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Spectroscopic investigation of flavoproteins: Mechanistic differences between (electro)chemical and photochemical reduction and oxidation

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ABSTRACT

The spectroelectrochemical investigation of proteins is an important method to study their redox properties and to elucidate protein ET pathways. Such studies are often more complicated for proteins than for small molecules because the redox-active cofactor is not easily accessible and/or the proteins are quite sensitive. For the determination of redox potentials it is necessary to work under equilibrium conditions which can be achieved by the presence of redox mediators. The proteins can be reduced and oxidized by chemical, electrochemical, and photochemical methods, each of which are briefly discussed. Differences in the mechanism of chemical and photochemical reduction of flavoproteins are pointed out. Photochemical reduction by irradiation with blue-light in the presence of EDTA can lead to the formation of flavoprotein semiquinone radicals which otherwise could not be observed.

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Redox enzymes catalyze the oxidation or reduction of a specific substrate or a group of substrates with similar structural and electronic properties. Depending on their catalytic reaction these enzymes can be applied in amperometric biosensors [1–3] or biofuel cells [3–5]. Prior to any possible application, the electrochemical and catalytic properties of the enzymes have to be elucidated. Many redox-active proteins have been discovered and investigated without knowing their distinct enzymatic function. To elucidate the enzymatic function and further characterize the proteins, their redox potential has to be determined. This is possible by potentiometric or spectroelectrochemical investigations in solution [6–11]. The protein can be reduced (or oxidized) either chemically or electrochemically. Redox-active proteins are composed of an electrochemically almost inactive apoprotein in which one or more cofactors are embedded. Since the cofactors can be shielded by the apoprotein, electron transfer (ET) is quite often hampered or not possible at all. In order to enhance ET, a mixture of small ambiphilic redox-active molecules can be added [6,7]. These redox mediators, which transport charges in their partially oxidized/reduced state, establish the electrochemical equilibrium which is necessary for the determination of redox potentials. In order to prove whether direct ET to redox-active proteins is possible, and to further elucidate the ET pathway, spectroelectrochemical measurements can be performed also in the absence of any mediators [9,12–15]. Alternatively to chemical or electrochemical redox investigations, proteins can be reduced or oxidized photochemically [16–21]. This method has been used frequently to study the kinetics of intraprotein and interprotein ET mechanisms [20,22]. In this contribution an introduction to the potentiometric and spectroelectrochemical investigation of redox-active proteins in solution is presented and differences in the mechanism of electrochemical and photochemical reduction of flavoproteins are discussed.

During an ordinary redox titration the protein solution is stirred in a cuvette equipped with a reference electrode in the presence of the mediator mixture under oxygen-free conditions [6,7,19]. The redox potential is adjusted by the stepwise addition of small amounts of reducing or oxidizing agent. Most frequently sodium dithionite (reducing agent) and ferricyanide (oxidant) are used. During the ongoing redox process, usually the UV/vis absorption is monitored and the spectra are evaluated at a wavelength at which the change in absorption is sensitive to the ratio between oxidized and reduced protein states. Other spectroscopic methods such as IR or ESR spectroscopy can be applied as well [6,7,10,15,23]. Additional contributions from the mediator mixture have to be taken into account because the absorption spectra of the redox mediators also may change depending on their redox state. Therefore, the redox mediators have to be applied in a concentration that is sufficiently low to allow the distinct monitoring of the protein redox process. At the same time, the mediator concentration should be high enough to establish fast electrochemical equilibrium. If possible, components for the mediator mixture should be chosen, which undergo only minor spectral changes in the wavelength region, in which the protein absorption is monitored. Since redox mediators

