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Title: The alkaline transition of blue copper proteins, Cucumis sativus plastocyanin and Pseudomonas aeruginosa azurin

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Abstract: Autoreduction of Cucumis sativus plastocyanin and Pseudomonas aeruginosa azurin took place at alkaline pHs, having been accompanied by the decrease in the intensities of the charge transfer band, Cys-S-(π)→Cu(II) at 597 and 626 nm, and the Cu(II)-EPR signals with small AII values of 6.5 x 10-3 and 5.3 x 10-3 cm-1 for plastocyanin and azurin, respectively. Further, an extra Cu(II)-EPR signal with a large AII value of 21 x 10-3 cm-1 also reversibly emerged with increasing pH. Plastocyanin and azurin are in an equilibrium of the three forms at alkaline pHs

Dear Professor Richard Cogdell
Editor FEBS Letters

Thank you very much for your suggestion to tidy us some of English before to submit the revised manuscript to FEBS Letters (FEBSLETTERS-S-05-01041R1). English was polished up by the professional English correction service as follows.

About twenty “the” and “a’ were deleted or added (not indicated).
About fifteen commas were added (not indicated).
Every “pH’s” was converted to “pHs”.
Single form was changed to plural form and vice versa for some points.
Other alterations are as follows.
P2. Line 3: “has not been known” was change to “has not been hitherto understood”.
P2. Line 16: “solved” was deleted.
P2. Line 17: “that” which had been deleted in the original manuscript was added.
P2. Line 23 and others: “high alkaline pH’s” was changed to “highly alkaline pHs”.
P3. Line 3: “till” was changed to “until”.
P3. Results and discussion, Lines 7 and 11 The order of words were changed from “slightly shifted” to “shifted slightly” and “in only” to “only in”.
P3. Line 5 from bottom: “with the spin Hamiltonian parameters” was added after “signal” and “typical to” was changed to “typical of”.
P4. Line 6: “recovered as that” was changed to “recovered to the level of that”.
P4. Line 11: “not only Pc was reduced” was changed to “not only was Pc reduced,”.
P4. Line 17: “the form to give” was changed to “the form giving”.
P4. Line 18: “In order to ascertain that” was converted to “In order to ascertain whether”.
P5. Line 21: “As conclusion” was changed to “In conclusion”.
P5. Line 3 from bottom: “under study” was changed to “being studied as to”.

Titles of cited papers were checked to be correct.

Sincerely yours,
Takeshi Sakurai

*** Response to Reviewers**

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Manuscript

The alkaline transition of blue copper proteins, *Cucumis sativus* plastocyanin and *Pseudomonas aeruginosa* azurin

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Abstract **Autoreduction of *Cucumis sativus* plastocyanin and *Pseudomonas aeruginosa* azurin took place at alkaline pHs, having been accompanied by the decrease in the intensities of the charge transfer band, Cys-S$^-$(π)→Cu(II) at 597 and 626 nm, and the Cu(II)-EPR signals with small A_{II} values of 6.5 x 10^{-3} and 5.3 x 10^{-3} cm^{-1} for plastocyanin and azurin, respectively. Further, an extra Cu(II)-EPR signal with a large A_{II} value of 21 x 10^{-3} cm^{-1} also reversibly emerged with increasing pH. Plastocyanin and azurin are in an equilibrium of the three forms at alkaline pHs.**

Keywords: Blue copper protein; plastocyanin; azurin; autoreduction

Footnote

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Abbreviations: EPR, electron paramagnetic resonance; Pc, plastocyanin; Az, azurin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DPPH, 1,1-diphenyl 2-picrylhydrazyl

