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Light induced redox reactions involving mammalian ferritin as photocatalyst

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Abstract

Under excitation by visible light the iron storage protein ferritin catalyses the reduction of cytochrome c and viologens as well as the oxidation of carboxylic acids, thiol compounds, and sulfite. The photochemically active element of ferritin is its mineral ferrihydrite semiconductor core. Band-gap excitation of these microcrystals leads to generation of electron-hole pairs that are sufficiently long-lived and reactive to engage in redox reactions with components of the medium. Photoreduction of cytochrome c and viologens occurs due to electron transfer from the conduction band of the iron oxide cluster through the protein shell surrounding the ferritin core. Laser photolysis coupled with time-resolved kinetics spectroscopy showed the electron transfer to propylviologen sulfonate to proceed in the microsecond time range. In the absence of electron acceptor at pH < 7, light excitation results in photodissolution of the iron oxide cluster with concomitant formation of Fe(II). These novel findings concerning the photocatalytic activity of ferritin with its inherent biological implications are discussed.

Keywords: Mammalian ferritin photocatalyst; Propylviologen sulfonate reduction; Cytochrome c reduction; Light-induced redox reactions

1. Introduction

One of the principal forms in which iron is maintained in living organisms is in microparticles of hydrated iron oxide bound by a protein shell, apoferritin. These iron-containing proteins, i.e., ferritins, along with another storage protein, hemosiderin, form the majority of non-heme iron containing entities found in practically all types of living organisms (animals, plants, bacteria, etc.). For example, almost one fourth of the total body iron in normal, healthy human beings, with ferritin serum concentrations alone ranging from 12 to 300 $\mu g\ l^{-1}$, can be accounted for in these two storage forms. When ferritin is isolated from mammalian liver or spleen, typically 10–20% and at times even greater than 30% of its molecular mass can be attributed to its iron content. Although they vary in structural details, ferritins have similar molecular architectonics: 24 protein subunits, assembled in cubic 4 3 2 point symmetry, form a practically spherical shell of roughly 12.5 nm external diameter with a central cavity of 7–8 nm internal diameter sequestering one or generally more microparticles of a hydrous ferric oxide [1–3]. The iron core of mammalian ferritins, in particular, has been demonstrated to have the crystalline structure of the natural mineral ferrihydrite of composition $5Fe_2O_3 \cdot 9H_2O$ or, more precisely, a hydrous-ferric oxide containing phosphorous bonds of the composition $(FeOOH)_8(FeO{:}PO_3H_2)$. These iron complexes contain nearly 60% iron by mass and, thus, a maximum of 4500 iron atoms can be stored within a single ferritin entity due to limitations imposed by the cavity's internal diameter [3–9].

Under the appropriate redox conditions, the protein shell of ferritin is permeable to iron. Ferritins, consequently, perform two main functions: (i) storing of iron to prevent the accumulation of toxic free-iron (detoxification of reduced iron) in the biological system and (ii) release of this stored iron when necessary to cells for cellular processes, such as synthesis of heme and other iron containing proteins, and for other redox-type processes within the cell. It has been established that hydrated iron oxide particles are formed within the protein shell of ferritin following oxygenation of the endogeneous ferrous ions by molecular oxygen [10,11,1–9]. Iron mobilization from ferritin occurs as a result of the reductive dissolving of the iron core; in this process, the organic compounds chelating the iron and reductants present in the cytoplasm are involved. The processes of ferritin core formation and dissolution have been intensively studied [1–9]; how-

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ever, the exact mechanism is still open to debate. The detailed mechanism involving ferritin and agents dissolved in the cytoplasm is also not clear, although in the literature there are numerous studies related to this topic [1–9]. Ferritin can, however, be considered not merely as a metalloprotein, but as a true enzyme exhibiting ferroxidase activity [12,13].