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are only active close to their own midpoint potential (this is the potential at which equal amounts of oxidized and reduced form are present), the midpoint potentials of the individual components of a mediator mixture should cover the potential range of interest. For the determination of protein midpoint potentials it is of utmost importance to perform a redox titration in both directions (stepwise reduction and reoxidation) leading to the same potential values for the investigated protein. This indicates that equilibrium conditions are achieved, and neither protein nor components of the mediator mixture undergo degradation during the experiment. When protonation/deprotonation steps are involved in the reduction/oxidation process, the corresponding midpoint potentials are pH dependent [6]. Depending on the complexity of the investigated redox system, the titrations have to be carried out with different mediator mixtures of varying concentration at different pH values in order to follow the expected pH dependent behavior. One disadvantage of a redox titration is that a relatively large amount of protein solution is required, especially if the measurement has to be repeated under different conditions. Furthermore, the usage of dithionite as reducing agent leads to the formation of strongly acidic products which may change the pH and even lead to the decomposition of the proteins. Some of these problems can be avoided by spectroelectrochemical measurements whereupon the redox potential is adjusted electrochemically. A three electrode setup (working, counter, and reference electrode) is required. The potential is applied at the working electrode with respect to the reference electrode and the current is passed between working and counter electrode. Any reduction process at the working electrode is coupled to an oxidation process at the counter electrode and vice versa. These electrodes should be separated in order to avoid spectral contributions of undesired degradation products which might be generated at the counter electrode. For protein spectroelectrochemistry different cell types have been developed with the main goal being to minimize the sample volume which is required for a single experiment [11,13,24–30]. By applying a gold capillary as working electrode, the sample volume can be reduced down to 20 μL, and, at the same time, an optical path length of 1 cm is available [13,24]. When proteins are investigated (spectro)electrochemically, some tend to adsorb on bare electrode surfaces. This process can be followed by degradation. Protein adsorption can be avoided by the use of modified electrodes. Surface modification can be performed for example by adsorption of thiol derivatives on gold electrodes [8,12–14,31]. The thiolate layer can serve as a protecting layer or even enhance the ET between electrode and protein when redox mediators are linked to the outer part of the thiol derivatives in a flexible manner [8].

Especially when flavoproteins are investigated, the photochemical reduction by irradiation with blue-light is an interesting alternative to chemical or electrochemical reduction [16,18]. In aqueous solution at neutral pH-free flavins are only stable in the oxidized (quinone) or in the doubly reduced state (hydroquinone, singly or doubly protonated) [8]. This is because the midpoint potential for the reduction of the neutral flavosemiquinone radical to the flavohydroquinone is more positive than the reduction of the flavoquinone to the flavosemiquinone radical anion [8,19,32]. The electrochemical reduction of free flavins in aqueous solution occurs as overlapping two electron process following an "ece" mechanism, i.e. as the sequence of an electrochemical step (ET), a chemical step (protonation), and a second electrochemical reaction step, leading to the flavin hydroquinone as the only observed product [19]. Depending on the pH, the flavin hydroquinone ($pK_a = 6.2$) is stable as radical anion or as neutral radical [33]. For flavin mononucleotide with an overall redox potential of $E_{fq/fhq} = -205$ mV at pH 7, the distinct potentials of first and second reduction steps were determined as $E_{fq/fsq} = -238$ mV and $E_{fsq/fhq} = -172$ mV, respectively [32]. In some flavoproteins, the singly reduced flavo-semiquinone radical or radical anion can be stabilized due to interactions with the apoprotein [34]. For instance, midpoint potentials of $E_{fq/fsq} = -137$ mV and $E_{fsq/fhq} = -382$ mV were reported for flavodoxin at pH 7 [34]. For glucose oxidase, a flavoenzyme, which is widely used in glucose biosensors and biofuel cells, midpoint potentials of $E_{fq/fsq} = -63$ mV and $E_{fsq/fhq} = -65$ mV at pH 5.3 and $E_{fq/fsq} = -200$ mV and $E_{fsq/fhq} = -240$ mV at pH 9.3 were determined [35]. The different flavin redox states, which can be distinguished by their absorption spectra [8,18], are shown in Fig. 1. There is only a minor difference in the spectra of the neutral and anionic flavin hydroquinone, which are both colorless [36].

The mechanism for the photochemical reduction of free flavins by irradiation with blue-light in the presence of ethylenediaminetetraacetic acid (EDTA), which is depicted in Scheme 1, is well known [17,37–39].

