC} remains in the solution after irradiation. Solutions were analyzed by HPLC. A typical chromatogram is shown in Figure 2 and displays four new products after photooxidation, labeled L_{TC}A–L_{TC}D.

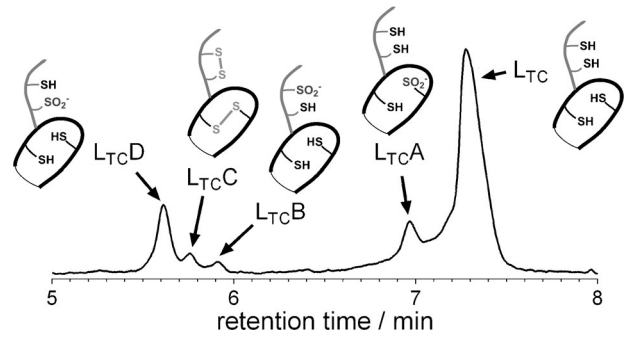


Figure 2. The chromatogram obtained after photooxidation of Zn·L_{TC} in phosphate buffer in D_2O (pD 7.0).

All these peaks were collected and analyzed by electrospray ionization mass spectrometry (ESI-MS). L_{TC}C is the already known bis-disulfide form of L_{TC} with two disulfides in each CXXC motif.[16] The reasons are: 1) the mass spectrum of L_{TC}C, which displays a peak at *m*/*z* 785.3 (3+), corresponds to the loss of 4 Da compared with L_{TC}, 2) the disappearance of L_{TC}C from the chromatogram when TCEP (tris(carboxyethyl)phosphine, a disulfide reducing agent) is added to the solution (Figure S1 in the Supporting Information, SI), and 3) the retention time of L_{TC}C. L_{TC}A, L_{TC}B, and L_{TC}D displayed the same mass spectra with a 32 Da mass increase (*m*/*z* 797.3 (3+)) compared with L_{TC} (*m*/*z* 786.6 (3+)) suggesting the formation of products that have incorporated two oxygen atoms. Each oxidation product was digested by endopeptidases and analyzed by ESI-MS. For L_{TC}D, the digestion by both trypsin and GluC gave a peak at *m*/*z* 508.0 (1−) corresponding to the addition of 32 Da to the VCQE digestion fragment. It loses 66 Da during MS/MS experiment in agreement with the loss of a SO_2H_2 fragment, thereby evidencing the formation of a sulfinate on Cys 13. Similarly, for L_{TC}B, the sulfinate formation on Cys 16 was evidenced by a peak at 695.9 (1+) that is, +32 Da compared with the CSKKE fragment. For L_{TC}A, the sulfinate is formed in the cycle as evidenced by a peak at 1171.5 (1+) corresponding to a +32 Da increase compared with the peptide c(PCE-(PGR)NCGK^{D}P). Unfortunately, as the cycle cannot be digested, it was not possible to determine which cysteine is oxidized. Nevertheless, inspection of the structure of Zn·L_{TC} in solution as determined by NMR[19] shows that the Cys 2 sulfur is totally shielded from solvent in contrast to Cys 5, which is highly exposed. Thus, we can infer that the sulfinate of L_{TC}A is on Cys 5. In all cases, the peaks attributable to nonoxidized peptidic fragments were observed as detailed in the SI.

$^1O_2$ oxidation of Zn·L_{TC} yields 90% sulfinate-containing peptides and 10% bis-disulfide peptides as deduced from the integration of the HPLC chromatograms. This result contrasts strongly with the photooxidation of glutathione, which yields S–S-bond-containing products (disulfide or thiosulfinate ester) as major products (>70%).[8] The same trend was observed for the photooxidation of free L_{TC} in D_2O buffered at pD 7.0 that yields 65% disulfide- and 35% sulfinate-containing peptides (Figure S6, SI). Therefore, the binding of Zn^{2+} to the cysteines favors the formation of sulfinate over disulfide.

