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Characterization in aqueous medium of an FMN semiquinone radical stabilized by the enzyme-like microenvironment of a modified polyethyleneimine†

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The elusive flavin semiquinone intermediate found in flavoproteins such as cryptochromes has been obtained in aqueous solution by single electron reduction of the natural FMN cofactor using sodium ascorbate. This species was formed in the local hydrophobic microenvironment of a modified polyethyleneimine and characterized by UV-Visible, fluorescence and EPR spectroscopies.

Flavin derivatives such as riboflavin, flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) are ubiquitous organic cofactors involved in various biological processes relying on hydride or single electron transfers.^{1,2} Along these processes the isoalloxazine heterocycle may adopt three different redox states; fully reduced, semi-quinone or fully oxidized (Scheme 1, right) depending on protein activities.³ In most systems, the oxidized form is commonly reduced by NAD (P)H cofactors *via* a hydride transfer and the resulting reduced form can either activate dioxygen for oxidation catalysis^{4,5} or deliver single electrons to other redox partners *via* the formation of a semiquinone radical.⁶ Interestingly, this semiquinone state may also be formed by light induced electron transfer, upon light irradiation of the oxidized form followed by the one electron oxidation of a neighboring amino acid.⁷ Such radical semiquinone intermediates are involved in many important biological processes such as DNA repair,⁸ circadian rhythms⁹ or bird migration compasses.¹⁰ They are stabilized by specific interactions with amino-acids present in the local microenvironment provided by their host protein, but are very unstable in aqueous solution because of their rapid disproportionation into the oxidized and reduced forms. Stabilization of such intermediates outside the protein scaffold would be very interesting to improve their characterization, but also to better

understand the chemistry of these elusive species. Yet, apart from a few examples of chemically modified flavin derivatives,^{11–13} only one long-lived hydrated FMN radical has been characterized so far.¹⁴ It was formed after laser irradiation and stabilization inside an agarose gel matrix to minimize the diffusion and therefore inhibit the intermolecular electron transfers responsible for its disproportionation in solution. Thanks to this approach, both the neutral semiquinone (FMNH[•]) and the anionic semiquinone (FMN^{•−}) radicals were thoroughly characterized by EPR and ENDOR spectroscopies. However, solution characterizations such as UV-Visible absorption and fluorescence spectroscopies were missing.

In previous studies, we showed that the incorporation of the oxidized FMN into the local microenvironment of modified polyethyleneimine (PEI) (Scheme 1, left) allowed its two electrons reduction by NADH in aqueous solution.¹⁵ The resulting reduced FMNH₂ could then, either efficiently transfer electrons to redox partners under anaerobic conditions,^{15,16} or catalyze the Baeyer–Villiger reaction under aerobic conditions.¹⁷

In this communication, we show that the replacement of NADH by sodium ascorbate completely changed the reactivity

Scheme 1 Structure of the modified polyethyleneimine (PEI) with guanidinium and octyl groups (left) and of the FMN at its three different redox states (right).

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of the system and led to the formation of the usually very elusive FMNH[•] intermediate which is here stabilized by the simple locally hydrophobic micro-environment of the modified PEI. To the best of our knowledge, this is the first characterization of such semiquinone FMNH[•] intermediate into a homogenous aqueous solution. This opens interesting perspectives for the development of new bio-mimetic systems that could stand as promising candidates for sustainable radical catalysis in aqueous solution.

