



Fluorescence assay for monitoring Zn-deficient superoxide dismutase in vitro

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Abstract

A method has been developed for selective detection of the zinc-deficient form of Cu, Zn superoxide dismutase (SOD1) in vitro. Zinc-deficient SOD1 mutants have been implicated in the death of motor neurons leading in amyotrophic lateral sclerosis (ALS or Lou Gerhig's disease). Thus, this method may have applicability for detecting zinc-deficient SOD1 mutants in human ALS patients samples as well as in a transgenic mouse model of ALS and in cultured motor neurons. We determined previously that structural analogs of 1,10 phenanthroline, which react specifically with Cu(I), react with the active Cu(I) of SOD1 when zinc is absent, but not when zinc is also bound, as evidenced by the fact that the reaction is inhibited by pretreatment of the enzyme with zinc. We report herein that bathocuproine, or its water-soluble derivative bathocuproine disulfonate, react with zinc-deficient SOD1 to form a complex which fluoresces at 734 nm when excited at 482 nm. Fluorescent intensity is concentration dependent, thus we propose to use fluorescent confocal microscopy to measure intracellular levels of zinc-deficient SOD1 in situ.

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the relentless death of motor neurons, resulting in progressive paralysis and atrophy of all voluntary muscles, including muscles of mastication, swallowing, and breathing. Survival after diagnosis is

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typically less than 5 years and there is no effective treatment.

Mutations to superoxide dismutase (SOD1) have been found in roughly 2% of ALS cases and this remains as the only known cause of ALS. However, there are no clinical or histopathological differences between SOD1-mediated and non-SOD1-mediated disease, leading many investigators to conclude that all forms of ALS may have a common, SOD1-based etiology. We have put forth a hypothesis which explains: (1) how more than 90 different single amino acid mutations can all result in the same toxic phenotype, (2) why motor neurons are particularly susceptible, and (3) how wild-type SOD1 can become toxic in the same manner as mutant enzymes and thereby cause all forms of ALS. The hypothesis holds that the balance between normal and toxic functioning of SOD1 hinges upon whether or not zinc is bound in the active site of the enzyme. Therefore, the ability to specifically identify and quantify zinc-deficient SOD1 is strategically important for further characterization of SOD1-mediated toxicity in ALS.

SOD1 normally functions to catalytically remove the oxygen radical superoxide ($O_2^{\bullet -}$) and, thereby, limit superoxide-dependent toxicity. SOD1 is present in cells at a very high concentration ($\geq 10 \mu\text{M}$ [1]) relative to most enzymes. The active site of SOD1 has one copper ion and one zinc ion bound by a common histidyl residue. The copper ion undergoes a one-electron reduction and reoxidation during one complete catalytic cycle and is essential for enzyme activity whereas the zinc ion does not redox cycle and has historically been considered to serve a mainly structural role.

The precise biochemical mechanism by which SOD1 mutants cause ALS remains to be elucidated. One of the leading hypotheses extends from findings that zinc binding affinity is substantially decreased in mutant enzymes; from these findings, it is reasonable to infer that the mutations alter the protein's conformation and de-optimize the zinc binding ligands [2–4]. Initially, it was not clear how a change in zinc binding affinity could translate into a toxic gained function. However, recent studies strongly suggest that bound zinc acts to maintain a rigid and tightly constrained active site such that compounds larger than superoxide

are excluded from interacting with the copper ion. Put another way, zinc may function to help maintain substrate specificity of SOD1 and avoid promiscuous reactions with other low molecular weight reductants and oxidants.

In the absence of zinc, bulkier reductants such as ascorbic acid, urate, NADH, and glutathione all are capable of entering the active site and reducing Cu(II) to Cu(I) [5]. The net effect of this abnormal copper reduction is to permit the enzyme to transfer electrons from cellular antioxidants to molecular oxygen and thereby generate superoxide, i.e., to run in reverse. The rate of this reverse reaction is limited by the competing forward reaction (rate constant for superoxide reacting with C(I)–SOD1 is $2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$), thus the equilibrium disfavors the production and release of free superoxide from Cu(I)–SOD1 and oxygen. However, superoxide reacts with nitric oxide (NO) eight times faster than it reacts with SOD1 and the reaction of superoxide and NO produces the potent cytotoxin and nitrating agent peroxynitrite. NO is a small, uncharged molecule, which can readily enter the active site of SOD1 and can actually shift the equilibrium in favor of superoxide production from Cu(I)–SOD1 and oxygen; the net result is a 'peroxynitrite synthase' activity (of zinc-deficient SOD1) that is largely driven by the concentration of NO.