1. Introduction

Blue copper proteins such as plastocyanin (Pc) and azurin (Az) function as electron shuttles in energy conversion systems, although the biological role of blue copper protein classified into phytocyanin has not been hitherto understood. Pc is widely distributed in the thylakoid membranes within the chloroplasts of higher plants, green algae and cyanobacteria, and transfers electrons from photosystem II to photosystem I [1]. Az is in the periplasmic space of several Gram-negative bacteria, such as *Alcaligenes*, *Achromobacter*, *Pseudomonas*,and *Methylomonas* [2]. Among the blue copper proteins, Pc from higher plants and Az from *Pseudomonas aeruginosa* have been extensively studied for their structure, spectroscopic properties and electron transfer reactions. The copper ion in Pc and Az is coordinated by 1Cys2His1Met [3], and additionally by a carbonyl group in a main chain for the latter [4], in a distorted tetrahedral geometry and a pseudotrigonal bipyramidal geometry, respectively.

Crystal structure analyses of Pc in the Cu(I) form revealed that one of the His ligands is eliminated from the coordination sphere and protonated at pH < 3.8 [5]. On the other hand, crystal structure analyses of Az in the Cu(II) form at pH 5.5 and 9.0 [6] and the NMR studies showed that a conformational change takes place due to the protonation/deprotonation equilibrium of the uncoordinated His35 with p*K*a ~ 7, while the absorption maximum is constant in the pH range 5 – 11 [7].

Irrespective of these changes on the structures of Pc and Az at acidic to neutral pH, we have observed that the blue color of multicopper oxidases, such as laccase and bilirubin oxidase containing the type I Cu, whose structure and properties are similar to the Cu in blue copper protein, becomes pale at highly alkaline pHs [8,9]. A similar phenomenon has not been reported for blue copper proteins, except for spinach Pc at the early stage of study [10]. In the present study, we demonstrate the detailed absorption and EPR spectral changes of *Cucumis sativus* (cucumber) Pc and *P. aeruginosa* Az from neutral to alkaline pH.

2. Materials and methods

Pc was purified from acetone powder of cucumber peelings using DEAE-cellulose chromatography and Sephadex G-75 gel chromatography as reported previously [11]. *P. areruginosa* Az purchased from Sigma was dissolved in 0.1 M phosphate buffer, pH 7.2, and dialyzed against the same buffer. The purities of Pc and Az were checked by SDS-PAGE.

The pH value of the Pc and Az solutions (700 μl) was changed from 6.6 to 11.0 and 7.1 to 10.6, respectively, by the stepwise additions of ca. 1 μl of sodium hydroxide, monitoring with a

Horiba pH meter F-12 attached to a microelectrode 6069MP-10C. After each changing of the pH, the Pc and Az solutions were incubated for 30 to 60 min at 4 °C untill the decrease of the absorption band at 597 nm for Pc and that at 626-628 nm for Az stopped. The absorption spectra were measured on a JASCO Ubest-50 spectrometer at room temperature. EPR spectra were measured on a JEOL RE1X X-band spectrometer at 77 K and at room temperature. The total amount of the EPR-detectable Cu(II) was determined by double integration using Cu-EDTA as a standard. Signal intensities due to the differences in tuning conditions were calibrated using 1,1-diphenyl 2-picrylhydrazyl (DPPH) as an external standard. The experimental error was ca. 10%.

3. Results and discussion

Pc exhibits an absorption spectrum with the two prominent bands at 597 and 775 nm (Fig. 1A). The former band has been assigned as coming from the Cys-$S^-(\pi)\rightarrow$Cu(II) charge transfer and the latter from the d-d transitions [12,13]. This absorption feature of Pc did not change prominently at neutral pHs. However, the intensities of these bands conspicuously decreased at pH >9.5. At pH 11.0, their absorption intensities reached ca. 40% of those at pH 6.6. In contrast, the absorption maximum of 597 nm was kept constant between pH 6.6 and 10.3, although it shifted slightly to a shorter wavelength with increasing pH, such that it was 594 nm at pH 11.0. The d-d band at ca. 775 nm also did not shift at pH < 10.3, although it became difficult to determine the exact absorption maximum at pH > 10.3 because this band was considerably broad. Fig. 1B shows the molar extinction coefficient of the Cys-$S^-(\pi)\rightarrow$Cu(II) charge transfer band as a function of pH. The decrease in the absorption intensity of Pc at high pH has been reported only in a study on the spinach Pc at the early stage [10], in spite of the many spectroscopic studies on Pc. In order to ascertain that Pc was autoreduced, the Pc at pH 11.0 was reacted with a small amount of ferricyanide, and it was observed that the absorption spectrum analogous to that at pH 6.6 was spontaneously restored (not shown).