Iron oxides and hydroxyoxides are semiconductors and, thus, may exhibit photocatalytic activity [14]. The ability of iron oxide colloids to photocatalyze hydrogen production [15,16], to oxidize iodide [17], carboxylic acids [18], phenol [19], sulfite, and sulfur dioxide [20] has been shown. Band-gap excitation of particles of iron oxide in the presence of chloride anions [21] or organic compounds [22] led to the dissolution of the particles with concomitant formation of Fe^{2+}. Thus, the photocatalytic properties of colloidal iron oxides have been extensively examined. Although several studies on the chemically induced reductions and oxidations and on the electrochemical characteristics of ferritin have appeared prior to and in the last decade [23–28], very little is known concerning the photoredox behaviour of ferritin. The irradiation of horse spleen ferritin with UV-A light resulted in Fe^{2+} formation [29]. Ferritin isolated from pea seeds likewise exhibited iron photoreduction and simultaneous ferroxidase-mediated reincorporation into the core [30].

In the present paper light-induced redox reactions involving mammalian ferritin as photocatalyst are investigated. Using a time-resolved laser photolysis method the dynamics of electron transfer from the ferritin core through the protein shell to cytochrome c or viologens have been unraveled. Continuous illumination confirmed the high activity of the iron oxide semiconductor clusters contained in ferritin to induce redox reactions.

2. Materials and methods

Experiments were performed using horse spleen ferritin (800 Fe atoms per molecule) in a sterile, filtered aqueous solution of 0.15 M NaCl, 100 mg ml^{-1} with < 1% Cd content (as % of ferritin) from Sigma. Horse heart cytochrome c, TES (N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid), MES (2-(N-morpholino)ethane sulfonic acid), CAPS (3-(cyclo-hexylamino)-1-propane sulfonic acid), oxalic, citric and malonic acids, glutathion, cysteine and sulfite were obtained from Fluka and sodium tartrate from Merck. Sephadex G-25 beads for filtration were purchased from Sigma. Propylviologen sulfonate (PVS) was synthesized according to Ref. [31]. The *o,o'*-xylene double-bridged viologen (DV^{4+}) was synthesized in the laboratory of S. Hünig at the Institute of Organic Chemistry, University of Würsburg. Millipore Q quality water was used in the preparation of all solutions.

Ferritin was incubated on ice in 10 mM EDTA solution for 1 h and then passed through a Sephadex G-25 column equilibrated with 0.3 M NaCl (pH 7.0) to remove loosely associated iron according to the procedure followed by Saito, et al [6]. Total iron was determined in general by the method of Crichton [7] with only minor modifications. Continuous illumination was performed using an XBO 450 W Xe lamp (Osram) equipped with appropriate cut-off filters and a 15 cm water jacket to remove IR irradiation. Prior to irradiation the solutions were bubbled with trace oxygen purified argon. A JK-2000 frequency-doubled ruby laser ($\lambda = 347$ nm) coupled with a time-resolved kinetic spectroscopy technique for detection of transient species was used in the laser flash photolysis experiments. UV-visible absorption spectra were recorded on a Hewlett-Packard 8452 A spectrophotometer.

3. Results

3.1. Photoreduction of cytochrome c

Illumination of the inorganic semiconductors TiO_2, ZnO and CdS resulted in the reduction of the enzymes hydrogenase [32–34], dehydrogenase [35], and cytochrome c [36]. We, thus, decided to attempt the photoinduced reduction of cytochrome c by ferritin. It should be noted that ferritin behaves as a true photocatalyst only in the presence of exogeneous electron donors acting as scavengers for valence band holes produced by light excitation in the ferrihydrite core. If no such electron donor is available the protein itself may act as a source of electrons, as is developed below and in the discussion section.