Because zinc-deficient SOD1 can abnormally utilize cellular antioxidants, oxygen, and NO to catalytically produce peroxynitrite means that even a small fraction of the total cytosolic SOD1 existing in a zinc-deficient state would result in continuous production of this potent cytotoxin. The mutations may serve merely to increase the probability that the mutants would exist in a zinc-deficient state. In this way, all 90 or so single amino acid mutations associated with ALS could result in formation of the same toxic phenotype via effects on zinc binding affinity. Delivery of zinc-deficient SOD1 into primary motor neurons in culture has been shown to initiate NO-dependent, apoptotic cell death [5]. Interestingly, even wild-type SOD1 was found to be toxic when deficient in zinc, providing additional evidence that toxicity was a function of the absence of zinc and not the mutation per se. This finding offers the intriguing

possibility that all forms of ALS can be explained on the basis of a gained function for SOD1 since one need only up-regulate the biosynthesis of other zinc binding proteins to out-compete apo wild-type SOD1 for zinc and result in formation zinc-deficient wild-type enzyme.

Evidence of a role for NO and peroxynitrite in ALS exists in the form of increased levels of both free and protein-bound nitrotyrosine in lower motor neurons of transgenic mice and in both upper and lower motor neurons in individuals with ALS [1,6–8]. However, additional support for this hypothesis in the form of actual measurement of zinc-deficient SOD1 in situ, has remained elusive. The essential problem relates to changes in metallation states upon cell lysis; zinc-deficient SOD1 readily rebinds zinc and is likely to exist in a zinc-deficient form only because of dynamic competition with other zinc binding proteins for available cytosolic zinc. A method for measuring zinc-deficient SOD1 in intact cells would avoid the problems associated with changes in metallation state upon cell disruption.

Direct measurement of zinc-deficient SOD1 in situ would not only permit this hypothesis to be tested, but would also provide a way to monitor the effects of any treatments aimed at decreasing the levels of zinc-deficient enzyme within neurons. We have identified two properties of zinc-deficient SOD1 that serve as the basis for a sensitive and highly selective fluorescence-based assay. Zinc-deficient SOD1 differs from the zinc-containing enzyme in two important respects: (1) it is rapidly reduced by reductants such as ascorbate and (2) it reacts with the Cu(I) specific chelator bathocuproine disulfonate (BCDS). Reaction of zinc-deficient Cu(I)–SOD1 with BCDS results in formation of a visible complex with a maximum of absorption at 482 nm; excitation of this complex results in a fluorescent signal in the region of 680–750 nm. Conversely, reaction of BCDS with zinc-deficient SOD1 quenches the intrinsic fluorescence of BCDS (ex/em = 320/400 nm). Because the fluorescence characteristics of the BCDS/SOD1 complex are essentially identical to those of free Cu(I)–BCDS, we are cannot distinguish between BCDS complexes with free Cu(I) and Cu(I)–SOD1. However Cu(I) ions do not exist in a free

form in cells and, even if free Cu were present, their reaction with BCDS would not be inhibited by pre-treatment with zinc as would the reaction of BCDS with zinc-deficient SOD1. Therefore, the existence of zinc-inhibitable fluorescence of a BCDS–Cu(I) complex would strongly indicated the presence of zinc-deficient SOD1 in cells.

2. Material and methods

D124N (replacement of aspartate by asparagine at position 124) is a site-directed human SOD1 mutant which has been shown to bind zinc with much less affinity than does wild-type enzyme [9]. D124N binds zinc poorly even in the presence of free zinc and is, therefore, used as a model enzyme to investigate the spectral properties of the zinc-deficient enzyme. Recombinant D124N SOD1 was prepared by expressing the mutated protein in *E. coli* as described previously [3,4].

As a source of free Cu, we used CuSO₄ at the appropriate concentrations. Zinc-deficient SOD1 and CuSO₄ were reduced to the respective Cu(I) forms by ascorbic acid followed by addition of BCDS. The pH of solution was maintained at 7.2 with 100 mM potassium phosphate buffer.

Absorption spectra were measured with Shimadzu UV–vis–NIR Spectrophotometer. Frequency doubled radiation at 480–490 nm of LiF:F₂⁺** tunable laser (400–600 nm) was used for fluorescence excitation. The average power of radiation was about 10 mW at repetition rate of 26 Hz and 50 ns pump pulse duration. A spectrometer (Acton Research 150) equipped with a TEC CCD camera was used for fluorescence detection at a 90° angle to the excitation light.

3. Results

3.1. Absorption

The addition of BCDS to CuSO₄ or zinc-deficient SOD1 which had been reduced by ascorbic acid, led to formation of colored complexes. The absorption spectra have a specific band at 482 nm with a FWHM of about 80 nm.

