Protein Photocleavage

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Site-Selective Photocleavage of Proteins by Uranyl Ions**

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Dedicated to Professor J. C. Scaiano on the occasion of his 60th birthday

The uranyl ion (UO_2^{2+}) has a high affinity for the phosphate groups of DNA/RNA,[1] and this affinity was exploited to photocleave these biopolymers at the uranyl-binding sites.[2–5] Earlier success in our laboratory with site-specific photocleavage of proteins by organic molecules[6] and metal complexes[7] led to the idea that photoactive[8] metal–oxo species such as UO_2^{2+} should be capable of cleaving proteins with high selectivity. However, even though there is ample evidence to suggest that UO_2^{2+} binds to proteins,[9,10] it is not known if UO_2^{2+} can photocleave the peptide backbone. It is also not known if binding of UO_2^{2+} at specific sites would result in high selectivity for the protein cleavage. In addition, the ease with which UO_2^{2+} can be directed to metal-binding sites on proteins and the activation of the cation at visible wavelengths indicated that this would be a promising approach for footprinting metal-binding sites. In this report, high-affinity binding of UO_2^{2+} to proteins and their site-selective photocleavage, with visible light, is demonstrated for the first time.

Serum albumin (SA) and transferrin are known to bind UO_2^{2+},[11,12] and these proteins therefore provided good initial targets to test the above approach. Quantitative data on the binding of UO_2^{2+} to bovine SA (BSA), however, were lacking; these were obtained from calorimetric and spectroscopic studies. Isothermal titration calorimetry of the addition of a solution of UO_2^{2+} (0.5 mM) to BSA (0.1 mM; see Supporting Information) indicated exothermic binding (Figure 1). Best fits to the data required a two-site binding model[13] and indicated a high-affinity site (binding constant (K_b) of $(1.6 \pm 0.7) \times 10^7\,M^{-1}$) with moderate exothermicity (binding enthalpy (ΔH) of (-4.7 ± 0.7) kcal mol^{-1}) and a low-affinity site ($K_b = 2.8 \pm 2.5 \times 10^5\,M^{-1}$) with weak exothermicity ($\Delta H = -(1.2 \pm 0.8)$ kcal mol^{-1}).

Binding of UO_2^{2+} to BSA was also supported by absorption, circular dichroism (CD), and fluorescence studies. The CD spectra, for example, of a mixture of BSA (1 mM) and UO_2^{2+} in increasing concentrations (0–0.8 mM) showed the growth of new bands at 440 and 510 nm (Figure 2). These evidences established the binding of UO_2^{2+} to BSA.

Photocleavage of BSA by UO_2^{2+} was achieved by irradiating a mixture of BSA and UO_2^{2+} at 420 nm (Figure 3). Three distinct product bands at 35, 33 and 30 kDa were obtained in yields (11 ± 0.5), (4 ± 0.6), and (11 ± 1) % (lane 5), respectively. No photocleavage was noted without UO_2^{2+} (lane 3) or without light (lane 4). Photocleavage was observed at wavelengths up to 460 nm, although the yields were lower (data not shown).

To demonstrate the general nature of the photocleavage, other proteins such as human SA (HSA), porcine SA (PSA), glucose oxidase, and transferrin were also tested. These are cleaved with high selectivity (see Supporting Information). As negative controls, we also tested the photoreactivity with proteins such as α-lactalbumin, hemoglobin, and myoglobin, which do not bind the uranyl ion. These latter proteins are not cleaved by UO_2^{2+}, a result that is consistent with our expectations. Therefore, binding is essential for the cleavage

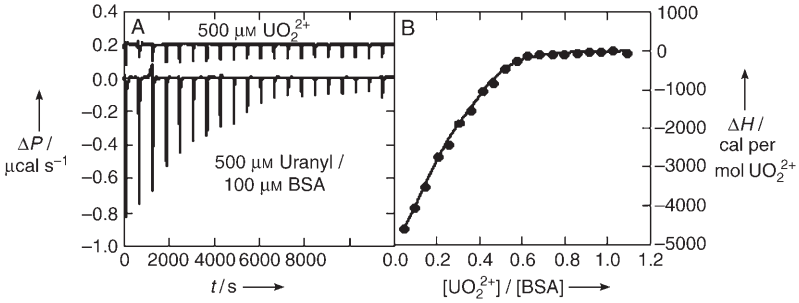

Figure 1. A) Exothermic binding of UO_2^{2+} to BSA (in water, pH 5.5); B) a two-site-binding fit to the data with binding constants of $1.6 \times 10^7\,M^{-1}$ and $2.8 \times 10^5\,M^{-1}$. ΔP = differential power.