To assess the efficiency of the oxidation of Zn·L_{TC} by $^1O_2$, kinetic studies were performed. $^1O_2$ can chemically react with Zn·L_{TC} to yield L_{TC}A, L_{TC}B, L_{TC}C, and L_{TC}D (rate constant k_r) or it can be physically quenched by Zn·L_{TC} (rate constant k_q). The rate of total $^1O_2$ removal by Zn·L_{TC} ($k_T = k_r + k_q$) was measured by singlet oxygen luminescence quenching experiments (Figure 3A and Table 1, see SI for experimental details). The measurements of k_r were performed by photooxidation competition experiments between Zn·L_{TC} and water-soluble anthracene-based $^1O_2$ traps. These traps react with $^1O_2$ to form stable endoperoxides (Scheme 1). In such experiments, the ratio of the rate constants k_{r1} and k_{r2} of two competitors C_1 and C_2 is given by Equation (1) where $[C_i]_0$ and $[C_i]_f$ are concentrations of compound C_i before and after photooxidation, respectively.

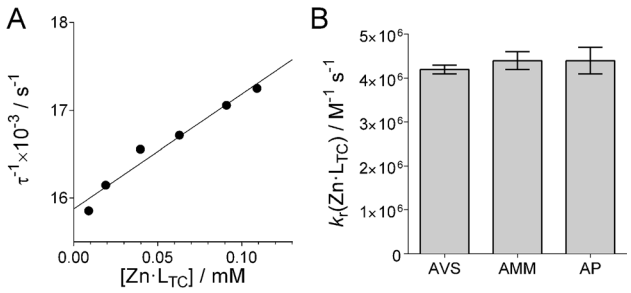
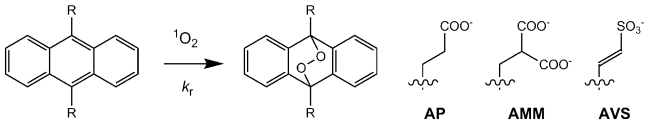


Figure 3. A) Singlet oxygen luminescence quenching experiment for Zn·L_{TC}: plot of (lifetime)$^{-1}$ versus [Zn·L_{TC}]. B) Evaluation of k_r for Zn·L_{TC} by competition experiments with anthracene-based traps monitored by HPLC.

Table 1: Rate constants of total $^1O_2$ removal (k_T) and chemical reaction with $^1O_2$ (k_r) of anthracene traps and peptides in phosphate buffer (pD 7.0), 298 K.

	$k_T \times 10^6$ [$M^{-1}s^{-1}$]	$k_r \times 10^6$ [$M^{-1}s^{-1}$]
AP	17 (2)	13 (2)
AMM	13 (1)	5.4 (8)
AVS	44 (7)	2.8 (4)
Zn·L_{TC}	14 (1)	4.3 (4)
L_{TC}(AAAA)	11 (1)	< 0.1
EGWGK	23 (2)	4.6 (5)



Scheme 1. Structure of the anthracene-based $^1O_2$ traps and their chemical reaction with $^1O_2$.

$$\frac{k_{r1}}{k_{r2}} = \frac{\ln\left(\frac{[C_1]_f}{[C_1]_0}\right)}{\ln\left(\frac{[C_2]_f}{[C_2]_0}\right)} \quad (1)$$

Three different traps were used for these competition experiments: AP (9,10-bis(propionate)anthracene), AMM (9,10-bis(methylmalonate)anthracene), and AVS (9,10-bis-(vinylsulfonate)anthracene) (Scheme 1). AVS is more soluble in water and its synthesis is more facile than that of AP and AMM.[20] We have determined the k_r values of these anthracene traps in our buffer conditions (phosphate pD 7.0) by measuring the quantum efficiency of their photooxidation (Table 1). Values for AP and AMM are identical within experimental error to those at pH 7.4 reported by Kuznetsova et al.[21] To our knowledge, the k_r value of AVS was not previously reported in the literature. Among these three traps, k_r increases in the order AVS < AMM < AP and spans over approximately one order of magnitude. HPLC was used to determine k_r for Zn·L_{TC} by competition with the anthracene traps. All three anthracene traps gave the same value of k_r for Zn·L_{TC} [$(4.3 \pm 0.4) \times 10^6 M^{-1}s^{-1}$] within the experimental error (Figure 3B). Hence, the chemical reaction represents approximately one third of the rate of total $^1O_2$ removal and the physical quenching the remaining two third ($k_q \approx 10^7 M^{-1}s^{-1}$).