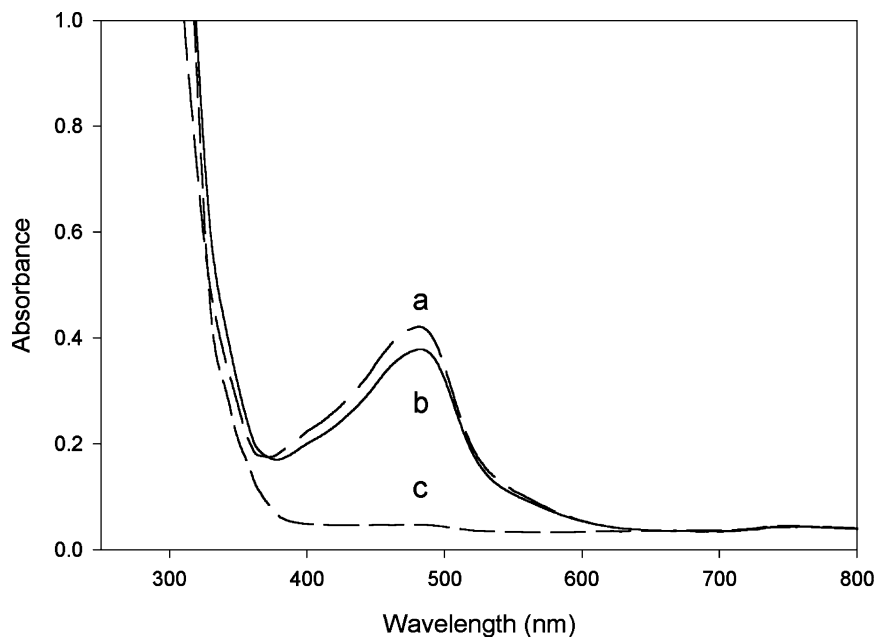


Fig. 1. Absorption spectra of (a) mixture of D124N SOD1 0.8 mg ml^{-1} , BCDS 0.1 mM and ascorbic acid 0.05 mM , (b) mixture of 0.05 mM CuSO_4 , 0.1 mM BCDS, and 0.05 mM ascorbic acid (c) mixture of metal replete wild-type SOD1 (0.8 mg ml^{-1}), 0.1 mM BCDS, and 0.05 mM ascorbic acid, in 100 mM potassium phosphate buffer.

These spectra are shown in Fig. 1a and b. BCDS is known to form high affinity 2:1 tetrahedral complexes with free Cu(I). As is readily apparent, the absorption spectrum of $(\text{BCDS})_2\text{-Cu(I)}$ is identical to that obtained with BCDS and zinc-deficient Cu(I)-SOD1. It is noteworthy that BCDS has no absorption at 482 nm . Therefore fluorescence of BCDA will not be excited under resonance excitation of $(\text{BCDS})_2\text{-Cu(I)}$ complexes.

UV-vis spectra indicated that BCDS does not react with metal replete (i.e., zinc- and copper-containing) wild-type SOD1 (Fig. 1c). We have determined previously that the reaction of BCDS with the active site copper atom is prevented by the presence of enzyme-bound zinc; no reaction with BCDS is seen even when the copper is reduced by ascorbate prior to adding zinc.

3.2. Fluorescence

The absorption band at 482 nm of the $(\text{BCDS})_2\text{-Cu}$ complex is not in resonance with BCDS. However, the same wavelength of laser

radiation excites a relatively strong fluorescence emission of BCDS, as can be seen in Fig. 2.

$(\text{BCDS})_2\text{-Cu(I)}$ complex formation is accompanied by the appearance of a new band centered at 734 nm with FWHM 150 nm and diminishing of background fluorescence of free BCDS. At a copper concentration twofold greater than that of BCDS (or greater), the fluorescence of BCDS was completely quenched and we observed the fluorescence of only the $(\text{BCDS})_2\text{-Cu(I)}$ complex. The spectrum of the $(\text{BCDS})_2\text{-Cu(I)}$ complexes is shown in Fig. 3.

Similar features appear in the fluorescence emission spectra of BCDS complexes with D124N (zinc-deficient) SOD1. However, in contrast to the experiments with free Cu(I), the fluorescence of BCDS was not quenched completely even at concentrations of zinc-deficient SOD1 several times higher than that of BCDS. The overall spectrum shown in Fig. 4 is a superposition of fluorescence signal of BCDS, the $(\text{BCDS})_2\text{-Cu(I)}$ complex, and Raman scattering of water at 3400 cm^{-1} (576 nm). One can see that fluorescence of the $(\text{BCDS})_2\text{-Cu(I)}$ complex strongly

