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Evidence of inter- and intra-molecular crosslinking of tyrosine residues of calmodulin induced by photo-activation of ruthenium(II)

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Tris(2,2′-bipyridyl)ruthenium(II) upon illumination with light at a wavelength of 450 nm in the presence of an electron acceptor induces dityrosine crosslinking in proteins.

Introduction

The ability of metal–ligand complexes (MLC) to participate in electron-transfer reactions finds numerous applications in physics, biophysics, electrochemistry, biology and medicine.[1–4] One of these MLCs, tris(2,2′-bipyridyl)ruthenium(II), or Ru(II), has unique photophysical features. The absorption of light is associated with the transfer of an electron from the ruthenium atom to one of the three bipyridyl groups, and in the excited state the electron can be easily donated to other molecules (electron acceptors). The loss of an electron converts the chemically neutral Ru(II) into Ru(III), which is a strong oxidant able to oxidize the other molecules and, particularly, amino acid residues such as tyrosine and tryptophan in peptides.[5] Recently, it has been reported that, upon illumination with light at a wavelength of 450 nm, Ru(II) could catalyze the crosslinking of proteins.[6,7] One of the possible mechanisms of protein crosslinking could be the formation of a covalent bond between two tyrosine residues (dityrosine formation). To examine this hypothesis we performed the crosslinking of calmodulin induced by photo-activation of Ru(II). Calmodulin has been selected for these experiments because it has only two tyrosine and no tryptophan residues, and dityrosine formation can be monitored by the appearance of specific fluorescence with a maximum at 410 nm.[8,9] In the present work we have demonstrated for the first time that Ru(II) can catalyze dityrosine crosslinking in proteins. Using a CCD camera and interference filters we were able to detect dityrosine fluorescence directly from the polyacrylamide gel.

Materials and methods

Reagents

Tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate, ammonium persulfate (APS), sodium azide (NaN_3), superoxide dismutase from bovine erythrocytes (SOD) and bovine brain calmodulin were purchased from Sigma (St. Louis, MO). Ethyl alcohol (spectrophotometric grade) was from Aldrich (Milwaukee, WI).

Photo-crosslinking reaction

The samples of 100 μl containing 30 μM calmodulin and 0.1 mM $Ru(bpy)_3Cl_2$ and 1 mM APS or 0.3 mM $Ru(bpy)_3Cl_2$ and no APS were placed in a 4 × 4 mm square quartz cuvette and illuminated for 1 or 5 min, respectively, with blue light at a wavelength of 450 nm, which was selected from an arc lamp (PTI A1000 model, PTI, Inc., Canada). The light power at the site of the cuvette was 3 mW cm^{-2}. The buffer solution contained 1 mM $CaCl_2$, 75 mM KCl, 5 mM sodium phosphate (pH 7.5). 0.1 mM $Ru(bpy)_3Cl_2$ and 1 mM APS were added just before illumination. After illumination the Ru(II) and APS were removed by passing calmodulin through a Sephadex G-50 spin column. The crosslinking products were analyzed by SDS polyacrylamide electrophoresis. Bands in the gel containing dityrosine were visualized using a CCD camera C5985 (Hamamatsu, Inc., Japan) under UV illumination through a 335 nm interference filter and emission selection through a 390 nm cut-off filter. After the fluorescence picture had been taken, the gel was stained with Coomassie Blue.

Fluorescence measurements

Fluorescence spectra were recorded using a digital phase-modulation spectrofluorimeter ISS K2 (ISS Inc., Champagne, IL). Emission was excited at 270 or 325 nm. The intensity of calmodulin fluorescence, after treatment and purification in a Sephadex G-50 spin column, was corrected for a dilution factor.

Results and discussion

The main goal of our study is to provide experimental evidence to prove that photo-activated Ru(II) can induce dityrosine formation in proteins. As a model for the study of dityrosine formation we chose calmodulin, since it has only two tyrosine residues, which could form inter- and intra-molecular crosslinks, as has been demonstrated by other methods.[8,9] Dityrosine crosslinking can be monitored by the appearance of specific fluorescence with a maximum at 410 nm and an excitation maximum at 325 nm.[10–12] Any possibility of a contribution from tryptophan residues to the fluorescence can be excluded since calmodulin has no tryptophan residues. The intrinsic fluorescence spectra of calmodulin (λ_{ex} = 270 nm) before and after illumination in the presence of Ru(II) and APS, followed by gel filtration of protein, are presented in Fig. 1.

Calmodulin, excited at 325 nm, did not fluoresce in the range 360–480 nm, whereas after photoreaction with Ru(II), the typical dityrosine fluorescence appeared (Fig. 2). The presence of APS and Ru(II) without illumination or illumination of calmodulin alone at 450 nm wavelength caused no changes in protein fluorescence.

