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Spectroelectrochemical insights into structural and redox properties of immobilized endonuclease III and its catalytically inactive mutant

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ABSTRACT

Endonuclease III is a Fe-S containing bifunctional DNA glycosylase which is involved in the repair of oxidation damaged DNA. Here we employ surface enhanced IR spectroelectrochemistry and electrochemistry to study the enzyme from the highly radiation- and desiccation-resistant bacterium *Deinococcus radiodurans* (*Dr*EndoIII$_2$). The experiments are designed to shed more light onto specific parameters that are currently proposed to govern damage search and recognition by endonucleases III. We demonstrate that electrostatic interactions required for the redox activation of *Dr*EndoIII$_2$ may result in high electric fields that alter its structural and thermodynamic properties. Analysis of inactive *Dr*EndoIII$_2$ (K132A/D150A double mutant) interacting with undamaged DNA, and the active enzyme interacting with damaged DNA also indicate that the electron transfer is modulated by subtle differences in the protein-DNA complex.

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1. Introduction

Endonuclease III (EndoIII) is a bifunctional DNA glycosylase from the Base Excision Repair (BER) pathway which removes a wide range of oxidized pyrimidines from DNA [1,2]. It belongs to the HhH-GPD superfamily of DNA glycosylases, which is characterized by a common fold composed of a conserved Helix-hairpin-Helix (HhH) motif, followed by a glycine and a proline-rich loop preceding an aspartate residue, which is essential for catalytic activity [3]. The family consists of a range of structurally related DNA repair proteins which include the A/G-mismatch specific adenine glycosylase (MutY), 3-methyladenine glycosylase II (AlkA) and human 8-oxoG DNA glycosylase (hOgg1) [4,5]. Unlike other HhH-GPD enzymes, EndoIII and MutY house a [4Fe-4S] cluster. This is consistent with a growing evidence for the presence of Fe-S clusters in nucleic acid processing enzymes, such as nucleases, transcription factors, RNA polymerases and methyltransferases [6]. The mechanism by which glycosylases locate damaged DNA substrates that possess very subtle differences in comparison with undamaged DNA, and the role of the cluster in these processes, are still highly disputed [7]. Early spectroscopic experiments demonstrated that the [4Fe-4S] cluster in EndoIII from *E. coli* (*Ec*EndoIII) is in the +2 oxidation state and redox inactive in solution, which lead to speculations that it has a structural or a regulatory role [8,9]. This view has changed in the light of more recent results obtained employing cyclic voltammetry (CV). It was shown that the Fe-S centers of several DNA glycosylases become redox active upon binding to electrodes modified with DNA. They reveal similar midpoint potentials varying within 50–100 mV and a slow heterogeneous electron transfer (ET) (with rate constant k_{ET} of 1–10 s^{-1}). Based on these findings, the mechanism which envisages a role of the [4Fe-4S] center in DNA-mediated signaling within cells was proposed [10–15]. More recent studies obtained on EndoIII from *Deinococcus radiodurans* (*Dr*EndoIII$_2$) [16] and surface enhanced vibrational spectroelectrochemistry nevertheless provided contradicting results [17]. This approach ensures simultaneous monitoring of orientation of the immobilized protein (by surface-enhanced IR absorption, SEIRA), structure/redox state of the cluster (by surface-enhanced resonance Raman, SERR) and its thermodynamic properties (by CV) [17–19]. Importantly, all steps of protein immobilization, including monolayer formation and protein attachment can be monitored by SEIRA spectroscopy, which enhances the IR absorption intensity by up to two orders of magnitude for the molecules close to the surface [20]. These complementary methodologies allow for experimental testing of several specific assumptions of the current mechanistic model of endonucleaseIII. First, it was shown that *Dr*EndoIII$_2$ becomes redox active upon electrostatic interactions with either DNA or other negatively charged molecules, and that this process is therefore not necessarily DNA mediated [17]. The midpoint potential for the $[4Fe\text{-}4S]^{1+/2+}$ transition (~10 mV) and heterogeneous ET rates (~3 s^{-1}) are slightly modulated by the type of electrostatic interaction and fall into the range of