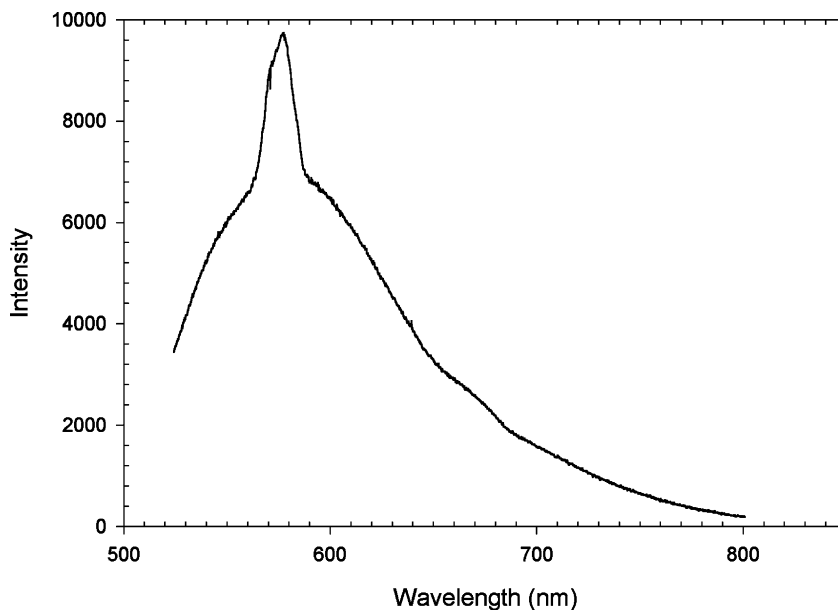


Fig. 2. Fluorescence spectrum of BCDS in 100 mM potassium phosphate buffer excited at 482 nm. The relatively narrow band at 576 nm corresponds to the Raman scattering of water.

overlaps with fluorescence of BCDS and could not be trivially resolved in the spectral region from 650

to 750 nm. We therefore performed the deconvolution procedure in order to resolve the distinct

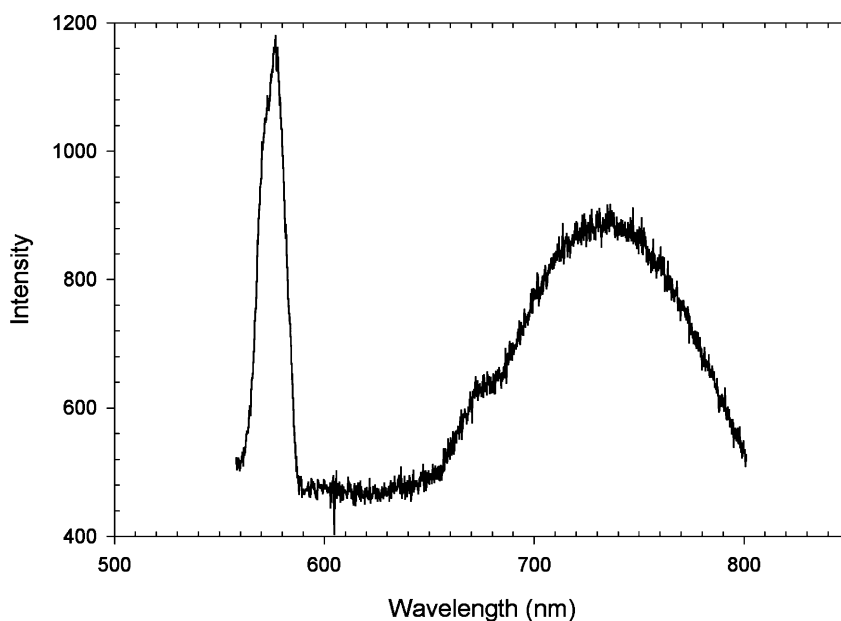


Fig. 3. Fluorescence spectrum of the BCDS complex with free Cu(I) in 100 mM potassium phosphate buffer excited at 482 nm. CuSO_4 was present at a concentration of 0.9 mM, BCDS at 0.3 mM, and ascorbic acid at 2.5 mM. The narrow line at 576 nm corresponds to the Raman scattering of water.

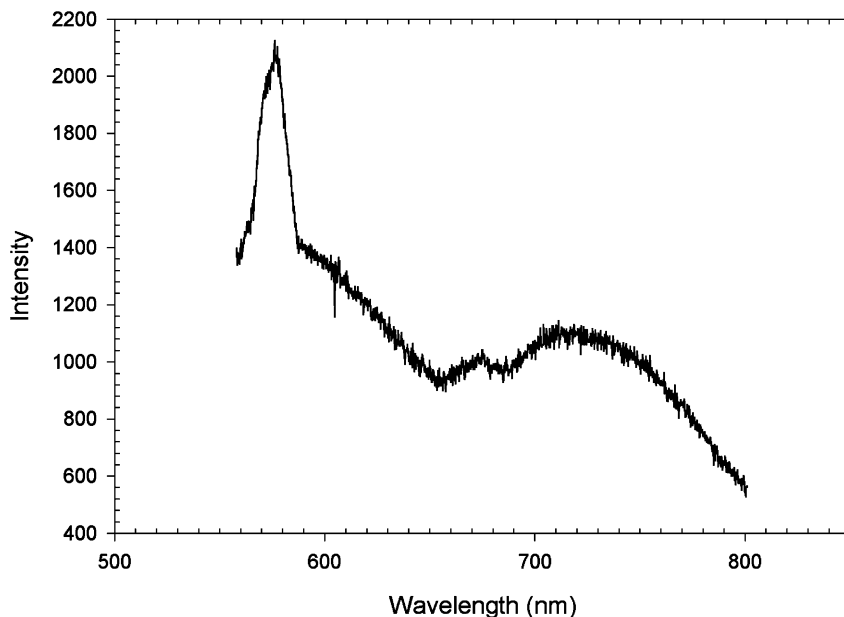


Fig. 4. Fluorescence spectrum of the BCDS complexes with D124N (zinc-deficient) SOD1 excited at 482 nm. D124N SOD1 was present at a concentration of 0.075 mM, BCDS at 0.3 mM, and ascorbic acid at 2.5 mM in 100 mM potassium phosphate buffer pH 7.2. The narrow line at 576 nm corresponds to the Raman scattering of water.

fluorescence of the $(\text{BCDS})_2\text{-Cu(I)}$ complex from fluorescence of BCDS itself.

