Calmodulin methionine residues are targets for one-electron oxidation by hydroxyl radicals: formation of S∴N three-electron bonded radical complexes

Thomas Nauser,[a] Michael Jacoby,[b] Willem H. Koppenol,[a] Thomas C. Squier[b] and Christian Schöneich[ac]

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The one-electron oxidation of calmodulin, studied on the microsecond timescale by pulse radiolysis, leads to methionine sulfide radical cations, which complex to adjacent amide groups to form three-electron bonded intermediates.

The one-electron (1e) oxidation of organic sulfides and methionine (Met) represents an important reaction mechanism *in vivo*.[1,2] Evidence for a Cu(II)-catalyzed oxidation of Met[35] in the Alzheimer's disease β-amyloid peptide (βAP) was obtained[3] despite unfavourable reduction potentials for the initial 1e-transfer (*cf.* the formal reduction potential of βAP-Cu(II), 0.5–0.55 V,[4] and the anodic peak potential of Met at pH 8.2, 1.26 V *vs.* Ag/AgCl[5]). Based on theoretical studies, Met radical cations were proposed as intermediates.[6] In the structure of βAP, Met radical cation formation may be facilitated by a preexisting close sulfur–oxygen (S–O) interaction between the Met[35] sulfur and the carbonyl oxygen of the peptide bond C-terminal to Ile[31].[7,8] This preexisting S–O bond suggests that the 1e-transfer from Met[35] to Cu(II) is supported through stabilization of the Met radical cation by the electron-rich carbonyl oxygen, generating an S∴O-three-electron-bonded[9] radical cation (Scheme 1, reaction 1).[7]

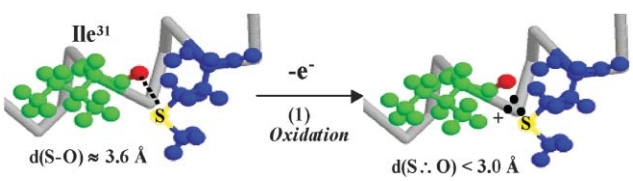
Pulse radiolysis data confirmed the formation and stabilization[10,11] of such S∴O bonded radical cations in model peptides on the microsecond timescale and their pH-dependent conversion into sulfur–nitrogen (S∴N)-bonded sulfide radicals (for a representative example, see Scheme 2, reaction 2).[10,11]

The catalytic role of preexisting S–O (or S–N) interactions may not only be important for the Cu(II)-dependent oxidation and neurotoxicity of β-amyloid peptide, but for the oxidation of Met in proteins, in general. Here we report the first experimental evidence for the formation of Met radical cations during the 1e-oxidation of a protein. We reacted the calcium-saturated forms of wild-type (wt) calmodulin (CaM–Ca_4) and Met-deficient mutant CaM–Ca_4 with pulse radiolytically generated hydroxyl radicals (HO•) [reaction 3] at pH 5, conditions under which the crystal structure of wt CaM–Ca_4 has been recorded[12] and the solvent accessible surface areas of the Met residues are known.[13]

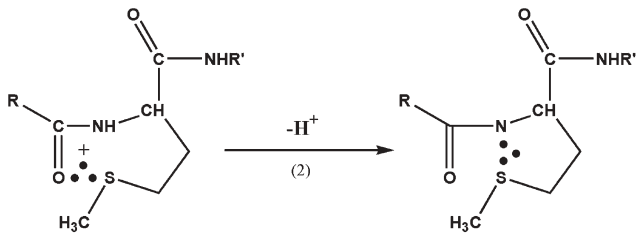
$$HO^{\bullet} + \text{CaM–Ca}_4 \rightarrow \text{products.} \quad (3)$$

Wild-type CaM contains nine Met residues, which become surface-exposed upon the binding of four equivalents calcium[14] and represent *ca.* 46% of the surface area of the hydrophobic patches important for the association of CaM to target proteins.[15] All nine Met residues are located within α-helices with at least one close interaction with a peptide bond carbonyl oxygen and/or amide nitrogen (*vide infra*).