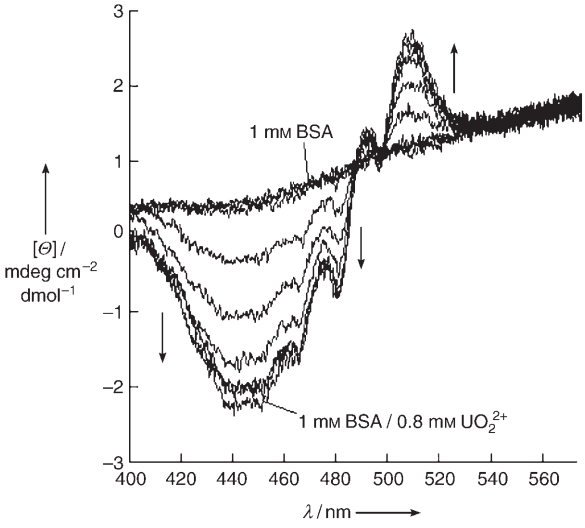

Figure 2. Circular dichroism spectra of BSA (1 mM) with increasing concentrations of UO_2^{2+} (0–0.8 mM).

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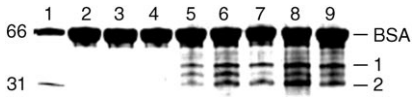

Figure 3. Visible-light induced cleavage of BSA (15 μM) by UO_2^{2+} (100 μM, 420 nm, 0.75 h, lane 5). Lane 1 contained molecular-weight markers (kDa), and all other lanes contained BSA. Lanes 4–9 contained UO_2^{2+}, lane 3 was the light control without UO_2^{2+}, and lane 4 was the dark control with UO_2^{2+}. The sample in lane 6 (100 μM UO_2^{2+}) was irradiated for 1.5 h. Lane 7 is similar to lane 5 but an additional portion of UO_2^{2+} (100 μM) was added after 0.75 h of irradiation. Lane 8 is similar to lane 7 except that the sample was irradiated for an additional 0.75 h after the second addition of UO_2^{2+}. The sample in lane 9 contained 200 μM UO_2^{2+} and was irradiated for 0.75 h. Labels 1 and 2 indicate the 35- and 30-kDa bands, respectively.

and free UO_2^{2+} is unlikely to be responsible for the observed photocleavage.

The reaction mixture was examined after photolysis to ascertain whether the uranyl ion is consumed during the reaction. The protein–uranyl complex retained its characteristic CD peaks, shown in Figure 2, even after the photoreaction; hence, the reaction is photocatalytic. This is consistent with the regeneration of the uranyl ion in its photoreactions with organic substrates.[14] The UV CD spectra of the BSA/uranyl mixture were also examined before and after irradiation (see Supporting Information). There was a decrease of only 8% in the 220-nm band between these spectra, a result indicating that the secondary structure of the protein is largely preserved. Even so, the product formation reached a maximum after around 0.75 h. Addition of fresh UO_2^{2+} to the reaction mixture and continued irradiation improved product yields (Figure 3, lane 8, yields of 20, 14, and 20%, respectively), but similar yields were also obtained when the same total concentration of UO_2^{2+} was used initially (lane 9, yields of 14, 9, and 14%, respectively).

To test the presence of photochemical intermediates in these reactions, quenching studies were carried out. Addition of 2-propanol quenched the reaction with a quenching constant of $0.35\,M^{-1}$. Since 2-propanol is known to quench the excited state of UO_2^{2+} at a rate constant (k_q) of $1.76\times10^9\,M^{-1}s^{-1}$,[15] interception of the excited state of free UO_2^{2+} (lifetime = 1.4 μs)[16] should have resulted in quenching constants greater than $10^3\,M^{-1}$ ($k_q\times$ lifetime). The much smaller value observed here ($0.35\,M^{-1}$) indicates that the cleavage results from UO_2^{2+} bound to the protein and not from free UO_2^{2+}.