Visible light illumination of cytochrome c solutions (absorption maximum of oxidized species at 528 nm) containing ferritin results in its reduction as shown by spectroscopic measurements (Fig. 1) with the rapid development of the reduced heme bands at 521 and 550 nm. At a 1 μM concentration of ferritin, 32 μM of cytochrome are fully

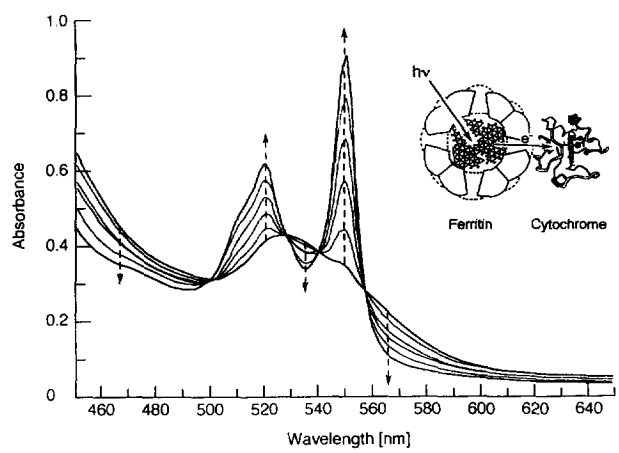

Fig. 1. Photoreduction of cytochrome c sensitized by ferritin. Time illumination (from the bottom upwards at 521 and 550 nm): 0, 15, 35, 55, 75, 115 min. De-oxygenated reaction mixture contained 1 μM horse spleen ferritin (from Sigma), 32 μM horse heart cytochrome c (from Fluka) and 50 mM TES (N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid), pH = 7.0. Illumination was performed by using a 450 W Xenon lamp equipped with a water filter and a 440 nm cut-off filter. Inset: schematic representation of proposed path for the photoreduction of cytochrome c.

reduced. As stated in the materials and methods section, each ferritin structure contains approximately 800 Fe atoms. However, as the Fe in the ferritin core is in the oxidant state (III), it cannot act as an electron donor. The iron remains in the Fe(III) state before and after the reaction. Hence, the photoreaction is catalytic with respect to iron. As stated previously, in the absence of exogeneous electron donors, the apoferritin structure is depleted of electrons, rendering the reaction non-catalytic with respect to the ferritin shell under these conditions. The rate of cytochrome photoreduction is dependent on light intensity as well as on the concentration of both ferritin and cytochrome. Ferritin strongly absorbs in the UV and visible regions as a result of light absorbance by the ferrihydrite mineral core. There are no designated characteristic bands in the absorption spectrum of ferrihydrate. The apoferritin absorbs light only in the UV-B region with an absorbance maximum at 280 nm. These absorbances result in a broad absorption band maximizing in the UV and diminishing in the visible with a relatively flat plateau between approximately 600 and 800 nm [23]. In order to elucidate the origin of the photoreceptor of the cytochrome photoreaction experiments were performed employing various spectral ranges of light excitation. Photoactivity of ferritin was witnessed in a wavelength domain corresponding to light absorption by the ferritin mineral core, i.e., at wavelengths shorter than 540 nm. Cytochrome photoreduction was observed under both aerobic and anaerobic conditions, and addition of sodium tartrate to the reaction mixture led to a marked increase in the reaction rate (Fig. 2). Oxalate, citrate and malonate enhances the cytochrome reduction as well. In the presence of glutathion, cysteine and sulfite a dark reduction of cytochrome c was observed, the rate of this reduction being enhanced by illumination. In the absence of electron donors (exogenous to the apoferritin structure, but within the surrounding medium), cytochrome reduction did not depend on the pH of the reaction medium within the range of 7–9; however, its rate decreased at pHs lower than 7. In the presence of sodium tartrate the rate of the photoreduction was independent of pH within the range of 5.5–8.5.