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values reported for other DNA glycosylases. Second, the first spectroscopic evidence was provided that the redox transition can be attributed to [4Fe-4S] cluster, which in fact shuttles between +1 and +2 states (and not +2/+3, as previously proposed [10–15]). Third, it was demonstrated that the orientation of the protein with respect to the electrode surface is crucial for heterogeneous ET, as the unfavorably oriented enzyme, achieved by attachment of *Dr*EndoIII$_2$ on OH-terminated and Ni-NTA – terminated self-assembled monolayers (SAMs), which could be directly observed in SEIRA spectra, is redox inactive even in the presence of DNA [17].

Here we have further extended this methodology to address other premises of the current mechanistic model, such as the effects of i) electric fields, ii) reductive potentials, iii) damaged/undamaged DNA substrate and iv) orientation of the enzyme in DNA/enzyme complexes, on protein structure, dynamics and ET. Parallel study of the native and the catalytically inactive double-mutant (K132A/D150A) *Dr*EndoIII$_2$ provides novel insights into the parameters that are relevant for a critical assessment of the current catalytic model of Fe-S containing DNA glycosylases. This evidence will further help us to construct a spectroscopically-based view of DNA repair by EndoIII enzymes.

2. Materials and Methods

2.1. Cloning, Expression and Purification of Native and Double Mutant of EndoIII$_2$

The gene encoding endonuclease III$_2$ (DR_0289) was amplified by PCR from genomic DNA of *Deinococcus radiodurans* DNA and inserted into pDest14 (Invitrogen) as described for *Dr*AlkA2, by using the following primers (Sigma Aldrich): FPEndoIII2TEV (5′-ATCACCATCACCATCACGAAAACCTGTATTTCCAGGGAGCAACTCGCAATTCTGCCTCCCCG-3′), RPEndoIII2 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTC TCAACCCTCGACATGCTCCAC-3′) and FRDrAll (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATG-3′) [21]. The inactive mutant (*Dr*EndoIII$_2$ K132A/D150A) was constructed by using the QuikChange® site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene) and using the forward primers: FPdrEIII$_2$K132A (5′-GGCGCGGGCCGCGCGACCGCCAATGTGGTGC-3′) and FPdrEndoIII$_2$D150A (5′-GCCATCGCGGTGGCGACCCACGTGGGCCGC-3′). The downstream primers were complementary and reverse to the primers described above. The mutation codons are underlined. Verification of the mutations was performed by BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Both the native and the mutant proteins were expressed in BL21(DE3)pLysS at 20 °C overnight in Power Broth medium (Molecular Dimensions). The cell harvesting, disruption and protein extraction and purification were performed as previously described [22]. Briefly, the cells were harvested by centrifugation and the extract was prepared by disrupting the cells during 3 freeze/thaw cycles followed by centrifugation. Protein purification from the extract was performed via a HisTrap HP column (GE Healthcare) equilibrated by 50 mM Tris-HCl pH 7.5, 150 mM NaCl buffer (buffer A). Fractions containing *Dr*EndoIII$_2$ were pooled and dialyzed overnight at 4 °C in buffer A in the presence of TEV protease, and afterwards loaded onto HisTrap column in order to remove the HisTag and the protease. The fractions containing the protein were pooled, concentrated and loaded on a Superdex75 10/300 size-exclusion column (GE Healthcare) equilibrated with buffer A. The extraction and purification of the mutant was performed according to the protocol for *Dr*EndoIII$_2$ including the first HisTrap purification step, but instead of TEV cleavage and subsequent HisTrap purification, the protein was passed through a PD10 (15 mL) column to remove Imidazole and NaCl prior to loading onto a Heparin SP column (5 mL). The resulting fractions from the Superdex75 and Heparin column were analyzed by SDS-PAGE, and fractions with >95% purity were pooled and concentrated to ~10 mg/mL prior to flash freezing in liquid nitrogen and storage at −80 °C.