Irradiation to the singlet state S_1 is followed by fast intersystem crossing to the triplet state T_1 which is formed in high quantum yield [40–43]. ET from EDTA and subsequent protonation results in the formation of a flavosemiquinone radical. Two flavosemiquinone radicals undergo fast disproportionation to oxidized and doubly reduced flavin. After the first ET step, EDTA releases one molecule of CO_2 [17]. Degradation of EDTA leads to the formation of a reactive

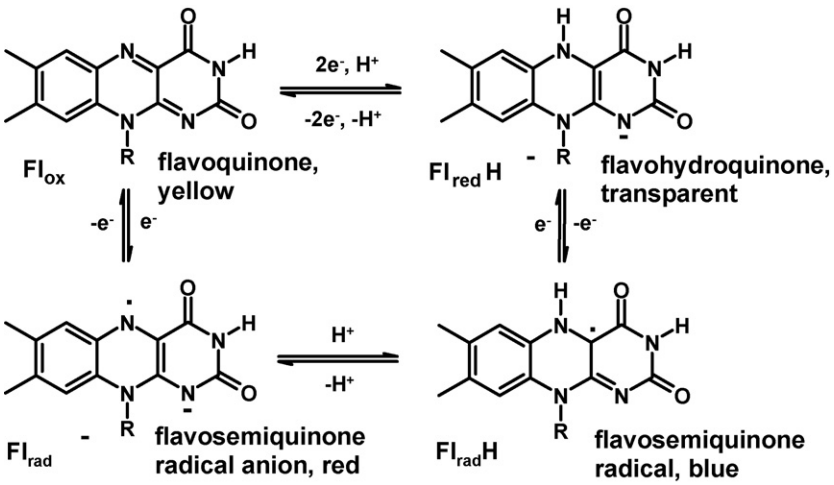


Fig. 1. The different flavin redox states.

radical which is able to donate a second electron. Beside EDTA, several other electron donors have been examined [17,44–48]. So far this mechanism is generally accepted for the photochemical reduction of free flavins in solution. As an alternative to chemical or electrochemical reduction, entire flavoproteins also can be reduced photochemically by irradiation with blue-light in the presence of electron donors such as EDTA. An interesting question is how the protein bound flavin is reduced in this case. It has been suggested that this reaction does not involve an excited state of the holoprotein [16]. Only free flavins outside the protein (which are always present in a solution of flavoproteins to some extent) are reduced photochemically. Thereafter these reduced flavins transport electrons to the protein bound flavins which are then reduced by an "interflavin oxidoreduction" [16]. Recent studies on a LOV1 C57G flavoprotein have shown that this mechanism is not generally valid [19]. LOV (light-oxygen-voltage sensitive) domains comprise the light sensitive part of many blue-light photoreceptor proteins [48–52]. In the LOV1 C57G variant the fast and efficient thioadduct formation following photoexcitation, which occurs in the wild type, is not possible because the photoreactive cysteine is replaced by glycine. The irradiation of this variant resulted exclusively in the formation of the stable neutral semiquinone radical form of the flavoprotein, whereas chemical reduction leads directly to the formation of the flavohydroquinone without the observation of a stable semiquinone intermediate [19]. The stepwise photochemical reduction of LOV1 C57G in the presence of EDTA (1 mM) is depicted in Fig. 2.