The EPR spectra measured at 77K also indicated that the content of Pc in the Cu(II) form decreased with increasing pH (Fig. 2). Only the Cu(II) signal with the spin Hamiltonian parameters, g_{II} = 2.24 and A_{II} = 6.5 x 10^{-3} cm^{-1} typical of blue copper protein is present in the spectrum at pH 6.6 (Fig. 2A) However, the intensity of the type I Cu signal began to decrease in harmony with the increase in pH. Unexpectedly, another Cu(II)-signal with the spin Hamiltonian parameters, g_{II} = 2.19 and A_{II} = 21 x 10^{-3} cm^{-1} increasingly emerged with increasing pH (Fig. 2B, the spectrum at pH 11.0), although its content was not high, even at pH 11.0. The total amount of EPR-detectable Cu(II) per protein molecule was apparently less than one, 0.6Cu(II) at pH 11.0 (0.5 Cu(II) for the signal with A_{II} = 6.5 x 10^{-3} cm^{-1} and 0.1Cu(II) for the signal with A_{II} = 21 x 10^{-3} cm^{-1}). When this Pc at pH 11.0 was reacted with a small amount of ferricyanide, the signal intensity due to the blue Cu center was almost recovered to the level of that at pH 6.6 (Fig. 2C), indicating that the content of the Cu species undetected by EPR originated from the autoreduction of Cu(II). Nevertheless, the new signal was still present at highly alkaline pHs. However, this extra signal disappeared when pH was returned to 6.6 (Fig. 2D). The corresponding EPR measurements at room temperature (data not shown) guaranteed that freezing was not the cause of the behaviors observed above. Therefore, all these results of the EPR spectra indicate that not only was Pc autoreduced, but the new EPR signal with the larger hyperfine splitting reversibly emerged. It has been reported that the blue Cu center of Pc [10] and type I Cu of laccase [8] and bilirubin oxidase [9] are autoreduced at highly alkaline pHs. Therefore, at highly alkaline pHs, Pc is in an equilibrium of the three forms, the Cu(II) form, the form giving the new EPR signal and the form not giving the EPR spectrum.

In order to ascertain whether the behavior observed above is not limited to Pc, we performed similar experiments on Az. The absorption spectrum of Az did not change appreciably between pH 7 and 9, as has been reported [7]. However, at pH > 9, the blue color of Az gradually faded, having reached a constant intensity within ca. 30 to 60 min. The extent of the decrease in the absorption band at 626-628 nm was more conspicuous at higher pHs, although the wavelength of the absorption maximum did not change (Fig. 3A). The molar extinction coefficient of Az as a function of pH is shown in Fig. 3B. The value of ε was 5,700 at pH 7.1, but it decreased to 4060 at pH 10.6. This indicates that 29% of Az was converted into the species to be colorless and/or to have a weak absorption intensity as to be masked by the residual oxidized Az. This change in the absorption spectra depending on pH was reversible when the increase in pH was stopped at pH 10.6, but became partly irreversible when the increase in pH was continued, because the protein molecule began to be fatally denatured at pH > 10.6.

The EPR spectra of Az at pH 7.1 and 10.6 are shown in Figs. 4A and 4B, respectively. The spectrum at pH 7.1 is the same as that has been reported hitherto with the spin Hamiltonian parameters, g_{II} = 2.27 and A_{II} = 5.3 x 10^{-3} cm^{-1} [7]. However, the EPR spectrum at pH 10.6 clearly indicates that another species with the spin Hamiltonian parameters, g_{II} = 2.18 and A_{II} = 21 x 10^{-3} cm^{-1} overlapped on the type I Cu signal. The intensity of this extra signal increased with increasing pH.