All fluorescence emission spectra were approximated as a set of bands with a Gaussian shape. At first, the fluorescence of BCDS was deconvolved (Fig. 5). Two broad bands at 574 and 662 nm correspond to the fluorescence of BCDS. The narrow 576 nm band corresponds to the Raman scattering of a water at 3400 cm^{-1} .

Next, the fluorescence of BCDS complexes with free Cu(I) was processed. As one can see in Fig. 6, the fluorescence of BCDS is completely quenched and a new 734.7 nm band, corresponding to the fluorescence of $(\text{BCDS})_2\text{-Cu(I)}$ complexes is observed, accompanied by Raman scattering of water at 576 nm.

Knowing the positions and FWHM of each band allowed us to fit the fluorescence of various BCDS complexes with zinc-deficient SOD1. We locked parameters that are responsible for band position and FWHM and fit the fluorescence of BCDS complexes with zinc-deficient SOD by amplitude only. The results of this procedure are shown in Fig. 7. The calculated curve fits the

experimental data with a least squares goodness of fit (r value) better than 0.99 in all cases. Data obtained from fitting procedure represent the actual fluorescence intensity of complexes.

Finally the dependences of fluorescence intensity as a function of complexes concentration can be determined as it done for our instrument in Fig. 8. Similar calibration curve could be build for any other spectroscopic detection system. BCDS fluorescence band at 573.98 nm and fluorescence band of BCDS complexes with zinc-deficient SOD1 at 735 nm are well separated and therefore the ratio method could be used for quantitative measurements as well.

4. Discussion and conclusions

We have demonstrated that complexes of BCDS with Cu(I) and zinc-deficient SOD1 display a characteristic fluorescence band at 734 nm with FWHM of 150 nm under resonance excitation at 482 nm. This fluorescence band could be used for qualitative and quantitative determination of zinc-

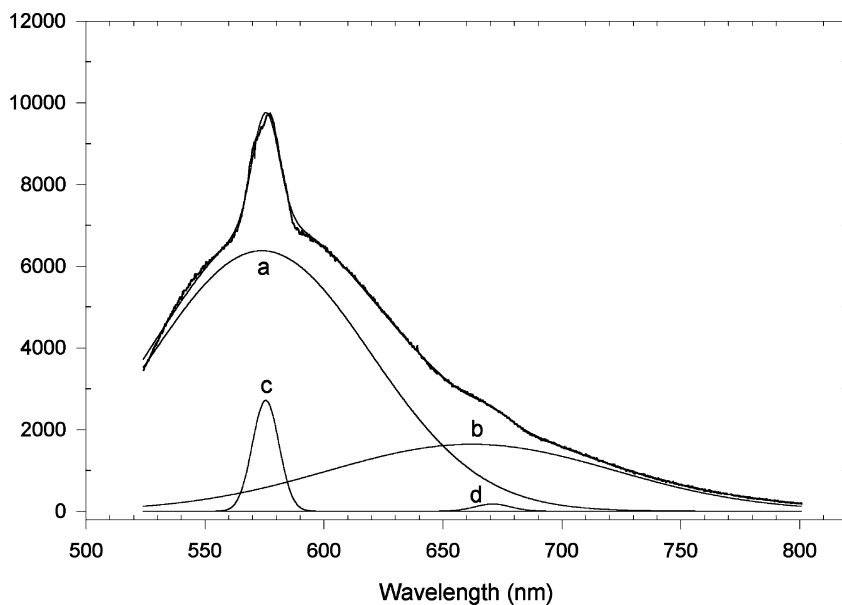


Fig. 5. Deconvoluted spectrum of BCDS fluorescence. Bands (a) and (b) centered at 573.98 nm and 662.18 correspond to BCDS fluorescence, (c) centered at 576 nm corresponds to Raman scattering of water, (d) 671 nm band is an artifact which is present in all spectra.