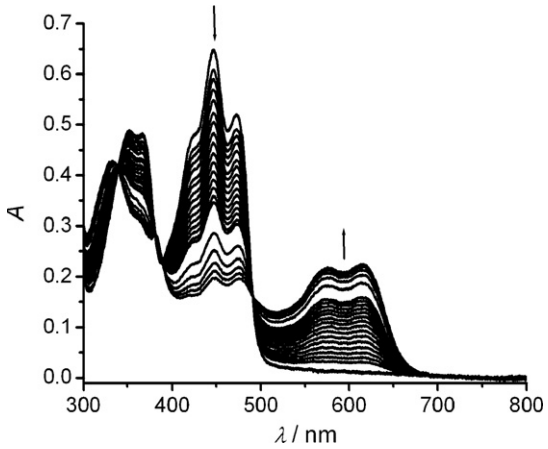
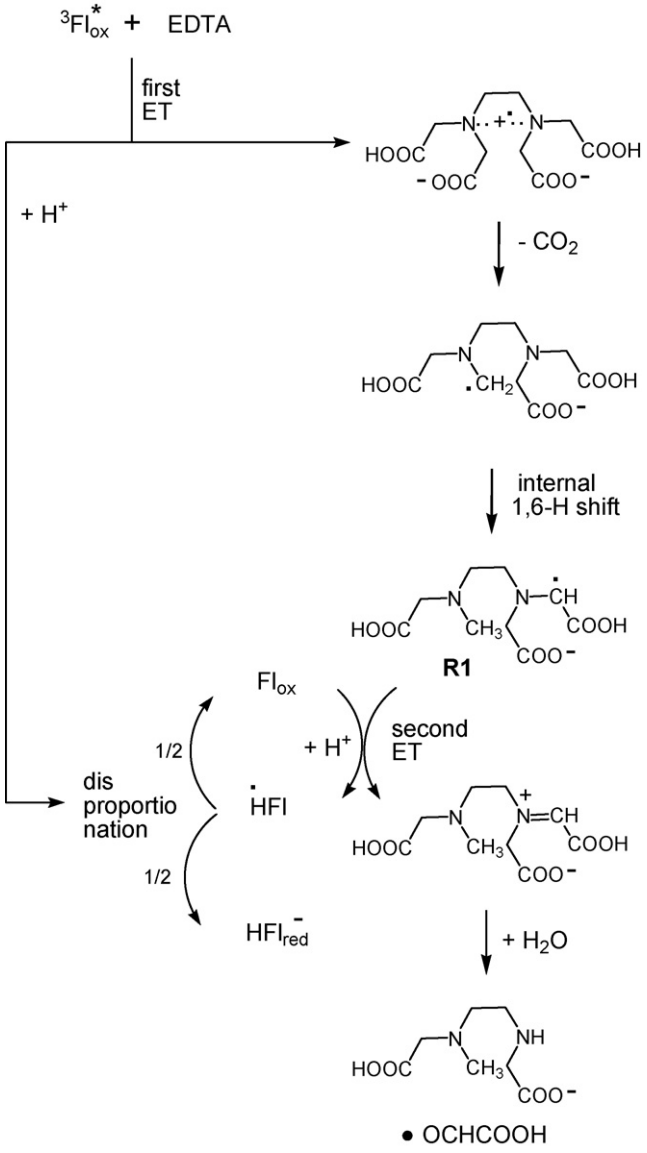


Fig. 2. Stepwise photochemical reduction of LOV1 C57G in the presence of EDTA (1 mM), from Nöll et al. [19]. Copyright Wiley–VCH Verlag GmbH & Co. KGaA. Reproduced with permission.



Scheme 1. Mechanism of flavin photochemical reduction in the presence of EDTA, from Nöll et al. [19]. Copyright Wiley–VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Further experiments were carried out in order to prove that in the case of LOV1 C57G chemical and photochemical reduction follow different mechanisms [19]. There are some indications that this difference in reduction mechanism might exist for other flavoproteins as well. The photochemical reduction of flavoproteins by irradiation with blue-light in the presence of EDTA was first carried out in 1966 [18]. This publication reported that the rate of semiquinone formation upon photochemical reduction is greater than for example from a mixture of oxidized and reduced flavoprotein [18]. When glucose oxidase was reduced photochemically, depending on the pH either the neutral flavoprotein semiquinone radical ($pK_a = 7.5$) [18] or the negatively charged radical anion was formed (see Fig. 3). While the radical anion was stable, the neutral radical could be reduced further to the flavoprotein hydroquinone by extended illumination. Interestingly, the flavoprotein semiquinone radical was not observed as an intermediate, when the reduced flavoprotein hydroquinone was reoxidized stepwise by admission of small amounts of oxygen [18].

Many proteins have been reduced by irradiation in the presence of EDTA or other electron donors and 5-deazaflavines [20,22]. In this case, the electrons can be transferred by free 5-deazaflavin semiquinone radicals in an "interflavin oxidoreduction" like reaction. Since the midpoint potential for the 5-deazaflavin quinone/semiquinone radical is very negative (about −650 mV vs. NHE) [53], this method allows also the reduction of various proteins other than flavoproteins [20,22]. After irradiation electron donation leads to the formation of a highly reductive anionic 5-deazaflavin semiquinone radical which is able to transfer a single electron to a protein redox center. Alternatively the 5-deazaflavin semiquinone radicals can undergo disproportionation [20,22,54] or dimerization [55–58]. The resulting species are less reductive than the 5-deazaflavin semiquinone radical [55]. When protein solutions containing 5-deazaflavins are irradiated in the absence of additional donors, ET from a protein redox center may occur and thereby result in protein oxidation [20,22].