2.2. Spectroscopy

UV–Vis spectra of 100 μM *Dr*EndoIII$_2$ double mutant (in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl) were acquired with a Shimadzu UV-1800 spectrophotometer.

For resonance Raman (RR) spectroscopic experiments, about 2 μL of 0.5 mM *Dr*EndoIII$_2$ and *Dr*EndoIII$_2$ double mutant in the resting state (20 mM Tris-HCl, pH 7.5, 50 mM NaCl) were introduced into a liquid-nitrogen-cooled cryostat (Linkam), mounted on a microscope stage and cooled down to 77 K. Spectra from the frozen sample were collected in backscattering geometry using a confocal microscope coupled to a Raman spectrometer (Jobin Yvon U1000) equipped with 1200 1/mm grating and a liquid-nitrogen-cooled CCD detector. The 413 nm line from a krypton ion laser (Coherent Innova 302) was used as excitation source. Typically, spectra were accumulated for 60 s with a laser power of 6–8 mW at the sample. The background scattering was removed by subtraction of a polynomial function.

All FTIR and surface enhanced IR absorption (SEIRA) data are presented as difference spectra after subtraction of a reference spectrum. All SEIRA spectra were recorded from 4000 to 1000 cm^{-1} at a spectral resolution of 4 cm^{-1} on a Bruker Tensor 27 spectrometer equipped with a liquid-nitrogen-cooled MCT detector [17]. All measurements were done at 10 °C while purging the sample compartment with dry air. Typically, each spectrum was composed of around 400 scans, and 10–40 spectra were co-added and further analyzed. SEIRA measurements were performed in Kretschmann-ATR configuration, using a trapezoidal silicon (Si) crystal, under an IR-beam incident angle of 60°. SEIRA electrodes were prepared by electroless deposition of a gold film on the reflecting layer of the silicon crystal [23]. The gold film enhances the IR absorption of molecules in the vicinity of the surface and, at the same time, acts as the working electrode in a three electrode spectro-electrochemical arrangement. The formation of a SAM on gold film electrodes was followed in time until no further changes in the spectra of the monolayer were observed. After subsequent buffer exchange and reaching the equilibrium state, several spectra were co-added and used as a background for obtaining the difference spectra of the following process, i.e. protein attachment. After functionalization of the Au surface (Section 2.4), protein was added to the SEIRA spectroelectrochemical cell (0.1–10 μM final concentration) and left to incubate typically for 40–90 min. Protein solution was afterwards removed and the cell was thoroughly rinsed with measuring buffer (50 mM Tris HCl, 50 mM NaCl, pH 7.6). Similarly, the protein SEIRA spectra were used as a background when DNA binding was monitored. Several electrodes were measured for each experiment; all SEIRA spectra (and voltammetric signals measured in SEIRA cell) employing *Dr*EndoIII$_2$ attached to SAM modified surfaces were reproducible, irrespective of the concentration of *Dr*EndoIII$_2$ and temperature of the protein incubation solution [17].

2.3. Cyclic Voltammetry

CV experiments were performed under argon atmosphere in a three electrode electrochemical cell arrangement. Different setups were used for coupling CV with SEIRA and for CV experiments only. In each case Ag/AgCl (3 M, KCl) served as reference electrode (+0.21 V vs. SHE), and a platinum wire as a counter electrode. As working electrodes, Au coated silicon crystal and Au electrodes (BASi) were used in SEIRA and CV, respectively. Electrode potentials were controlled by a Princeton Applied Research 263A potentiostat. The buffer used for the measurements of EndoIII$_2$ contained 50 mM Tris HCl, 50 mM NaCl at pH 7.6 (pH 8.5 for the mutant). Scan rate dependence was performed in the range of 2–1000 mV/s, typically in the potential window from −0.3 to +0.5 V, and the ET rate constant was determined using Laviron method [24,25]. Prior to CV measurements, the SAM coated electrodes were immersed for 40–60 min into 0.1–0.3 mM enzyme solution (in 5 mM phosphate buffer, pH 7.5), or alternatively, a droplet of concentrated

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