In order to identify the cleavage site on BSA, amino acid sequencing of the photofragments was carried out (see Supporting Information). N-terminal sequencing of the 35-kDa fragment indicated the residues Asp-Thr-His-Lys-Ser, which match with the known N-terminal residues of BSA. Sequencing of the 30-kDa fragment indicated residues Lys-Asn-Tyr-Gln, which are internal to BSA. Thus, the newly formed N terminus is sequencible, and this is useful in the identification of the cleavage site. From the known primary sequence of BSA and the above sequencing data, we conclude that BSA is cleaved at Val314–Cys315.

The minor 33-kDa band (lane 5, Figure 3), which increases in intensity with extended irradiation time, is too small in yield to be sequenced, and this band is likely to be the secondary photoproduct from the 35-kDa fragment. The 2-kDa band, from the secondary cleavage of the 35-kDa band, is probably eluted out of the gel. If the 33-kDa band were the result of direct cleavage of BSA, then the corresponding 32-kDa band would be present; however, this is not observed.

To test whether UO_2^{2+}-induced photocleavage is useful for mapping metal-binding sites on BSA, the photocleavage was carried out in the presence of Ca^{II}, Zn^{II}, Cd^{II}, or Ni^{II} (2 mM metal ion). Only Ni^{II} completely inhibited the formation of the 35-, 33-, and 30-kDa bands, while Zn^{II} showed modest reductions in the product formation (see Supporting Information). This inhibition of photocleavage is not due to increased ionic strength because addition of 100 mM $NaNO_3$ to the reaction mixture had no effect on the yield. In addition, CD studies show that addition of Ni^{II} or Zn^{II} substantially reduced the intensities of the 440- and 510-nm CD bands of the UO_2^{2+}/BSA complex. Inhibition, therefore, is probably due to competitive binding of Ni^{II} or Zn^{II} to the metal-binding site or due to allosteric interactions across the protein. CD studies also show that UO_2^{2+} does not bind at the primary Cu^{II} site[17] on BSA.

From the above data and from the known 3D structure of HSA,[18] as a model for BSA, it is proposed that UO_2^{2+} binds to the cluster of ligands Asp311, Asp313, Cys315–disulfide, and His366. Among these, Asp311 and His366 appear to be essential for the photoreaction. For example, Asp311 of BSA is replaced by serine in HSA and this protein is not cleaved by UO_2^{2+} at Val314–Cys315. Similarly, His366 in BSA is replaced by Phe in PSA and this protein is also not cleaved by UO_2^{2+} at this site (see Supporting Information). The lack of cleavage of HSA and PSA at these sites strongly suggests that Asp311 and His366 in BSA are important for the observed photocleavage.

The protein cleavage most likely proceeds through steps which are similar to those proposed for DNA cleavage[1] and the photoxidation of hydrocarbons by the uranyl ion.[14] In our case, hydrogen-atom extraction from the Val314 side chain by the uranyl ion in the excited state is a strong possibility.[14,16] The reaction of the resulting alkyl radical with oxygen could lead to peptide-bond cleavage, as suggested earlier.[6,7] Further studies are in progress to consider these details.

In conclusion, UO_2^{2+} photocleaves a number of proteins, with high selectivity, and the primary cleavage site on BSA is Val314–Cys315. Ni^{II}, and to a lesser extent Zn^{II}, inhibit binding of UO_2^{2+} and the subsequent photocleavage. The binding site involved is likely to be the secondary Ni^{II} site on BSA and not the N-terminal site.[19] This is the first demonstration of protein photocleavage by a metal–oxo species with visible light. Photochemical footprinting with UO_2^{2+} is a promising method for investigating cohabitating metal-binding sites on proteins and biological interfaces.

Experimental Section

A VP-ITC isothermal titration calorimeter (MicroCal, Northampton, MA) was used to inject 20 portions of 500 μM UO_2^{2+} (14 μL) into 100 μM BSA at 15 min intervals (25°C, pH 5.5). The resulting data were fitted by using the Origin software (MicroCal, Northampton, MA). For the photoreactions, mixtures of 15 μM protein (see

Supporting Information) and 200 μM $UO_2(NO_3)_2$ were prepared in 0.5–5 μM $NaNO_3$ (100 μL, pH 5.5) and irradiated at 420 nm for 0.75 h, and the products separated by 9% sodium dodecylsulfate PAGE as described previously.[6] The gels were stained by Coomassie Brilliant Blue, and the product bands were quantified by using NIH Image v1.1.6 software (NIH, U.S.A).