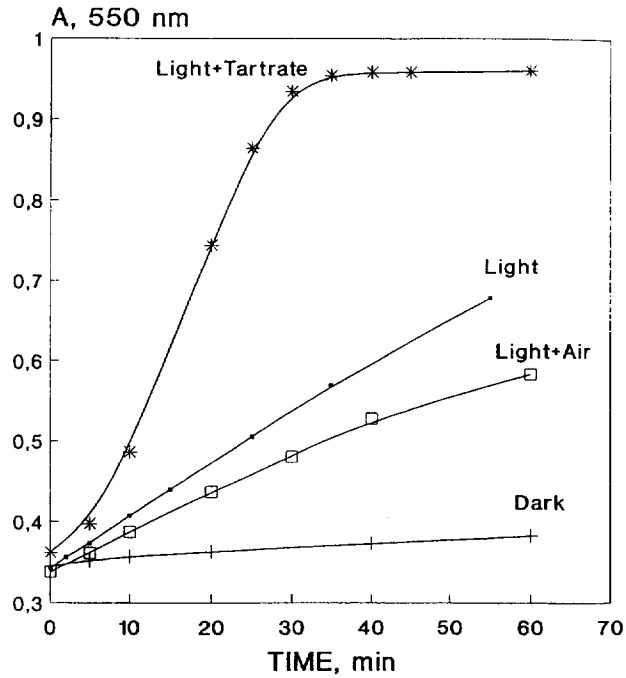

Fig. 2. Effect of tartrate and oxygen on photoreduction of cytochrome c sensitized by ferritin. Conditions are as described in Fig. 1. The solution was not de-oxygenated in the case (Light + Air) and 0.01 M sodium tartrate was added in the case (Light + Tartrate).

3.2. Photoreduction of viologens

Viologens, quaternary salts of N,N′-R,R′-4,4′ bipyridinium cations, are widely employed in the studies of photophysical and photochemical properties of inorganic semiconductors, first, due to easily discernible changes in the absorption spectra of their oxidized and reduced forms, and, secondly, because their reduction is independent of the pH of the medium. In experiments with ferritins, viologens have previously been employed for the reduction of the ferritin iron core and elucidation of its redox properties [37,38]. The *o,o′*-xylene double-bridged dimer viologen, DV^{4+} [39], with a redox potential of $E^\circ = 0.0$ V(NHE) and propylviologen sulfonate, PVS° ($E^\circ = -0.36$ V(NHE)), were used as electron acceptors for investigation of ferritin photocatalytic properties. In contrast to cytochrome photoreduction, ferritin-catalyzed reduction of DV^{4+} and PVS° is observed exclusively under anaerobic conditions. Photoreduction of DV^{4+} occurs in the absence of an additional electron donor, although the maximum obtained concentration of photoreduced DV^{4+} in this case did not exceed 1 μM. The presence of tartrate at a concentration of 0.02 M markedly enhanced the reaction rate and allowed complete DV^{4+} reduction. The photoreduction of DV^{4+} was observed between pH 6 and 8 and inhibited at pH < 5.5. The photoreduction of PVS° was observed only at pH greater than 9.0 (Fig. 3), no reduction being observed at lower pH. Using the time-resolved laser pulse photolysis technique, kinetic curves for PVS° photoreduction on a microsecond time scale (Fig. 4) were obtained. Analysis of these kinetic curves demonstrated PVS° photoreduction within 4 μsec after the laser flash ($k_{obs} = 10^6\ s^{-1}$). It should be noted that with the laser excitation of $\lambda = 347$ nm used in our experiments, as in continuous photolysis by light at wavelengths greater than 330 nm, viologen reduction is also observed in the absence of ferritin. This reduction is apparently related to the photoactivation of the PVS°/tartrate complex in this wavelength domain. The photochemistry of the charge transfer complex of viologens with carboxylic acid has been the subject of several studies [40,41]. However, not only was the extent of the reduction considerably less than in the presence of ferritin, but the reaction's kinetic parameters were different as well (Figs. 3 and 4). Under visible light illumination at wavelengths longer than 440 nm, neither PVS°, nor DV^{4+} are reduced in the absence of ferritin, thus demonstrating the photocatalytic activity of ferritin.