The products of the photoreaction of calmodulin with Ru(II) were analyzed by SDS polyacrylamide gel electrophoresis. The

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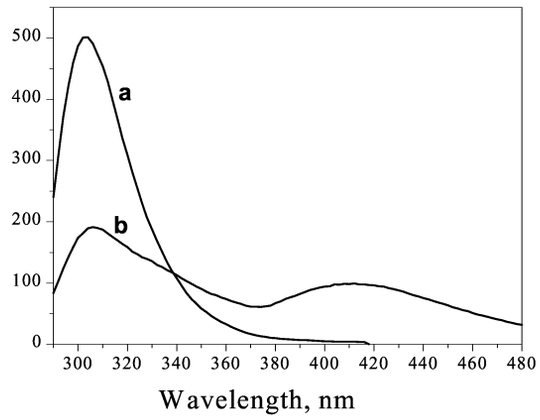

Fig. 1 Steady-state fluorescence spectra of calmodulin before (a) and after (b) exposure for 1 min to blue light (450 nm) in the presence of 0.1 mM Ru(II) and 1 mM APS followed by gel filtration in a Sephadex G-50 spin column [to remove Ru(II) and APS]. The excitation wavelength was 270 nm.

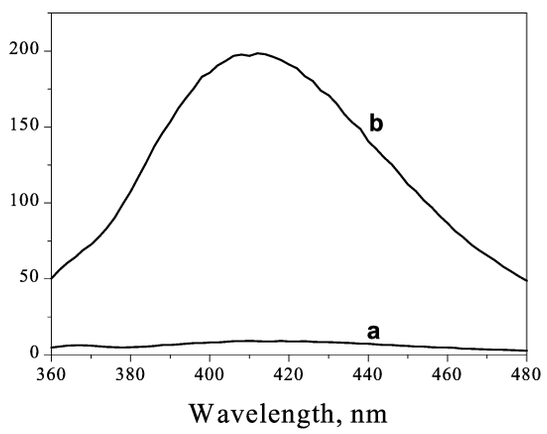

Fig. 2 Steady-state fluorescence spectra of calmodulin before (a) and after (b) exposure for 1 min to blue light (450 nm) in the presence of 0.1 mM Ru(II) and 1 mM APS followed by gel filtration in a Sephadex G-50 spin column [to remove Ru(II) and APS]. The excitation wavelength was 325 nm.

fluorescence and Coomassie Blue staining images of the same gel are shown in Fig. 3. The untreated calmodulin migrated as a single band with an apparent molecular weight of about 17 kDa; however, some minor contamination by S-100 protein was also seen (Fig. 3, A, lane 1). Ru(II), which migrates much faster than calmodulin peptides, was allowed to come out from the gel before electrophoresis was stopped, so that it would not interfere with the dityrosine fluorescence in the gel. The appearance of fluorescence in the monomers, dimers and oligomers after photoreaction with Ru(II) indicated the occurrence of inter- and intramolecular crosslinking of the tyrosine residues of calmodulin.

APS is known to act as an electron acceptor to generate Ru(III) from photoexcited Ru(II)*.[13,14]

$$\mathrm{Ru(II)^*} + \mathrm{APS} \longrightarrow \mathrm{Ru(III)} + \mathrm{SO_4^{2-}} + \mathrm{SO_4^{-\bullet}} \qquad (1)$$

The transition from Ru(II) to Ru(III) could be easily monitored because it is accompanied by a change in the color of the solution (from orange to green) due to the difference in the absorption spectra of the two compounds. In contrast to Ru(II) the Ru(III) is a non-fluorescent compound. To study the possible role of sulfate radicals we carried out the reaction in the presence of 0.5% ethanol, which is known to act as an efficient quencher of the sulfate radical.[15] We did not observe any significant differences in the degree of dityrosine fluorescence in

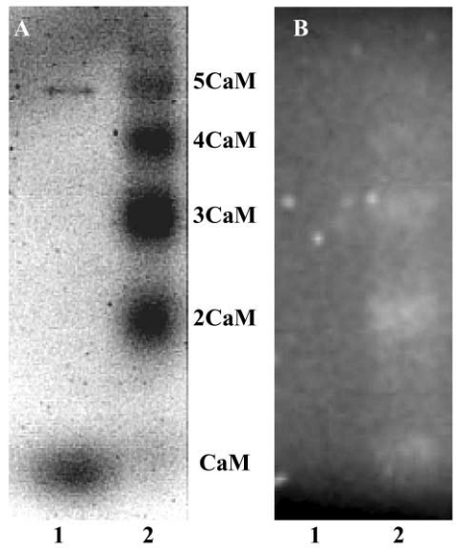

Fig. 3 Coomassie Blue (A) and fluorescence (B) images of the gel. Lanes: 1, calmodulin; 2, calmodulin treated with Ru(II) and light. Monomers, dimers and higher orders of oligomers are denoted as CaM, 2CaM, 3CaM, *etc.*

the absence or presence of ethanol (data not shown). Therefore, we concluded that APS serves as a cofactor in the crosslinking reaction, and the sulfate radical does not play a critical role in dityrosine formation.