Additional information on the photocleavage of other proteins by UO_2^{2+}, BSA/UO_2^{2+} CD spectra before and after irradiation, and the cleavage inhibition by Ni^{II} and Zn^{II} are available in the Supporting Information.

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[1] P. E. Nielsen, *Methods Mol. Biol.* **2001**, *148*, 111–119.
[2] S. M. Fonseca, H. D. Burrows, M. G. Miguel, M. Sarakha, M. Bolte, *Photochem. Photobiol. Sci.* **2004**, *3*, 317–321.
[3] G. S. Bassi, N. E. Mollegaard, A. I. H. Murchie, D. M. J. Lilley, *Biochemistry* **1999**, *38*, 3345–3354.
[4] P. E. Nielsen, C. Jeppesen, O. Buchardt, *FEBS Lett.* **1988**, *235*, 122–124.
[5] D. Wittberger, C. Berens, C. Hammann, E. Westhof, R. Schroeder, *J. Mol. Biol.* **2000**, *300*, 339–352.
[6] C. V. Kumar, A. Buranaprapuk, *Angew. Chem.* **1997**, *109*, 2175–2177; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 2085–2087.
[7] C. V. Kumar, J. Thota, *Inorg. Chem.* **2005**, *44*, 825–827.
[8] a) A. B. Yusov, V. P. Shilov, *Russ. Chem. Bull.* **2000**, *49*, 1925–1953; b) A. L. Buchachenko, I. V. Khudyakov, *Russ. Chem. Rev.* **1991**, *60*, 555–567.
[9] a) C. Monteilhet, C. Zelwer, J. L. Risler, *FEBS Lett.* **1974**, *46*, 101–105; b) J. Wang, R. S. Houk, D. Dreessen, D. R. Wiederin, *J. Am. Chem. Soc.* **1998**, *120*, 5793–5799.
[10] a) F. Takusagawa, S. Kamitori, S. Misaki, G. D. Markham, *J. Biol. Chem.* **1996**, *271*, 136–147; b) G. M. Ananyev, A. Murphy, Y. Abe, G. C. Dismukes, *Biochemistry* **1999**, *38*, 7200–7209.
[11] a) J. A. Muntz, E. S. G. Barron in *Toxicology of Uranium* (Ed.: A. E. Tannenbaum), McGraw-Hill, New York, **1951**, pp. 182–198.
[12] R. G. Reed in *Transport by Proteins* (Eds.: G. Blauer, H. Sund), de Gruyter, New York, **1978**, pp. 57–78.
[13] J. Tellinghuisen, *Anal. Biochem.* **2003**, *321*, 79–88.
[14] W.-D. Wang, A. Bakac, J. H. Espenson, *Inorg. Chem.* **1995**, *34*, 6034–6039.
[15] G. Sergeeva, A. Chibisov, L. Levshin, A. Karyakin, *J. Chem. Soc. Chem. Commun.* **1974**, 159–160.
[16] S. J. Formosinho, H. D. Burrows, M. G. Miguel, M. E. D. G. Azenha, I. M. Saraiva, A. C. D. N. Ribeirio, I. V. Khudyakov, R. G. Gasanov, M. Bolte, M. Sarakha, *Photochem. Photobiol. Sci.* **2003**, *2*, 569–575.
[17] CD studies with a mixture of Cu^{II} (2 mM) and BSA (1 mM) show that the CD bands that are assigned to Cu^{II} bound to the N terminus (a positive peak at 475 nm and a negative peak at 556 nm) are not diminished by the presence of UO_2^{2+} (2.2 mM).
[18] S. Sugio, A. Kashima, S. Mochizuki, M. Noda, K. Kobayashi, *Protein Eng.* **1999**, *12*, 439–446.
[19] W. Bal, J. Christodoulou, P. J. Sadler, A. Tucker, *J. Inorg. Biochem.* **1998**, *70*, 33–39.

