COMMUNICATION

Surface enhanced vibrational spectroscopic evidence for an alternative DNA-independent redox activation of endonuclease III†

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Surface enhanced vibrational spectro-electrochemistry of endonuclease III provides direct evidence that the [4Fe–4S] cluster is responsible for the enzyme redox activity, and that this process is not exclusively DNA-mediated, as currently proposed. We report the first surface enhanced resonance Raman spectrum of a $[4Fe-4S]^{2+}$ cluster containing enzyme.

Deinococcus radiodurans is an extremely radiation and desiccation resistant bacterium, which can withstand 200-fold higher irradiation doses than other bacteria.[1,2] The resistance mechanism is not known, but an efficient DNA repair machinery is considered to play a key role in it. Endonuclease III (EndoIII) is a [4Fe–4S] cluster containing DNA repair enzyme from the helix–hairpin–helix family of DNA glycosylases, crucial for removal of oxidation damaged bases in DNA.[3,4] Early evidence indicated that Fe-S clusters in these enzymes are not amenable to oxidation or reduction in solution and suggested structural or regulatory roles of the cofactor.[5,6] More recent electrochemical studies of glycosylases immobilised on DNA-modified electrodes indicated that the [4Fe–4S] cluster is activated towards oxidation upon binding to DNA. It was proposed that the cluster exerts a function in DNA-mediated signalling for detection of DNA lesions,[7–12] but based on data obtained by electrochemical approaches, which cannot provide any information about the molecular origin of the electrochemical signal. Here we employed surface enhanced resonance Raman (SERR) spectroscopy and surfaced enhanced IR absorption (SEIRA) spectro-electrochemistry to demonstrate that the cluster of the immobilised EndoIII from *D. radiodurans*

(*Dr*EndoIII) is prone to reduction, which is not necessarily DNA-mediated.

*Dr*EndoIII was electronically coupled to metal electrodes modified with bifunctional alkanethiol-based self assembled monolayers (SAMs).[13] The cluster integrity of *Dr*EndoIII immobilised on the 11-mercapto-undecanoic acid (MUA)-coated nanostructured Ag working electrode was probed by SERR spectroscopy. For *Dr*EndoIII, which has a broad electronic absorption band centered at 410 nm, both the plasmonic and the resonance enhancement conditions are well matched using 413 nm excitation.[14] The negatively charged MUA surface readily interacts with the highly positively charged DNA binding site of *Dr*EndoIII (*vide infra*) that includes the active site pocket and the [4Fe–4S] cluster (Fig. S1, ESI†), which is in glycosylases optimised for a fast and precise accommodation of the negatively charged DNA substrate.[4,15–17]

Upon protein immobilisation and removal of the loosely bound molecules by rinsing with buffer, the functionalised flat disk Ag electrode was inserted into a pre-cooled cryostat that was rapidly cooled to 77 K. The SERR spectrum of the Ag–MUA–*Dr*EndoIII construct was measured at 77 K since the intrinsically weak RR bands of Fe–S clusters can only be detected at low temperatures.[18,19] The spectrum shows well defined modes at 337, 363 and 384 cm^{-1} (Fig. 1a), indicative of a [4Fe–4S] cluster in the +2 oxidation state, which in proteins can change either to +1 or +3 state as in, *e.g.* ferredoxins and HiPIPs, respectively. It closely resembles the RR spectral pattern observed in frozen *Dr*EndoIII solution (Fig. 1b), which reveals bands at 337 cm^{-1}, 384 cm^{-1} and 390 cm^{-1} (Fe–S stretching involving the bridging S) and at 359 cm^{-1} and 366 cm^{-1} (Fe–S stretching involving the terminal S), demonstrating the structural integrity of the cofactor upon enzyme immobilisation. The electrode construct was afterwards brought to ambient temperature, reduced chemically with 1 mM sodium dithionite, rinsed with buffer, frozen again and measured by SERR spectroscopy. No features of the $[4Fe-4S]^{2+}$ (or $[3Fe-4S]^{+}$) cluster were observed in the spectra (Fig. 1c), indicating either its reduction to a RR-silent state which lacks resonance enhancement at 413 nm excitation or degradation/desorption.

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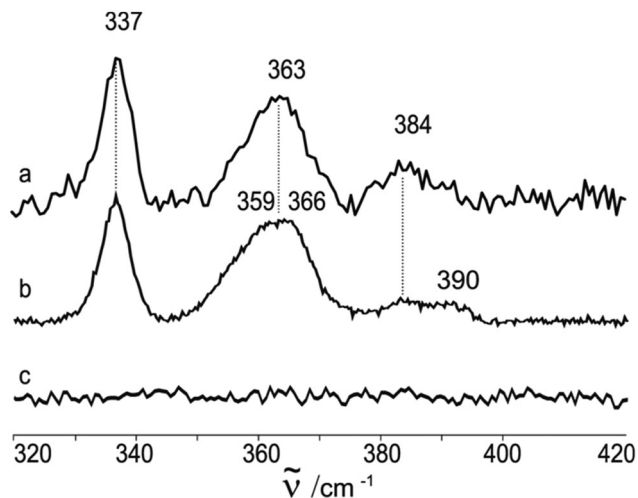
Fig. 1 SERR and RR spectra of *Dr*EndoIII. (a) SERR spectrum of *Dr*EndoIII immobilised on MUA, upon (c) reduction with sodium dithionite; (b) RR spectrum of 0.5 mM resting state *Dr*EndoIII. Spectra are measured with 413 nm excitation at 77 K using 1.5 mW and 8 mW in the SERR and RR experiment, respectively.