From the nature of the reaction products L_{TC}A, L_{TC}B, L_{TC}C, and L_{TC}D, it is clear that the chemical reaction only involves the zinc-bound sulfur groups, whereas the physical quenching may originate from both the ZnS_4 core and its peptidic envelope. To assess the contribution of each component, L_{TC}(AAAA), the 4-Cys-to-Ala mutant of L_{TC} was synthesized (Figure 1). L_{TC}(AAAA) corresponds to the peptidic envelope surrounding the ZnS_4 core in Zn·L_{TC}, that is {Zn·L_{TC}-ZnS_4}. A value of $(11 \pm 1) \times 10^6 M^{-1}s^{-1}$ was measured for the k_T of L_{TC}(AAAA). No chemical reaction was observed under the conditions used for the photooxidation of Zn·L_{TC} and the competition experiment with AVS showed the exclusive consumption of AVS. Thus we can estimate $k_r <$ $0.1 \times 10^6 M^{-1}s^{-1}$ for L_{TC}(AAAA). Hence, the physical quenching measured for L_{TC}(AAAA) and Zn·L_{TC} are identical within the limit of error. This means that in Zn·L_{TC}, the peptidic envelope is responsible for the physical quenching of $^1O_2$ and the ZnS_4 core is responsible for the chemical reaction.

The kinetics of the reaction of isolated amino acids with $^1O_2$ have been extensively studied and show that amino acids with sulfur atoms (Cys and Met) or aromatic rings (Trp, Tyr, His) are the most reactive. However, investigations with regard to the incorporation of those amino acids in proteins are scarce. Recently, Jensen et al. have reported the k_r values for tryptophan residues of various proteins in phosphate buffer (pD 7.4).[22] These values range from $3 \times 10^7 M^{-1}s^{-1}$ for solvent-exposed tryptophans down to below $10^5 M^{-1}s^{-1}$ for buried tryptophans. We believe that these values are overestimated by an order of magnitude and that the k_r value for solvent-exposed tryptophan would rather be ca. $3 \times 10^6 M^{-1}s^{-1}$.[23] This was confirmed by measuring the k_r value of the tryptophan in the five amino acids peptide EGWGK. A k_r value of $(4.6 \pm 0.4) \times 10^6 M^{-1}s^{-1}$ was obtained by competition with AMM and AVS (Table 1 and SI). Therefore, the ZnS_4 core of Zn·L_{TC} reacts chemically with $^1O_2$ at a rate that is similar to that of solvent-exposed tryptophan residues in proteins. Additionally, in contrast to tryptophans, zinc fingers are often exposed to solvent and thus, they appear as sensitive targets for $^1O_2$.

Finally, we focused on the consequences of the reaction of the ZnS_4 core on the stability of the zinc complex in order to determine if a treble-clef zinc finger would be destroyed under cellular conditions. For this purpose, Zn^{2+} binding studies were performed with the sulfinate-containing peptides L_{TC}A, L_{TC}B, and L_{TC}D as previously reported for L_{TC}.[19] The titration of L_{TC}A by Zn^{2+} in phosphate buffer (pH 7.0) was monitored by circular dichroism (CD) spectroscopy (Figure 4A). The evolution of the CD spectrum is very similar to that of the parent complex Zn·L_{TC} and attests to the formation of a 1:1 metal/peptide complex as the final species. Its CD