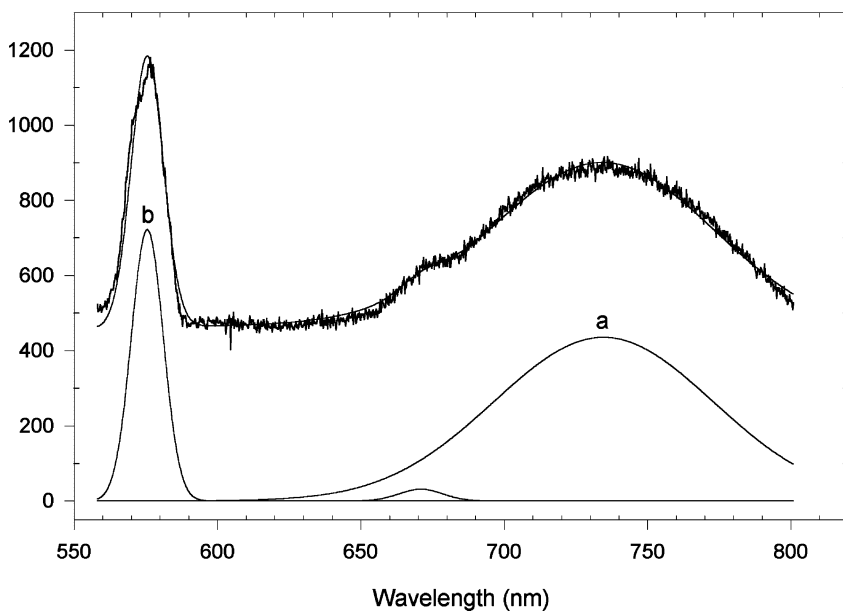


Fig. 6. Deconvoluted spectrum of (BCDS)₂-Cu(I) complexes fluorescence. Band (a) centered at 734.67 nm corresponds to fluorescence of (BCDS)₂-Cu(I), (b) 576 nm band corresponds to Raman scattering of water.

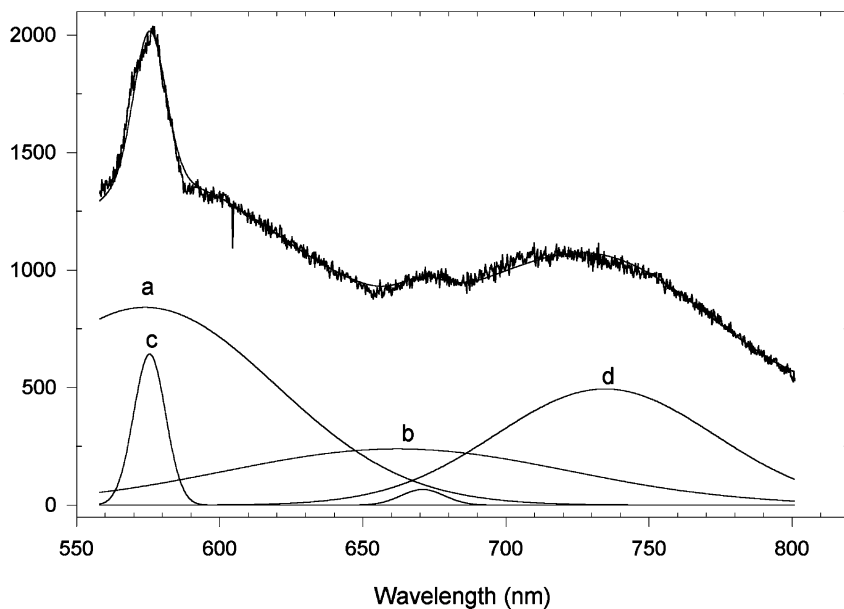


Fig. 7. Deconvoluted spectrum of the BCDS/Cu(I)–SOD1 (D124N mutant) complexes fluorescence. D124N SOD1 was present at a concentration of 0.075 mM, BCDS at 0.3 mM, and ascorbic acid at 2.5 mM in 100 mM potassium phosphate buffer pH 7.2. Bands (a) and (b) centered at 573.98 nm and 662.18 correspond to BCDS fluorescence, (c) 576 corresponds to Raman scattering of water, and (d) 734.67 nm corresponds to fluorescence of the BCDS/Cu(I)–SOD1 (D124N mutant) complexes.

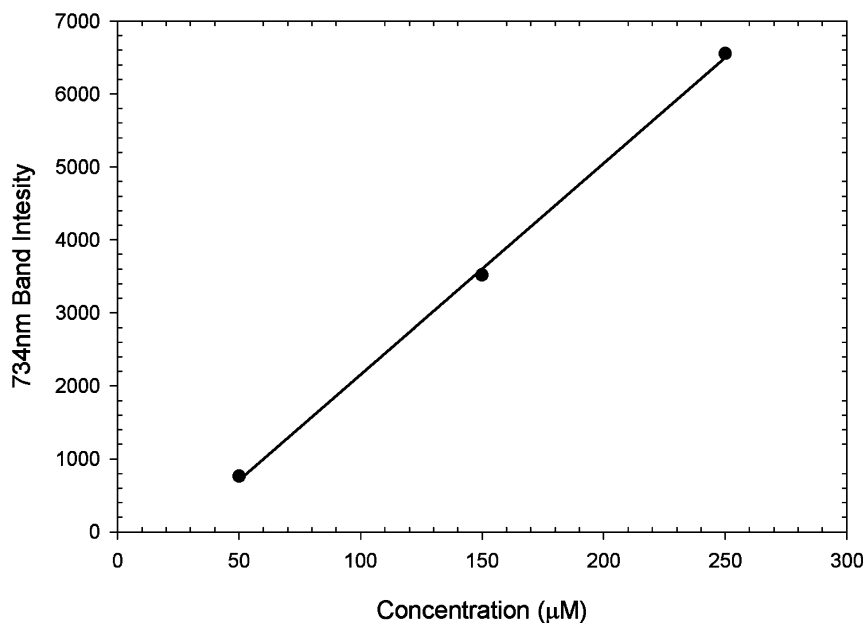


Fig. 8. Intensity of the 734 nm fluorescence band of the BCDS/Cu(I)–SOD1 (D124N mutant) complexes a function of concentration.