Fig. 1A shows the optical spectrum integrated over 500 ns between 0.6 and 1.1 μs after pulse irradiation[16–18] of an N_2O-saturated aqueous solution, pH 5, containing 6×10^{-5} M wt CaM–Ca_4. The spectrum is characterized by a broad maximum around 390 nm as expected for an (S∴N)-three-electron bonded sulfide radical. For comparison, Fig. 1A shows typical spectra for (S∴O)[11] and (S∴N)[19]-bonded sulfide radicals, recorded for small model sulfides, normalized to fit into the plot. The optical spectrum recorded for wt CaM–Ca_4 shows a close similarity to that characteristic for (S∴N) three-electron bonds. Importantly, no transient 390 nm absorbance was observed upon the reaction of HO• radicals with two Met-deficient CaM mutants, L7–Met[144,145] and L8–Met[144] (Fig. 1A). In these mutants, all Met residues were replaced by Leu, except the fairly unreactive[13] (towards oxidation by H_2O_2[13]) residues Met[144] in L8–Met[144] and Met[144,145] in L7. These experiments show that the transient 390 nm absorbance in the spectrum of wt CaM–Ca_4 is caused by the reaction of HO• with Met. Based on ε_{390}(S∴N) = 4500 M^{-1} cm^{-1},[19] the yield[20] of S∴N-bonded sulfide radicals is calculated as G(S∴N) = 1.2 ± 0.3. Taking $k_3 = 3 \times 10^{10}$ $M^{-1}s^{-1}$,[21–25] we calculate that at 0.85 μs after the pulse (the mean over the integration period between 0.6 and 1.1 μs after the pulse) the reaction of HO• with 6×10^{-5} M wt CaM–Ca_4 is *ca.*



Scheme 1 Assistance of the 1e-oxidation of Met[35] by Ile[31] in βAP.



Scheme 2 pH-dependent rearrangement of S∴O to S∴N bond of Met sulfide radical cation in a linear model peptide.

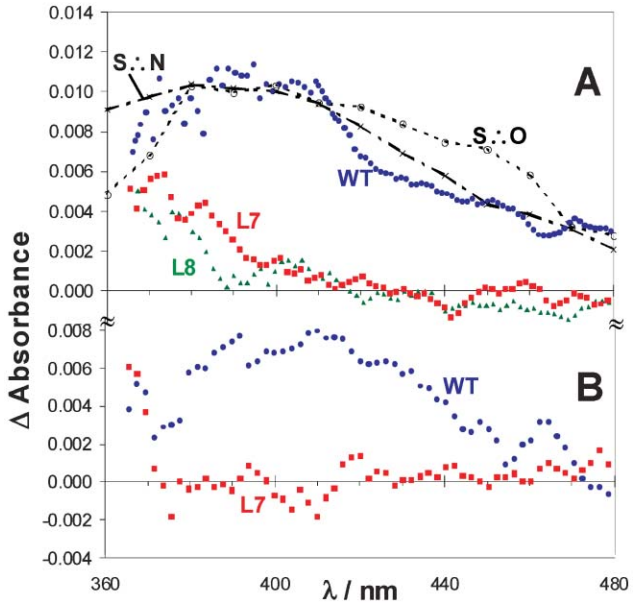
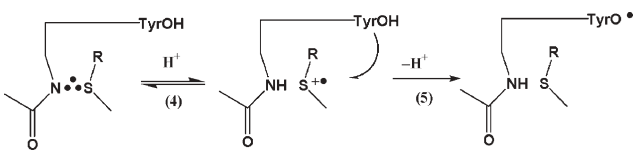


Fig. 1 Optical spectra obtained (A) 0.6–1.1 μs and (B) 5.0–5.5 μs after pulse irradiation (18 Gy) of N_2O-saturated aqueous solutions, pH 5, containing 6×10^{-5} M CaM–Ca_4 (wt and mutants L8–Met^{144}, L7–$Met^{144,145}$).

75% complete. This corresponds to $G = 0.75 \times 5.3 = 4.0$ of $HO^{\bullet}$ reacted with wt-CaM–Ca_4 and an efficiency of 30% for the $HO^{\bullet}$-dependent formation of (S∴N)-bonded complexes. A large fraction of the remainder $HO^{\bullet}$ will likely add to one of the surface-exposed[14] Phe residues, which are present on both wt and mutant CaM, contributing to the significant absorbance at $\lambda < 370$ nm observed for all protein variants.