The use of NADH as a cofactor for catalysis suffers from its prohibitive cost and its replacement by cheap reducing agents such as sodium ascorbate would therefore be an important improvement. Imada *et al.* showed that this was possible using N5 alkylated flavinium salts, suggesting that the flavin-derivative was fully reduced during the catalytic process.¹⁸ Yet, when 10 equivalents of sodium ascorbate were added to the above described artificial flavoenzyme made of FMN cofactors incorporated into a modified PEI under anaerobic conditions, the usual two electrons reduction of the FMN (Fig. 1a) could not be observed. Instead, a new chromophore appeared at 610 nm with a shoulder at 680 nm (Fig. 1b). This new species reached its maximum of absorbance after 10 hours of reaction and was stable for about two hours under anaerobic conditions before fading along the next 10 hours. In the absence of polymer, no chromophore could be observed upon addition of ascorbate to an FMN solution (Fig. 1c). According to literature, this new absorption band at 610 nm is reminiscent of the electronic absorption spectra of the semiquinone radical intermediates (FMNH[•]) previously characterized in proteins.^{19–23} Such intermediates show two bands of similar intensities at about 450–500 nm and 600–650 nm depending on the protein nature. Based on the molar extinction coefficient of FMNH[•] previously characterized in proteins ($\sim 4000 \text{ M}^{-1} \text{ cm}^{-1}$ for the band at 600–650 nm),²⁴ the absorbance of 0.07 measured at 610 nm would account for a concentration of about 17 μM (68% of the FMN in solution). The main difference between the spectrum observed here and the ones observed in proteins, is that the 450 nm band was not decreasing during the

process. Consequently, one may wonder if the new chromophore at 610 nm is indeed generated from the FMN cofactor. To answer this question, fluorescence spectroscopy was performed by irradiating the sample at 614 nm and an intense fluorescence band was measured at 686 nm (Fig. 2). These fluorescence parameters are in very good agreement with data previously obtained for flavin semiquinone intermediates in proteins²⁵ and confirm that this new chromophore is indeed a flavin derivative issued from the FMN cofactor. This fluorophore reached its maximum after about 2 h of reaction and was stable for another 2 h before fading along in the next 8 h (Fig. 3). Control experiments using NADH instead of the ascorbate or using the ascorbate in the absence of polymer did not generate any fluorescence. It is also worth to note that fluorescence experiments were performed in 96 well plates and could not be realized under anaerobic conditions, making the intermediate less stable compared to the UV-Visible experiments. Concerning the stability of the absorption band at

Fig. 2 UV-Visible spectrum of an aqueous solution of FMN (25 μM) and PEI_{guan-oct} (2.5 mM) 3 hours after the addition of sodium ascorbate (250 μM) (solid black line) and fluorescent spectrum of a solution of FMN (100 μM) and PEI_{guan-oct} (10 mM), 3 hours after the addition of sodium ascorbate (1 mM) and excitation at 614 nm (dashed red line).

Fig. 1 UV-Visible spectra of aqueous solutions of (a) FMN (25 μM) and PEI_{guan-oct} (2.5 mM in monomer) upon addition of NADH (250 μM) and monitored for 3 h; (b) FMN (25 μM) and PEI_{guan-oct} (2.5 mM in monomer) upon addition of sodium ascorbate (250 μM) and monitored for 10 h and (c) FMN (25 μM) upon addition of sodium ascorbate (250 μM) and monitored for 10 h. Conditions: anaerobic atmosphere (Ar), in distilled water, at room temperature. Starting spectra are red lines and final ones are black lines.

Fig. 3 Time course of the fluorescence emission for an aqueous solution of FMN (100 μ M) and PEI_{guan-oct} (10 mM) upon addition of ascorbate (1 mM) (green circles), for the same solution upon addition of NADH (1 mM) (red squares) and for an aqueous solution of FMN only (100 μ M) upon addition ascorbate (1 mM) (white triangles). (λ_{Ex} = 614 nm, λ_{Em} = 700 nm).

Fig. 4 EPR spectra of degassed DMSO solutions of FMN (1 mM) with PEI_{guan-oct} (10 mM) and ascorbate (10 mM) (solid red trace), of FMN (1 mM) with ascorbate (10 mM) only (solid green trace) and of FMN (1 mM) with PEI_{guan-oct} (10 mM) and NADH (10 mM) (dashed black trace). Conditions: Microwave frequency 9.63 GHz, microwave power 0.25 mW, modulation amplitude 8 Gauss, modulation frequency 100 kHz, gain 56 db, temperature 90 K.