deficient SOD1 in cells by means of fluorescence confocal microscopy (FCM). However, the quantum fluorescent yield is rather small; the smallest concentration of zinc-deficient SOD1 detected in our experiments was 50 μM . That is about one order of magnitude higher than physiological concentrations of SOD1 in cells. However, preliminary estimations of detection limit of microRaman/Fluorescence imaging system Dilor XY, which we plan to use for detection of SOD1 in cell culture, or a similar microscope based system equipped with liquid nitrogen cooled CCD camera with a ten times better detectability than thermoelectrically cooled CCD used for volumetric measurements, showed that physiological concentrations of SOD1 could be measured with a signal to noise ratio about 37 during 1 min signal accumulation. Detailed calculations of the detection limit can be found in supplementary data section. Therefore we expect that confocal microscope should provide the required sensitivity for detection of zinc-deficient SOD1 in cell culture. Thus, this method will be further refined for use in testing the zinc-deficient SOD1 hypothesis in ALS. Measurement of zinc-deficient SOD1 in situ (using FCM) would provide additional support for the fundamental hypothesis regarding the toxic form of SOD1 mutants in ALS and provide a way to monitor treatments designed to reduce the levels of the zinc-deficient enzyme.

The similarity of absorption and fluorescence spectra of BCDS complexes with free Cu(I) and with D124N (zinc-deficient) SOD1 suggests that a BCDS-D124N SOD1 complex did not exist at the time measurements were made. However, quenching of intrinsic BCDS fluorescence (ex/em = 320/400 nm) by free Cu(I) is twofold more efficient than an equal concentration of D124N (zinc-deficient) SOD1. This result is consistent with a 1:1 complex of BCDS with active site Cu(I), as compared to a 2:1 complex of BCDS with free Cu(I). Thus, the issue of the precise nature of the BCDS/SOD1 complex remains unresolved. Because copper is strongly pro-oxidant and causes oxidative injury to cells, mechanisms have evolved to prevent free copper from existing within cells. It has been estimated that there is less than one free copper atom per cell [10]. Thus,

formation of a fluorescent complex (ex/em = 482/734 nm) upon addition of BCDS to cells would likely reflect the presence of zinc-deficient SOD1. While the possibility that BCDS could extract the copper from other copper-binding proteins, it is most unlikely that such copper extraction would be inhibitable by pretreatment with zinc. Thus, a zinc-inhibitable formation of a fluorescent BCDS complex would be strongly suggestive of the presence of zinc-deficient SOD1 within cells. Ongoing experiments in our laboratory are designed to test this hypothesis.

Appendix A

It is assumed that we will use liquid nitrogen cooled (LNC) CCD camera with the following characteristics: 2000 \times 800 pixels, 30 \times 12 mm active area, 80% quantum efficiency at 740 nm, $S_d = 1.4 \times 10^{-4}$ (electron/pixel-second) dark current at operational temperature of 140 K, read out noise $N_r = 5$ electrons. The total noise N_t associated with acquisition of spectrum by CCD camera may be expressed as:

$$N_t = (N_r^2 + N_d^2 + N_{ph}^2)^{1/2} \quad (1)$$

where N_r is a read out noise, N_d is a dark charge noise and N_{ph} is a photon noise. The photon noise can be neglected due to a low intensity of the acquired signal. We examine detectability of the system for one minute accumulation time with pixel binning in vertical direction to a supersize pixel (200 pixels). After one minute accumulation the photon shot noise and total noise will be:

$$\begin{aligned} N_d^2 &= 1.4 \times 10^{-4} \text{ (electrons/pixel-s)} \times 60 \times 200 \text{ (pixels)} \\ &= 1.68 \text{ electrons} \end{aligned} \quad (2)$$

and

$$N_t = (5^2 + 1.68)^{1/2} = 5.17.$$

Since 5.17 counts rms usually means $5.17 \times 5 = 25.9$ counts peak-to-peak noise fluctuations. The smallest detectable signal will be the signal that is equal to this noise. Since the quantum efficiency of CCD camera is 80% the amount of photons

corresponding to the noise level will be 32.3. Thus detectability is $32.3(\text{photons})/60(\text{s}) \times 200(\text{pixels}) = 2.69 \times 10^{-3}$ (photons/pixel-s).