The double integration of the EPR spectrum at pH 10.6 indicated that the total amount of the EPR-detectable Cu(II) was 0.9 per protein molecule, of which 0.7Cu(II) came from the species with the small A_{II} value, and $0.2Cu^{2+}$ from the species with the large A_{II} value. The residual 0.1Cu was not detected both in the absorption and EPR spectra. Successive measurements indicated that 0.1Cu was not detected by EPR, suggesting that an EPR-undetectable species was present at highly alkaline pHs. The reaction of a small amount of ferricyanide with Az at pH 10.6 realized the increases in the absorption intensity at 626 nm and the type I Cu EPR signal, indicating that type I Cu had been autoreduced at highly alkaline pHs, similarly to the case of Pc.

As for the origin of the autoreduction, no reducing equivalent was present in the solution. Takabe et al. [10] reported that the autoreduction of spinach Pc was in parallel with the exposure of a hydrophobic site near the ligand Cys to the solvent. On the other hand, the theoretical studies repeatedly indicated that the radical center is both on the central Cu atom and S atom of the Cys residue as a ligand [14-16]. If the radical center moves from Cu(II) to S^-, $S^\cdot$ – Cu(I) is obtained. This species will not give the Cys-$S^-(\pi)\rightarrow$Cu(II) charge transfer band in the absorption spectrum and the EPR signal typical to the oxidized blue Cu center. The S radical directly bound to the metal ion may be EPR-undetectable because its relaxation time will be significantly affected by the adjacent Cu(I) ion. Younes et al. [17] have suggested the possibility that the Cu-S chromophore in blue copper proteins is delocalized, and that an equilibrium: RS^--Cu(II) = $RS^\cdot$-Cu(I) exists based on the model studies.

In conclusion, the Cu center in Pc and Az is in an equilibrium between the Cu(II) form, the Cu(I) form and the Cu(II) form to give the large A_{II} value in the EPR spectrum, and at highly alkaline pHs the latter two forms become increasingly favorable. At present, it seems difficult to speculate about the structure of the new Cu(II) species. Since the Cu ions in both Pc and Az are coordinated by 1Cys2His1Met, phytocyanins coordinated by 1Cys2His1Gln are being studied as to whether they also exhibit analogous behavior or not, although we have observed that cucumber plantacyanin, now also called cucumber basic blue protein, gives the two different rhombic EPR signals depending on the pH [18].

Figure Legends

Fig. 1. Absorption spectra of *Cucumis sativus* plastocyanin as a function of pH. (A)

Absorption spectra of Az at pH 6.6 and 11.0. (B) Change of the molar extinction coefficient, ε at 597 nm. Protein concentration, 20 μM. Cell path 1 cm.

Fig. 2. The X-band EPR spectra of *Cucumis sativus* plastocyanin at pH 6.6 (A), at pH 11.0 (B), B reacted with a small amount of ferricyanide (C), and C as returned to pH 6.6 (D). The protein concentration was 20 μM. Measurement conditions: temperature 77 K, frequency 9.19 GHz, power 3.125 mW, modulation 100kHz and 1.0 mT, amplitude 125, sweep time 4 min (8 min for the expanded spectra (x4)), filter 0.3 s (1 s for the expanded spectra (x4)).

Fig. 3. Absorption spectra of *Pseudomonas aeruginosa* azurin as a function of pH. (A) Absorption spectra at pH 7.1 and 10.6. (B) Change of the molar extinction coefficient, ε at 626-628 nm. Protein concentration, 28 μM. Cell path 1 cm.

Fig. 4. The X-band EPR spectra of *Pseudomonas aeruginosa* azurin at pH 7.1 (A) and pH 10.6 (B). Measurement conditions: temperature 77 K, frequency 9.19 GHz, power 3.125 mW, modulation 100kHz and 1.0 mT, Amplitude 125, sweep time 4 min (8 min for the expanded spectra (x4)), filter 0.3 s (1 s for the expanded spectra (x4)) .