The formation of dityrosine also occurred in the absence of APS; however, the yield of crosslinking product was low. In the absence of APS, molecular oxygen can play the role of electron acceptor.[16–18] The quenching of Ru(II)* by O_2 can occur by two mechanisms:

$$\mathrm{Ru(II)^*} + \mathrm{O_2} \longrightarrow \mathrm{Ru(II)} + \mathrm{O_2(^1\Delta_g)} \qquad (2)$$

$$\mathrm{Ru(II)^*} + \mathrm{O_2} \longrightarrow [\mathrm{Ru(III)} \cdots \mathrm{O_2^{\bullet -}}] \qquad (3a)$$

$$[\mathrm{Ru(III)} \cdots \mathrm{O_2^{\bullet -}}] \longrightarrow \mathrm{Ru(III)} + \mathrm{O_2^{\bullet -}} \qquad (3b)$$

The first mechanism [eqn. (2)] is an energy-transfer quenching that generates the ground state Ru(II) and singlet oxygen. The second one involves charge transfer to form a cage complex [Ru(III) $\cdots$ $O_2^{\bullet -}$] in the first stage [eqn. (3a)] and then formation of Ru(III) and the superoxide radical anion [eqn. (3b)]. In as much as $O_2(^1\Delta_g)$ lies 0.98 eV above the triplet ground state, and the 0–0 transition energy of the $Ru(bpy)_3$ is 2.15 eV, it is thermodynamically possible for Ru(II) to react with O_2 and generate $O_2(^1\Delta_g)$ by reaction (2).[17] On the other hand, the value of $E^\circ(O_2/O_2^{\bullet -})$ is -0.16 V (in aqueous solution), whereas the value of E°[Ru(III)/Ru(II)*] is -0.88 V, therefore the pathway represented by eqns. (3a) and (3b) is also thermodynamically possible.[17] Previously it was shown that neither singlet oxygen nor the superoxide radical is involved in the formation of dityrosine.[19] To verify this we investigated the effect on dityrosine formation of sodium azide (NaN_3) and superoxide dismutase (SOD), which are known to be good scavengers of singlet oxygen and superoxide, respectively. We found no effect of NaN_3 or SOD on the formation of dityrosine in the presence of APS (Fig. 4), it follows that reaction (1) is the predominant one under these conditions. In the absence of APS the SOD increased production of dityrosine by 20%, while NaN_3 had no effect on the reaction (Fig. 4). The enhancement of dityrosine production by SOD could be explained by a decrease in the of rate of recombination of superoxide with Ru(III) and transformation of Ru(III) to Ru(II) and superoxide to O_2. SOD increases the Ru(III) concentration and therefore accelerates the dityrosine production. Ru(III) is a strong oxidant capable of

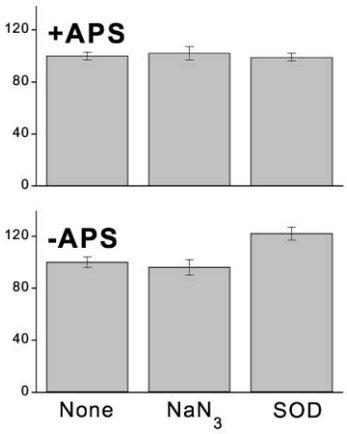

Fig. 4 Effect of additives (1 mM NaN_3 and 1.5 μM SOD) on the Ru(II)/light-mediated formation of dityrosine crosslinks in calmodulin. The numbers on the axes represent percent of area under the spectrum of dityrosine fluorescence relative to that in the absence of NaN_3 and SOD.

oxidizing amino acids, including tyrosine residues. The formation of tyrosine radicals is followed by dityrosine crosslinking.

We have shown for the first time that Ru(II) can induce dityrosine formation in proteins upon blue-light illumination. The results presented provide insights into the mechanism of photooxidation of proteins by ruthenium compounds. The ability of ruthenium compounds under light illumination to oxidize tyrosine residues and induce dityrosine crosslinking of calmodulin might have a practical application in the inhibition of calmodulin-mediated signal transduction in cancer cells. Our preliminary data indicated that, upon light illumination, Ru(II) can inhibit the activity of the matrix metalloproteinases, which have tyrosine residues in the active site.[20] We have also demonstrated the feasibility of the detection of protein dityrosine crosslinks directly in the polyacrylamide gel by fluorescence methods.

Acknowledgements

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