Let us compare the smallest detectable signal with fluorescence of 10 μM solution of BCDS-SOD1 complexes, that is considered to be an average physiological concentration of SOD1 in cells. We assume that confocal microscope equipped with 0.75 numerical aperture objective allows us to collect light from the volume equal to $X10^{-15} \text{ m}^3$. That corresponds to 10^{-17} moles or 6.02×10^6 molecules of SOD1, or 12.04×10^6 BCDS-SOD1 complexes. If the power of laser radiation in the focal spot of microscope is equal to 10 mW the number of excitation photon can be calculated as:

$$N_{\text{exc}} = \frac{10 \times 10^{-3} \text{ W} \times 480 \times 10^{-9} \text{ m}}{6.62 \times 10^{-34} \text{ J} \cdot \text{s} \times 3 \times 10^8 \text{ m/s}} \approx 2.42 \times 10^{16} \text{ photons/s} \quad (3)$$

and photon flux in the focal spot as

$$\Phi = \frac{N_{\text{exc}}}{S} = \frac{2.42 \times 10^{16}}{10^{-6} \text{ cm}^2} = 2.42 \times 10^{22} \frac{\text{photons}}{\text{s} \cdot \text{cm}^2} \quad (4)$$

where S is an area of the focal spot. The number of photons that BCDS-SOD1 complexes emit under 10 mW excitation at 480 nm is approximately equal to:

$$N_{\text{em}} = \sigma_{\text{ab}} \Phi N \eta \quad (5)$$

where σ_{ab} is an absorption cross-section, Φ is a photon flux and N is a number of BCDS-SOD1 complexes in the studied volume, η is a quantum efficiency of emission from the excited state. The quantum efficiency could be approximately estimated by comparing BCDS-SOD1 complexes fluorescence signal and Raman scattering of water. The ratio of the number of fluorescence photons to the number of Raman photons is equal to the ratio of area (S_{576}) under Raman band at 576 nm and area (S_{734}) under fluorescence band at 734 nm. The S_{734}/S_{576} ration for the 0.15 mM concentration of BCDS-SOD1 complexes corresponds to:

$$\frac{S_{734}}{S_{576}} = \frac{\sigma_{\text{ab}} \Phi N_0 \eta}{\sigma_{\text{R}} \Phi N_{\text{H}_2\text{O}}} = \frac{\sigma_{\text{ab}} N_0}{\sigma_{\text{R}} N_{\text{H}_2\text{O}}} \eta = 5.27 \quad (6)$$

Thus, quantum efficiency of emission from the excited state is equal to:

$$\eta = 5.27 \frac{\sigma_{\text{R}} N_{\text{H}_2\text{O}}}{\sigma_{\text{ab}} N_0} \quad (7)$$

where $N_{\text{H}_2\text{O}}/N_0 = 3.7 \times 10^5$ is a ratio of number of water molecules to the number of BCDS-SOD1 complexes in 0.15 mM solution. Hence, number of emitted photons can be obtained by substituting Eq. (7) in to Eq. (5).

$$N_{\text{em}} = \sigma_{\text{ab}} \Phi N \cdot 5.27 \frac{\sigma_{\text{R}} N_{\text{H}_2\text{O}}}{\sigma_{\text{ab}} N_0} = 5.27 \cdot \Phi N \sigma_{\text{R}} \frac{N_{\text{H}_2\text{O}}}{N_0} = 5.68 \times 10^5 \text{ photons/s} \quad (8)$$

The amount of photons collected by the microscope objective can be found as:

$$N_{\text{col}} = N_{\text{em}} \frac{\Omega_{\text{obj}}}{4\pi} T_{\text{obj}} = N_{\text{em}} T_{\text{obj}} \frac{(1 - (1 + NA^2)^{-1/2})}{2} = 3.98 \times 10^4 \text{ photons/s} \quad (9)$$

and corresponding light intensity

$$I = N_{\text{col}} \cdot h\nu = 1.07 \times 10^{-12} \text{ W/cm}^2 \quad (10)$$

Where Ω_{obj} is solid angle of collection for microscope objective, T_{obj} is microscope objective transmission at 740 nm and NA is microscope objective numerical aperture. The power transmitted through the spectrometer is given by:

$$P = \frac{W \cdot H \cdot A^2}{F^2} \cdot T \cdot I = 4 \cdot W \cdot H \cdot NA_{\text{spec}}^2 \cdot T \cdot I = 5.35 \times 10^{-17} \text{ W} \quad (11)$$

where $H = 5 \text{ mm}$ is slit height, $W = 200 \mu\text{m}$ is slit width, $T = 0.5$ is spectrometer transmission at 740 nm, $NA_{\text{spec}} = 0.05$ spectrometer numerical aperture, $I = 1.07 \times 10^{-12} \text{ W/cm}^2$ incident light intensity. The power $5.35 \times 10^{-17} \text{ W}$ corresponds to 199 photons per second at 740 nm. Since we illuminate 2000 pixels, the fluorescence signal per pixel-second will be 199 (photons/s)/2000 (super-size-pixels) = 0.1 (photon/pixel-s) compared to 2.69×10^{-3} (photon/pixel-s) detectability of the

instrument. It corresponds to a signal to noise ratio equal to X37 and further attest that BCDS-SOD1 complexes could be detected in a single cell.

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