450 nm during the reaction, this could be explained by a hypsochromic shift of the band usually observed around 500 nm in proteins towards lower wavelength. In such case, the formation of this new band would compensate the decrease of the original band of the FMN at 450 nm. To confirm this assumption, a differential spectrum was plotted by subtracting 68% of the original FMN spectrum to the spectrum shown in Fig. 1b. The obtained spectrum displayed maxima of absorptions at 620, 450 and 375 nm, which indeed appeared to be very similar to the one previously reported for FMN semiquinone in a protein environment (Fig. S1†).²⁴

Apart from their specific UV-Visible and fluorescence spectroscopic parameters, flavin semiquinone intermediates also have very specific electronic paramagnetic resonance parameters. EPR spectroscopy was thus performed on the intermediate previously observed and the 25 μ M solutions of FMN only gave a weak EPR signal. This was not very surprising considering the fact that only 17 μ M of the intermediate species was supposed to be formed according to UV-Visible spectroscopy. The concentration was thus increased up to 1 mM, but the solution was not homogeneous anymore and a green precipitate formed in the Schlenk line. This precipitate was dried-off under strict anaerobic conditions and resolubilized in DMSO. The EPR analysis of the solution gave an intense signal at $g = 2.0031$, while the control experiments using either NADH instead of ascorbate, or ascorbate in the absence of polymer, only gave very weak signals (Fig. 4).

This clearly demonstrated the formation of a green radical species absorbing at 610 nm with fluorescence emission at 700 nm upon excitation at 614 nm. Overall, these experiments show that the use of sodium ascorbate did not lead to the fully reduced FMNH₂ as it was the case with NADH, but actually led to the one electron reduction of the FMN cofactor into a flavin semiquinone intermediate with similar spectroscopic parameters as the FMNH[•] (Scheme 2). In theory, the 0.28 V standard potential of ascorbate is not low enough for the reduction

Scheme 2 Reactivity of FMN cofactors with NADH or ascorbate when incorporated into the local microenvironment of polyethyleneimine modified with guanidinium and octyl groups.

of the FMN cofactor (standard potential of -0.30 V). However, this latter potential is associated with a two electrons process, while the formation of FMNH[•] relies on a one e^- and one H^+ transfer. Interestingly, it was previously shown in literature that the thermodynamic and kinetic properties of the ascorbate were tuned by their local environment and that the ascorbate was, as stated by the authors “*stronger reductant – better able to donate a proton and an electron (or H^+) – in local environments with fewer hydrogen bond donors*”.²⁶ The locally hydrophobic microenvironment the modified PEI may therefore facilitate this PCET from the ascorbate ion to the FMN cofactor in order to form the FMN semiquinone radical within the polymer. Additionally, when incorporated into the local

microenvironment of the polymer, the standard potential of the FMN is shifted of 65 mV towards a higher potential,¹⁵ which will also facilitate the reduction process. Such changes were already observed in the case of natural enzymes²⁷ and Rotello *et al.* confirmed the importance of supramolecular interactions in these phenomenon using chemical models.^{28,29} Once trapped into this enzyme-like microenvironment, the FMNH[•] intermediate cannot freely diffuse in solution, precluding its rapid disproportionation and allowing its stabilization in aqueous solution even in the presence of dioxygen.‡

Conclusions

We demonstrate that the reaction of ascorbate with FMN cofactors incorporated into the local microenvironment of a modified polyethyleneimine allows the formation and stabilization of the FMNH[•] semiquinone radical in aqueous solution, which is usually only observed within natural flavoproteins. This species was characterized by UV-Visible, fluorescence and EPR spectroscopies and was shown to be stable for about two hours, even under aerobic conditions. We suggest that the incorporation of the FMN into the polymeric matrix impedes the free diffusion of the radical in solution, therefore precluding its disproportionation into FMN and FMNH₂.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

‡ Modified polyethyleneimine polymers were synthesized as previously described.^{15,30}

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