Subsequent cyclic voltammetry (CV) measurements of the same RR-silent Ag–MUA–*Dr*EndoIII construct at ambient temperature revealed that the protein was still bound to the electrode surface and redox active, with transition at $E^{0\prime} \sim 10$ mV (Fig. S2, ESI†). The redox transition occurs at the same potential as in the case of *Dr*EndoIII immobilised on MUA coated Au electrodes (Fig. 2), which reveals an apparent redox potential, $E^{0\prime}$, of 20 ± 10 mV and an electron transfer (ET) rate constant, k_{ET}, of 3.1 ± 0.4 s^{-1}, demonstrating that the [4Fe–4S] cluster of the protein which was exposed to the laser beam in SERR experiment, was in the reduced, native, RR-silent state. Our data therefore suggest that the Fe–S cluster in immobilised *Dr*EndoIII is prone to reduction, and that voltammetric signals can be ascribed to the [4Fe–4S]$^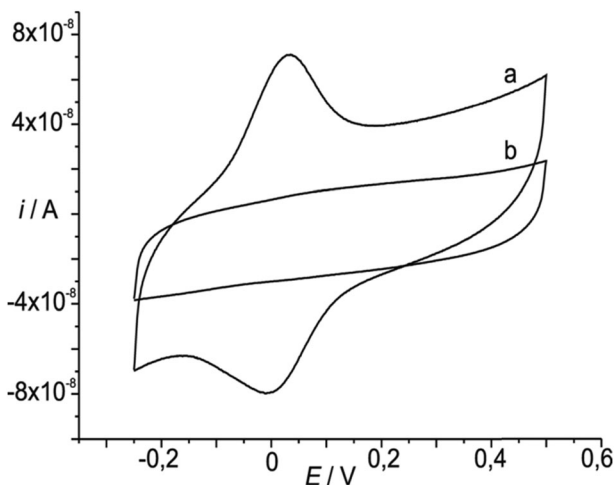{1+/2+}$, rather than to the [4Fe–4S]$^{2+/3+}$ couple as currently proposed.[7–12] In a control experiment *Dr*EndoIII was immobilised on Au electrodes modified with 15 base pair double-stranded DNA (15 bp dsDNA) terminated SAMs, previously employed in electrochemical studies of other DNA glycosylases.[7–11] The redox properties of *Dr*EndoIII on DNA-terminated SAMs ($E^{0\prime} \sim 10$ mV, $k_{ET} \sim 1.4$ s^{-1}) (Fig. S3, ESI†) are comparable with those on MUA. Moreover, they are consistent with data reported for *Ec*EndoIII, MutY and *Af*UDG glycosylases immobilised on DNA-modified Au electrodes ($E^{0\prime}$ in 50–100 mV range, k_{ET} in 1–10 s^{-1} range)[7–11] and *Ec*EndoIII attached to DNA-functionalised graphite electrodes ($E^{0\prime} = 20 \pm 10$ mV).[12] No CV signals were observed when *Dr*EndoIII was immobilised on Ni^{2+}-NTA SAMs *via* a His tag introduced at the N-terminus (Fig. S4, ESI†).[20,21]