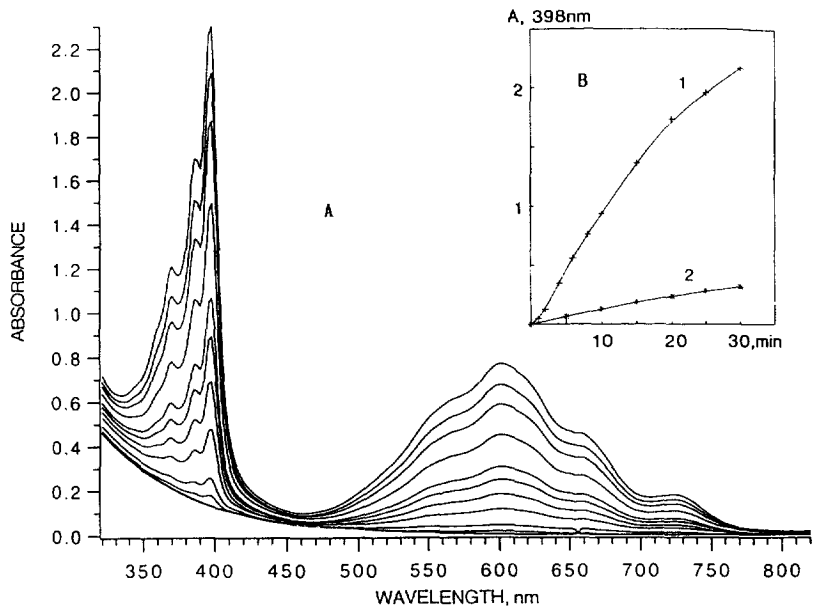

Fig. 3. (A) Photoreduction of propylviologen sulfonate sensitized by ferritin. Time illumination (from the bottom upwards at 398 and 602 nm): 0, 1, 2, 4, 6, 8, 10, 15, 20, 25, 30 min. De-oxygenated reaction mixture contained 0.2 μM ferritin, 1 mM propylviologen sulfonate, 0.1 M tartrate and 50 mM CAPS (3-(cyclohexylamino)-1-propane sulfonic acid), pH 10.0. Illumination was as in Fig. 1 with a 330 nm cut-off filter. (B) Kinetics' curves of propylviologen photoreduction in the presence of ferritin (**1**) and in the absence of ferritin (**2**).

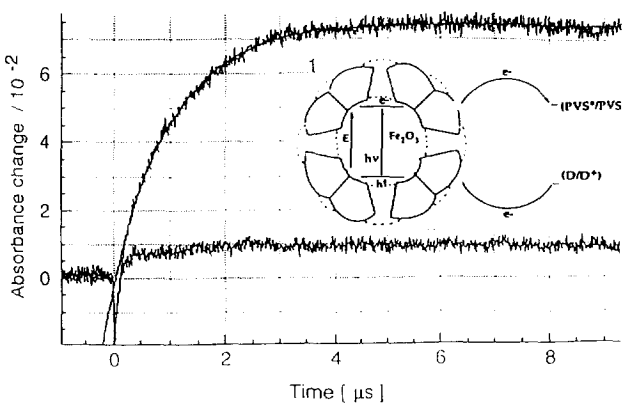

Fig. 4. Time profiles of the 602 nm absorption of reduced propylviologen sulfonate (PVS^-) formed by laser-pulse excitation ($\lambda_{exc}=347$ nm). De-oxygenated reaction mixtures were as described in Fig. 3; (**1**) in the presence of ferritin (**2**) in the absence of ferritin. The smooth curve drawn through the data trace **1** represents the best fit obtained by non-linear optimization of the parameters of a first order rate kinetic equation $\Delta A = C + B\exp(-At)$ with $A = 9.5 \cdot 10^5\ s^{-1}$, $B = -0.036$ and $C = 0.074$. Inset: schematic representation of proposed path for PVS's photoreduction.

4. Discussion

The photochemically active element of ferritin is its mineral core, allowing light absorption in the visible part of the spectrum. Ferrihydrite microcrystals forming the core, as other iron oxides, are semiconductor particles. Absorption of photons whose energy exceeds the width of the semiconductor bandgap causes the generation of electron-hole pairs energetic and long-lived enough to be involved in redox reactions with the components of the surrounding medium. The valence band holes oxidize the electron donors (carboxylic acid, thiol compounds, sulfite) added to the medium surrounding the ferritin structure or, in their absence, the ferritin protein shell itself and/or the buffer molecules. The electrons photogenerated in the valence band are involved in the interfacial electron transport reactions with electron acceptors, e.g., cytochrome c or viologens.