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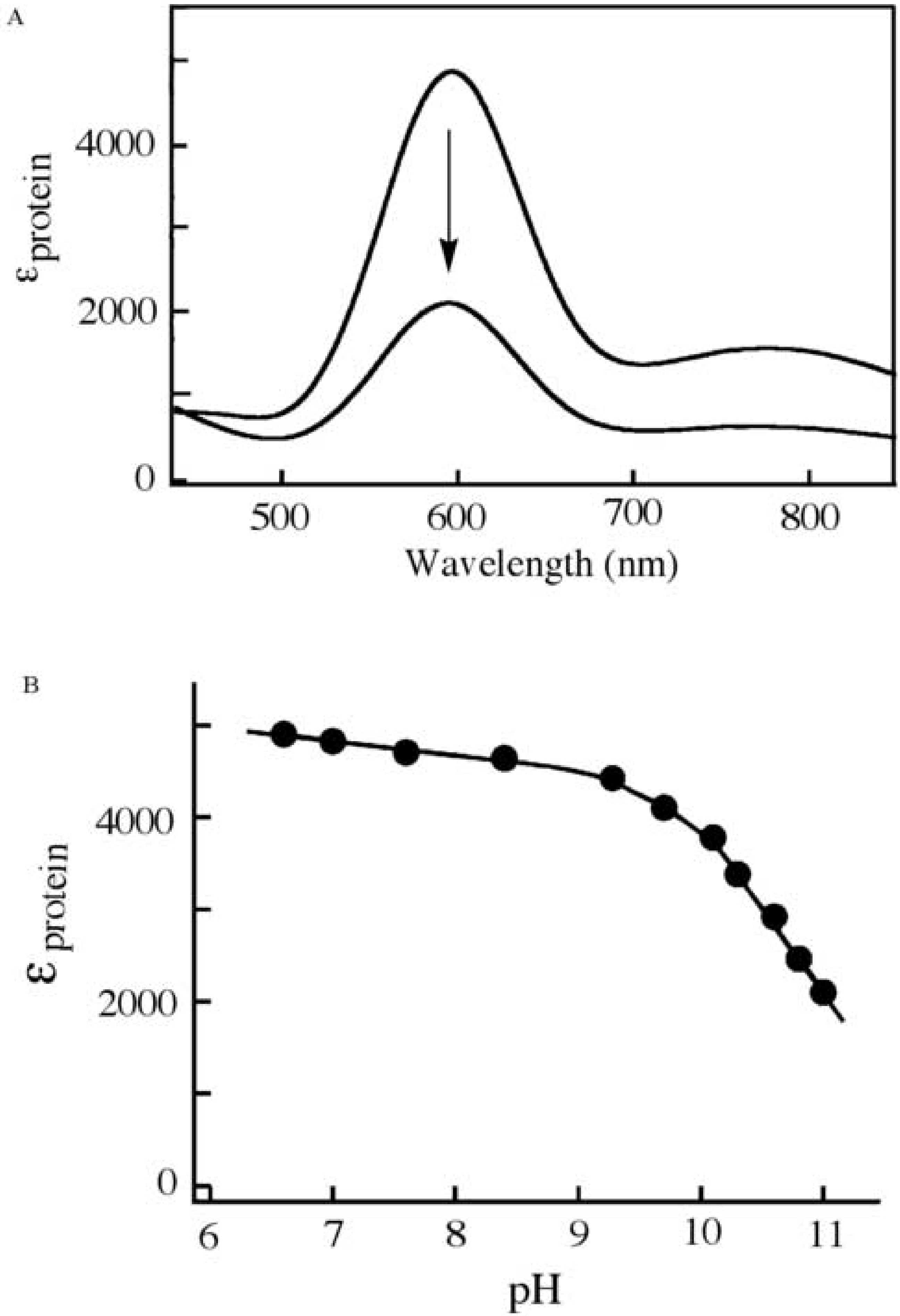