The orientation of *Dr*EndoIII with respect to the electrode coated with the employed SAM was probed by ATR-SEIRA spectroelectrochemistry.[14,20–22] *Dr*EndoIII was adsorbed on functionalised gold film deposited on silicon crystals, which at the same time serves as a working electrode and an amplifier of the spectroscopic signal of the immobilised protein molecules.[14,22] The spectra indicate that *Dr*EndoIII adsorbs on MUA, 15 bp dsDNA and Ni^{2+}-NTA terminated SAMs, adopting distinct orientations relative to the electrode surface (Fig. 3).[14,18,22] This is concluded from the variations of the intensity ratios of the amide I ($\sim$1650 cm^{-1}) and amide II ($\sim$1550 cm^{-1}) bands. A relatively low $I_{amide\ I}/I_{amide\ II}$ ratio (1.06) observed on carboxyl- and DNA-terminated SAMs corresponds to a predominantly parallel alignment of *Dr*EndoIII helices with respect to the
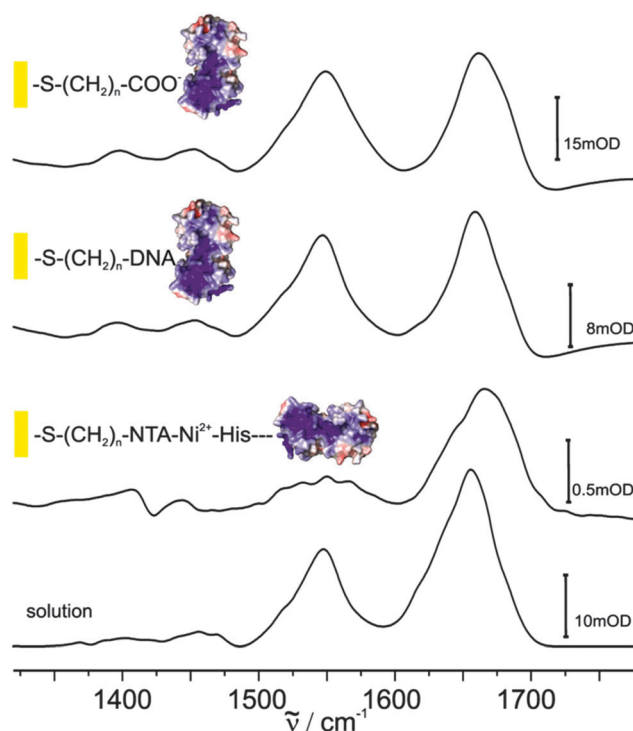

Fig. 2 CV of *Dr*EndoIII immobilized on MUA (v_{scan} 50 mV s^{-1}, Ag/AgCl reference and Pt counter electrode) (a) and a control MUA coated Au electrode (b).

Fig. 3 SEIRA spectra of *Dr*EndoIII immobilized under different orientations. The transmission FTIR spectrum of 0.7 mM protein in solution is shown for comparison.

electrode surface, suggesting that the SAMs interact with the highly positively charged DNA binding site of the enzyme. The high ratio (3.6) determined for Ni^{2+}-NTA SAM indicates that the α-helices, and thus the long axis of the protein, are largely oriented perpendicular to the electrode surface, corresponding to a close proximity of the cluster to the electrode and the DNA binding cleft exposed to the solvent. The FTIR spectrum of the enzyme in solution, measured in transmission mode (Fig. 3) reflects a random orientation distribution of the protein molecules ($I_{amide\,I}/I_{amide\,II}$ = 1.57). Taken together, CV and SEIRA results demonstrate that favourable orientation of the protein with respect to the electrode, which is achieved only for DNA and MUA SAMs, is crucial for heterogeneous ET in DrEndoIII. Moreover, orientation, $E^{0\prime}$ and ET rate constants are all similar for DrEndoIII immobilised on DNA and COOH modified electrodes, indicating that heterogeneous ET in EndoIIIs is not necessarily DNA-mediated. Other negatively charged molecules can induce redox activity of the [4Fe–4S] cluster in the immobilised enzyme.

We further tested if the presence of DNA can induce the redox activity of DrEndoIII immobilised on NTA, which leaves the DNA binding cleft exposed to the solvent (Fig. 3). An excess of normal or damaged 15 bp dsDNA was added to the SEIRA spectroelectrochemical cell accommodating immobilised DrEndoIII. SEIRA bands of the protein immobilised on Ni^{2+}-NTA undergo significant changes upon addition of DNA (Fig. S5, ESI†): the amide I downshifts from 1666 cm^{-1} to 1660 cm^{-1}, accompanied by an intensity decrease and the appearance of an additional component at 1641 cm^{-1}. Further bands at high frequencies can be assigned to C–H and N–H vibrations originating from the DNA duplex, indicating its binding to the protein. No CV signals were observed upon addition of undamaged or damaged DNA (data not shown), indicating that the presence of DNA alone is not sufficient for redox activation of immobilised DrEndoIII, as currently believed.[7–12] The orientation of the enzyme relative to the electrode surface (or the physiological redox partner) has to be correct, which ensures the directionality of ET and thus explains the lack of redox activation in solution even in the presence of DNA.[8,9,18] Addition of damaged or undamaged DNA had no effect on redox activity of DrEndoIII immobilised on MUA SAMs, since the electrode surface docking engages the DNA binding site and makes it unavailable for DNA.

In conclusion, we show that redox activation of DrEndoIII does not rely exclusively on DNA binding. We propose that in vivo the FeS cluster may be redox-activated by other charged species (e.g. proteins or ROS) and take part in cellular processes other than DNA damage search and identification. Clearly, further studies are required to test this hypothesis. The obtained insights will have a general impact on the understanding of the role of [4Fe–4S] clusters in DNA glycosylases, including the central enzyme for repair of ROS damage in humans, hNTH1, for which DrEndoIII represents a bacterial homologue. The first SERR spectroscopic characterisation of a Fe–S containing enzyme immobilized on a biocompatible surface in its native state that we report here will open new possibilities for studies of similar systems.

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