Fig. 1B shows the optical spectrum recorded between 5 and 5.5 μs after pulse irradiation of an N_2O-saturated aqueous solution, pH 5, containing 6×10^{-5} M wt CaM–Ca_4. At this time after the pulse, the reaction of $HO^{\bullet}$ radicals with CaM–Ca_4 is complete. The absorption maximum is now shifted to 405–415 nm, consistent with a time-dependent formation of tyrosyl radicals.[26] Importantly, pulse irradiation of L7–$Met^{144,145}$ did not generate a 405–415 nm absorption, suggesting that Met sulfide radicals are precursors of tyrosyl radicals (Scheme 3, reactions 4 and 5). This is in accord with data on electron transfer from Tyr to Met radical cation complexes in small model peptides.[26] Based on ε_{405}(tyrosyl radical) = 2600 M^{-1} cm^{-1},[27] the yield of tyrosyl radicals at 5–5.5 μs after the pulse is calculated as $G = 1.65$. This yield represents 31% of the total $HO^{\bullet}$ radical yield ($G_i = 5.3$). Based on a *ca.* 30% yield of S∴N during the reaction of $HO^{\bullet}$ with CaM–Ca_4 (*vide supra*), this final yield of tyrosyl radicals suggests that the conversion of (S∴N)-bonded protein radicals into protein tyrosyl radicals is quantitative.



Scheme 3 Formation of tyrosyl radicals.

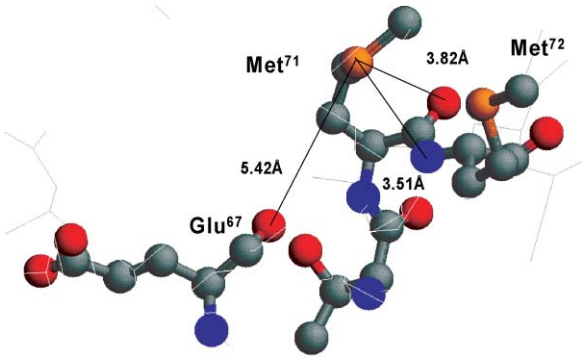


Fig. 2 Representative example of preexisting S–O and S–N interactions of Met^{71} in the crystal structure (PDB file 1CLL)[12] of wt-CaM–Ca_4.

The structure of wt CaM–Ca_4 (PDB file 1CLL)[12] reveals that all Met sulfide atoms are located in close vicinity to at least one peptide bond amide and/or carbonyl function. Fig. 2 shows a representative case for Met^{71} to demonstrate the close vicinity of the Met sulfur atom to its C-terminal peptide bond with a distance of 3.51 Å to the amide nitrogen and 3.82 Å to the amide carbonyl group. Other close interactions exist, for example, between the Met^{51} sulfur atom and the amide nitrogen of the peptide bond C-terminal to Glu^{47} (4.25Å) or the Met^{76} sulfur to the carbonyl group in the peptide bond C-terminal to Met^{72}. These two examples show close distances to functional groups of amino acid residues in position i-4, similar to the case presented for the Ile^{31}–Met^{35} interaction in βAP (*vide supra*). A comprehensive search of the Cambridge Structural Database indicated a high frequency of non-bonded S–O interactions between protein Met sulfides and peptide bond amides.[28] Hence, stabilization of Met sulfide radical cations by amides may be a general phenomenon in proteins.

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Thomas Nauser,[a] Michael Jacoby,[b] Willem H. Koppenol,[a] Thomas C. Squier[b] and Christian Schöneich[ac]
[a]*Laboratory of Inorganic Chemistry, ETH Zürich, Zürich, Switzerland, CH-8093*
[b]*Pacific Northwest National Laboratory, Richland, WA, USA*
[c]*Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS, USA. E-mail: schoneic@ku.edu; Fax: +1 (785) 864 5736; Tel: +1 (785) 864 4880*

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