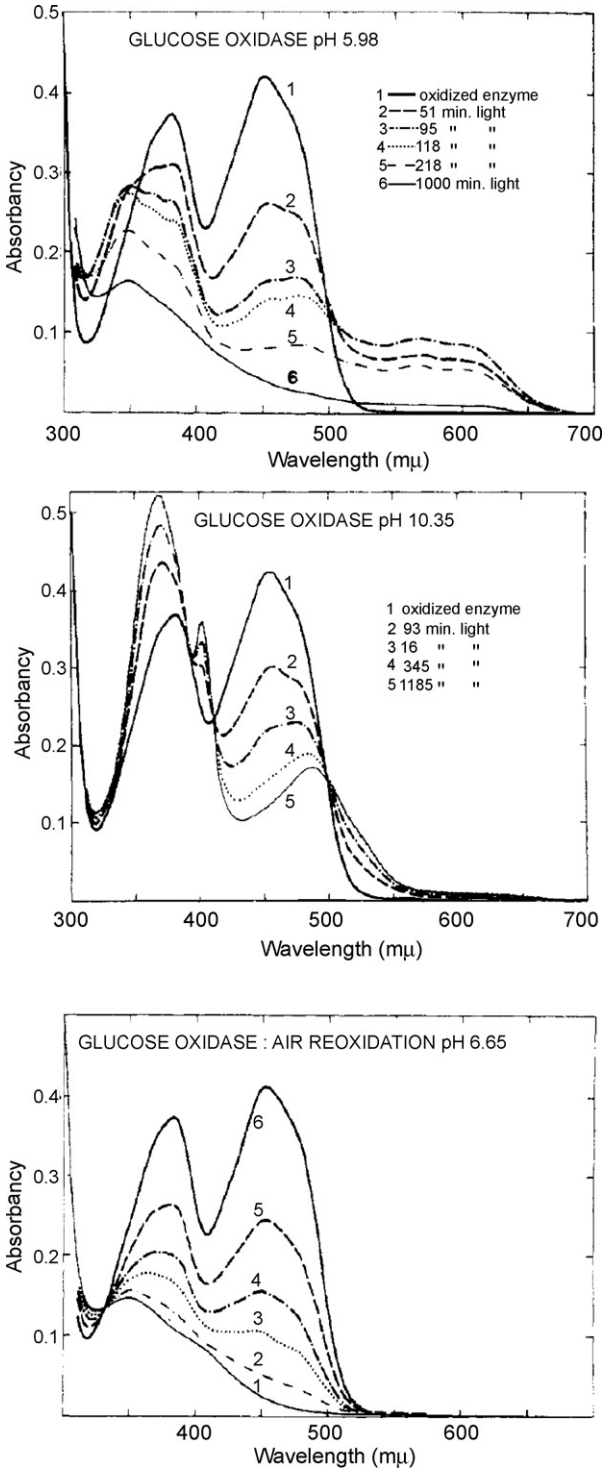


Fig. 3. Photochemical reduction of glucose oxidase at pH 5.3 (top), at pH 10.35 (middle), and reoxidation of photochemically reduced glucose oxidase at pH 6.65 (bottom). Reprinted with permission from Massey and Palmer [18]. Copyright 1966 American Chemical Society.

The spectroelectrochemical investigation of proteins is an important method to study their redox properties and to elucidate protein ET pathways. The proteins can be reduced and oxidized by chemical, electrochemical, and photochemical methods. Especially when flavoproteins are investigated, the photochemical reduction by irradiation with blue-light in the presence of EDTA can lead to the formation of flavoprotein semiquinone radicals which otherwise could not be observed. The photochemical reduction of redox proteins (in the absence of redox mediators) is not favored for the determination of redox potentials because it is a non-equilibrium reaction.

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