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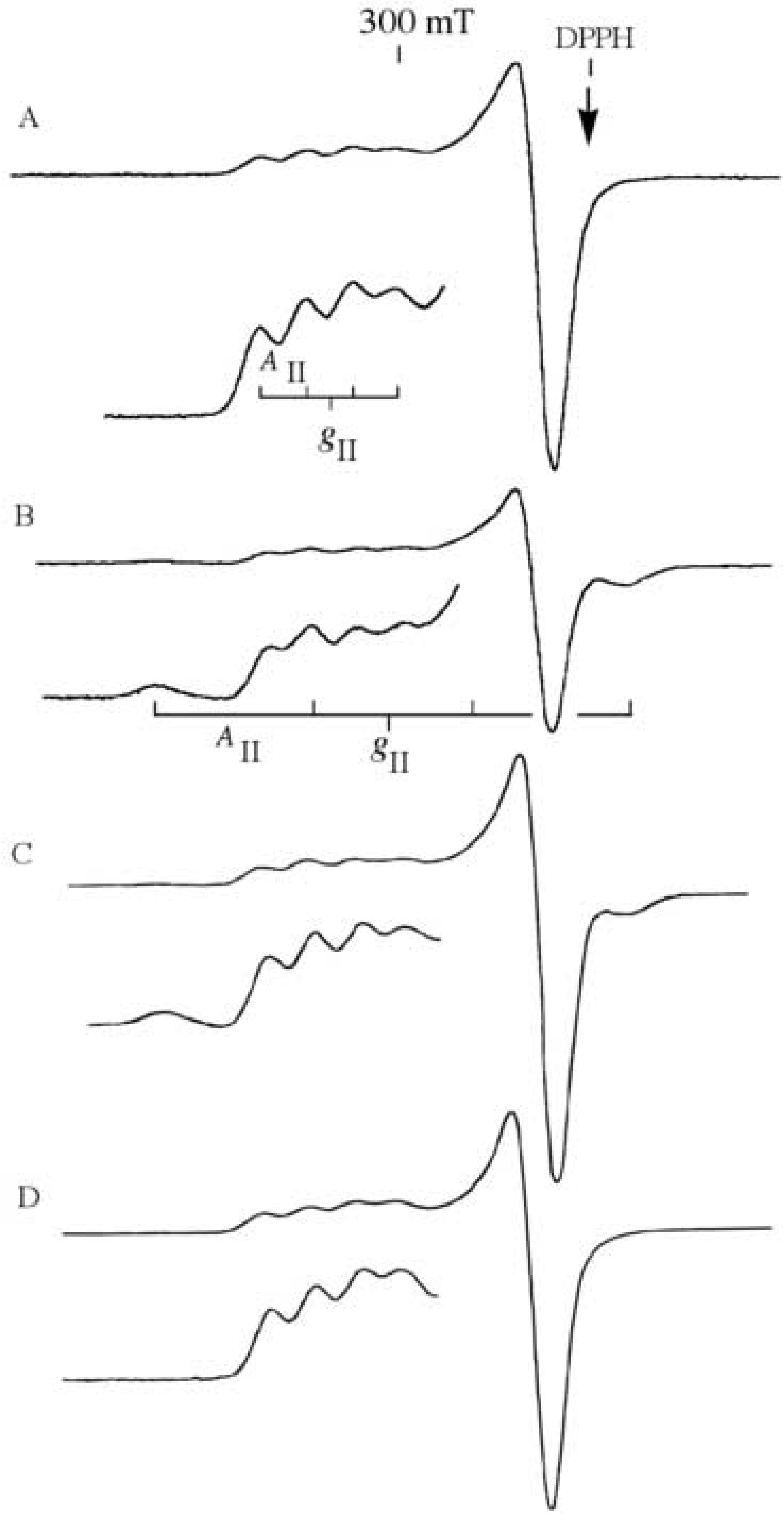