Since the ferritin mineral core is surrounded by a protein shell of ~2.5 nm thick, possessing intrasubunit channels of 0.4–0.5 nm diameter [1–3], the question arises as to the photoreduction mechanism involving cytochrome c and viologens, these molecules being much larger than the pore size. It has been shown earlier that the dark redox reactions involving the ferritin mineral core and large reductants (flavoproteins and ferredoxins) or oxidants (cytochrome c and Cu(II)-proteins) proceed in the absence of electron mediators or Fe^{2+} chelators [24]. The authors of these studies have suggested that long distance electron transfer between the ferritin mineral core and the external redox reagents occurs by electron tunneling through the ferritin protein shell. Reversible dissociation of ferritin polypeptide subunits was likewise postulated in order to explain the direct contact of large reagents with the ferritin core [9,25]. In this study, cytochrome reduction by the irradiated ferritin core was accounted for by direct electron transfer through the ferritin protein shell (Fig. 1, inset) as was assumed to occur in the dark reduction of cytochrome c by viologen reduced ferritin [24]. As an alternative mechanism, the participation of Fe^{3+} and Fe^{2+} ions in the electron transport may be proposed. These ions are bound by the polypeptide ferritin shell or are formed in the course of the photoinduced dissolution of ferrihydrite microcrystals and could be used to mediate electrons

from the ferritin core to electron acceptors. The use of ferritin treated with 1 mM EDTA (with subsequent purification on a Sephadex G25 column) proved that Fe^{3+} ions loosely bound to the shell were not involved in the photoreduction of cytochrome and viologens.

To confirm, or eliminate, the possibility of participation in the reaction of Fe^{2+} formed by ferrihydrite photocorrosion, we studied in more detail the ferritin photoreduction. Illumination of ferritin solutions containing electron donor, under anaerobic conditions, leads to a gradual ferritin bleaching (Fig. 5). An assay of the reaction medium for Fe^{2+} demonstrated complete photoreduction of Fe^{3+} to Fe^{2+}. Fe^{2+} ions formed during the reductive photodissociation are capable of reducing cytochrome and DV^{4+}. However, it should be noted that the processes of ferritin core photodissolution and the ferritin-photocatalyzed reduction of electron acceptors have different pH dependencies. The photocorrosion rate is diminished as the pH increases, and, at pH 7.5, photocorrosion is no longer observed. The rate of cytochrome c photoreduction in the absence of added donors is, however, constant between pH 7–9 and decreases upon acidification of the medium. In the presence of tartrate the photoreduction of cytochrome is independent of the acidity of the medium in the pH range 5.5–8.5. Ferritin-photocatalyzed PVS° reduction is observed only at pH > 9 under conditions where the photocorrosion of iron oxide is unfavorable. From these experimental observations, the conclusion can be drawn that cytochrome and viologens are reduced principally by direct transfer of electrons photogenerated in the ferritin mineral core, although the possibilities of the photoreduction with participation of the Fe^{2+} ion at pHs < 7 and the direct contact of cytochrome and viologens with the ferritin core due to reversible dissociation of ferritin polypeptide subunits cannot be excluded. In addition, under aerobic conditions, the superoxide-radical, whose formation is possible in the ferritin-photocatalyzed oxygen reduction ($E^\circ = -0.35$ V at pH = 7.0), may act as the cytochrome reductant.