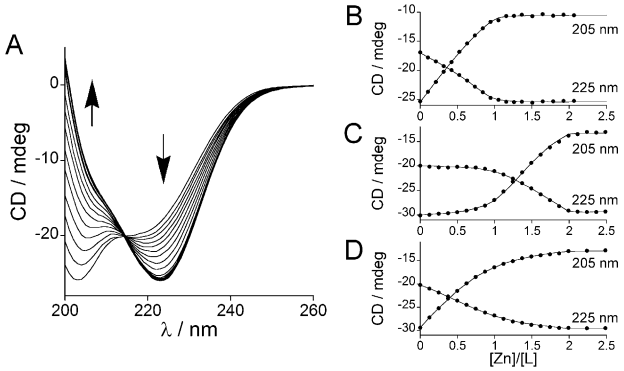


Figure 4. A) CD titration of L_{TC}A (20 μM) by Zn^{2+}. B) Evolution of the CD at 205 nm and 225 nm against the Zn^{2+}/peptide ratio for direct titration (A). C) Evolution of the CD at 205 nm and 225 nm for the titration of L_{TC}A (23 μM) and HEDTA (23 μM) by Zn^{2+}. D) Evolution of the CD at 205 nm and 225 nm for the titration of L_{TC}A (23 μM) and EGTA (23 μM) by Zn^{2+}. In (B), (C) and (D), solid lines correspond to the fits with the K_{11} and K_{12} values given in the text. Titrations were performed in phosphate buffer (20 mM, pH 7.0, 298 K). All spectra were corrected for dilution.

spectrum resembles that of $Zn{\cdot}L_{TC}$ thereby suggesting that $L_{TC}A$ folds the same way around Zn^{2+} in the 1:1 complex. However, the evolution of the CD spectrum at 205 nm and 225 nm shows a slight curvature below 1.0 equiv Zn^{2+}, which was not observed with $Zn{\cdot}L_{TC}$ (Figure 4B). This indicates the presence of another complex besides the 1:1 complex and a substantial destabilization of the 1:1 complex compared with the parent peptide L_{TC}. Factor analysis performed with the program SPECFIT[24] confirmed the existence of three absorbing species: the free peptide and the 1:1 and 1:2 complexes. To determine the apparent Zn^{2+} binding constants $K_{11}=[Zn(L_{TC}A)]/[Zn][L_{TC}A]$ and $K_{12}=[Zn(L_{TC}A)_2]/[Zn][L_{TC}A]^2$, titrations were performed in the presence of HEDTA and EGTA as competitors. Fitting these titrations with SPECFIT[24] (Figure 4C and D) yielded $K_{11}=10^{9.5(3)}\,M^{-1}$ and $K_{12}=10^{14.4(3)}\,M^{-2}$. Notably, K_{11} is approximately five orders of magnitude lower than that of $Zn{\cdot}L_{TC}$ ($10^{14.7(1)}\,M^{-1}$).[19] A similar behavior was observed for $L_{TC}D$ with K_{11} and K_{12} values of $10^{10.3(4)}\,M^{-1}$ and $10^{17.3(2)}\,M^{-2}$, respectively (see SI).[25] All this shows that the oxidation of a single cysteine to a sulfinate significantly decreases the Zn^{2+} binding affinity of L_{TC} by ca. 4–5 orders of magnitude. It is worth noting that the concentration of free zinc in cells was estimated to be buffered around 10–100 pM.[26,27] By decreasing the affinity of a treble-clef zinc finger from ca. $10^{15}\,M^{-1}$ down to $10^{10}\,M^{-1}$, $^1O_2$ oxidation would lead to Zn^{2+} release and unfolding of the zinc finger domain as a structural consequence.

In conclusion, we have shown that $^1O_2$ interacts exclusively through chemical reaction with the zinc-bound cysteines of $Zn{\cdot}L_{TC}$, a $Zn(Cys)_4$ treble-clef zinc finger model, to form sulfinates with a rate constant $k_r=(4.3\pm0.4)\times10^6\,M^{-1}s^{-1}$, which is competitive to that of the most reactive solvent-exposed tryptophans. Such an oxidation strongly destabilizes the zinc finger site. This study demonstrates that zinc finger proteins can be targets for $^1O_2$. It also shows the possibility that some zinc fingers may be involved in $^1O_2$ sensing, and the zinc finger site of ChrR[6] appears as a likely candidate for such a role. Interestingly, a small zinc finger protein was shown to be required for the induction of $^1O_2$-dependent gene expression and photooxidative stress resistance in several organisms.[28] The reactivity of other zinc fingers is now under investigation.

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