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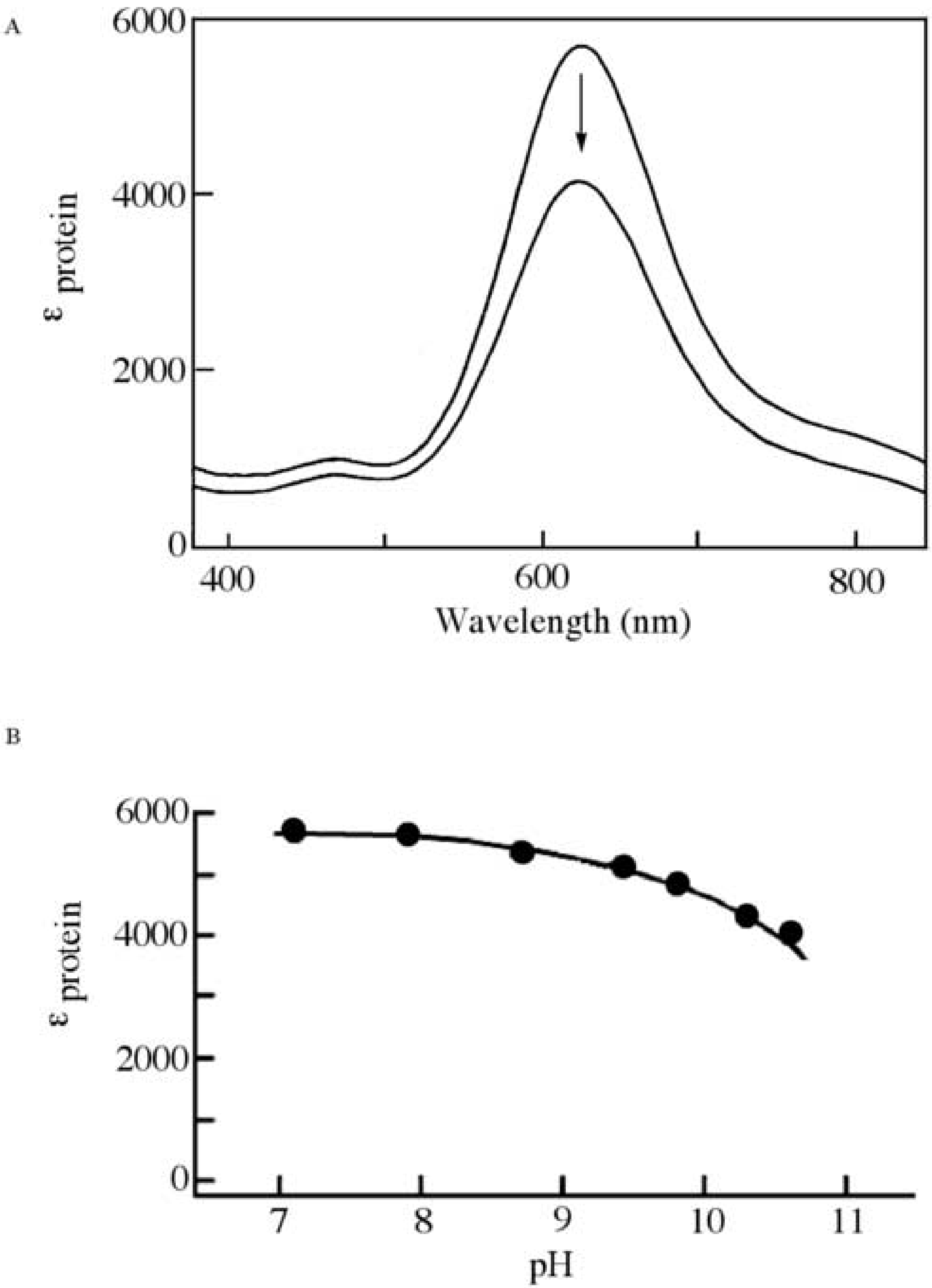

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