Mammalian ferritin from horse spleen undergoes an electrochemical or chemical reduction reaction in which each iron atom present is reduced by one electron [26–28]. The midpoint reduction potential for mammalian ferritin is −0.190 V [28]. Chemically reduced ferritin reduces cytochrome c ($E^\circ = 0.240$ V), plastocyanin ($E_{1/2} = 0.350$ V), stellacyanin ($E_{1/2} = 0.350$ V), O_2 and $Fe(CN)_6^{3-}$ [24]. Under irradiation, horse spleen ferritin can behave as a photocatalyst for interfacial oxidation–reduction reactions and is capable of photoreducing acceptors having redox potentials from +0.240 V (potential of cytochrome c) to −0.360 V (potential PVS°) vs NHE. There is a high probability of redox photoprocesses occuring in ferritin containing plant and bacterial cells. Undoubtedly, the possibility of exciting ferritin in mammalian cells by light irradiation is not great in vivo, although absorption of light by ferritin contained in blood and skin could be possible and most likely occurs. M. Aubailly, et al., [29] have shown that irradiation of mammalian ferritin with UVA (365 nm) induces reductive mobilization of Fe^{3+} to Fe^{2+} and subsequent photo-oxidation of proteins and lipoproteins in vitro. Since UVA (365 nm) radiation penetrates deeply into the dermis (50–60 μm), they concluded that ferritin present in fribroblasts and macrophages of dermis may participate in processes leading to skin photodamage induced by UVA and UV light resulting in photoaging and carcinogenesis. Investigations discussed in this article underline possible constructive photoprocesses that ferritins might contribute to in living organisms, e.g., photoreduction of cytochromes and other biological com-

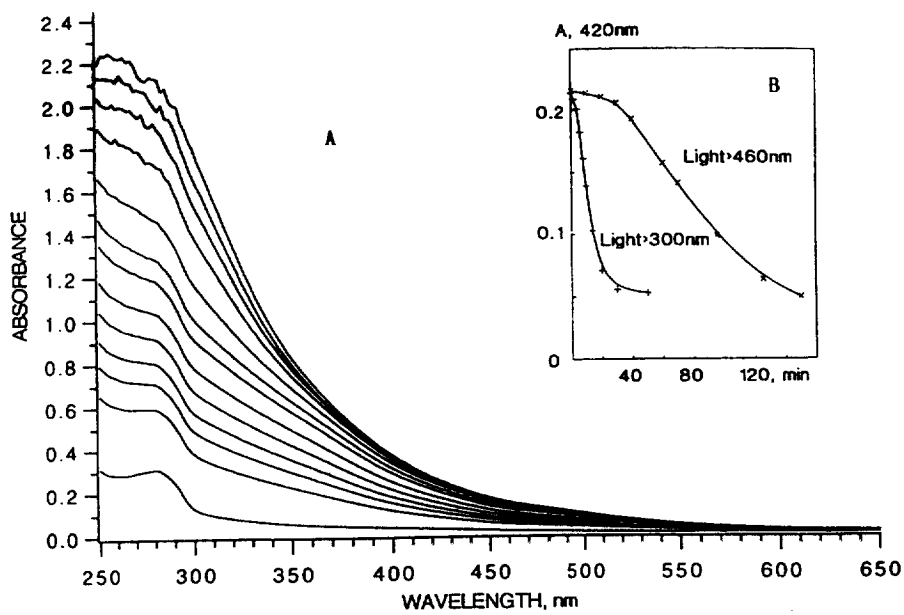

Fig. 5. (A) Photoreduction of ferritin. Time illumination (from the top downwards): 0, 2, 4, 6, 10, 15, 20, 30, 40, 60, 75, 155 min. De-oxygenated reaction mixture contained 0.5 μM ferritin, 0.02 M tartrate and 50 mM MES (2-(N-morpholino)ethane sulfonic acid), pH = 5.5. Illumination was as in Fig. 1 with a 300 nm cut-off filter. (B) Kinetics' curves of ferritin photoreduction under illumination by different wavelengths of light.

pounds having even more negative redox potentials. Insight is gained concerning the possible mechanisms of ferritin's participation in destructive photoprocesses under visible light, i.e., the photoactivated reductive mobilization of Fe^{2+} in the presence of electron donors and the photosensitized oxidation by ferritin of compounds such as carboxylic acids, thiocompounds, etc. Insight into ferritin's physiological functions as well as its inherent physical properties can be gained through a systematic study of photo-redox reactions involving light activation of the ferritin mineral core.

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