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Directed evolution of protein-based neurotransmitter sensors for MRI

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Abstract

The production of contrast agents sensitive to neuronal signaling events is a rate- limiting step in the development of molecular-level functional magnetic resonance imaging (molecular fMRI) approaches for studying the brain. High throughput generation and evaluation of potential probes is possible using techniques for macromolecular engineering of protein-based contrast agents. In an initial exploration of this strategy, we used the method of directed evolution to identify mutants of a bacterial heme protein that allowed detection of the neurotransmitter dopamine *in vitro* and in living animals. The directed evolution method involves successive cycles of mutagenesis and screening that could be generalized to produce contrast agents sensitive to a variety of molecular targets in the nervous system.

Keywords

Magnetic resonance imaging; directed evolution; protein engineering; cytochrome P450; dopamine

1. INTRODUCTION

With the continuing development of magnetic resonance imaging (MRI) contrast agents sensitive to molecular hallmarks of brain activity (1), a new generation of functional neuroimaging methods could combine noninvasive, whole-brain coverage with unprecedented specificity for neuronal processes. Design and synthesis of effective contrast agents for molecular fMRI remains a significant challenge, however. Protein engineering strategies applied to MRI-detectable proteins (2–5) might provide a means to accelerate this endeavor. Many proteins contain paramagnetic ions that can enable them to be visualized by MRI with longitudinal (T_1) or transverse (T_2) relaxation time weighting. Advantages of protein contrast agents, compared with conventional synthetic agents, include amenability to modification, ease and low cost of production, and the possibility of targeting and endogenous production in genetically modified cells or organisms.

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In recent work, we used the protein engineering technique of directed evolution to generate dopamine-sensing mutants of the cytochrome P450 BM3 heme domain (BM3h) (6). Our approach involved applying repeated cycles of random mutagenesis and screening, each step allowing us to identify BM3h mutants with enhanced affinity and selectivity for dopamine, compared with other potential ligands (Fig. 1). After four rounds of successive improvements, we obtained a variant with an 8.9 μM dissociation constant for dopamine and little responsiveness to other neurotransmitters. This molecule acted as an MRI contrast agent with T_1 relaxivity (r_1) values of 1.1 ± 0.1 and 0.17 ± 0.03 $mM^{-1}s^{-1}$ in the absence and presence of saturating dopamine concentrations, respectively. The agent responded semi-quantitatively to dopamine release from cultured PC12 cells *in vitro*, and allowed detection of dopamine dynamics in the brains of living rodents.

The directed evolution method is highly versatile (7), and could be used to prepare MRI contrast agents based on BM3h or other paramagnetic proteins; these probes could be selected for sensitivity to a wide variety of neurochemicals. Analytes of interest might include neurotransmitters other than dopamine, cytokines, or components of intracellular signal transduction pathways. Here we detail the protocol we applied to produce BM3h-based dopamine sensors (6), with possible variations noted where appropriate.

2. MATERIALS

2.1 Preparation of Random Mutagenesis Library

2.1.1 Library Construction

1. Plasmid containing parent gene for directed evolution (*see* Note 1).
2. Appropriate PCR primers for amplification of parent gene.
3. 20× dNTP stock (4 mM dATP, 4 mM dGTP, 10 mM dTTP, 10 mM dCTP).
4. *Taq* DNA polymerase with supplied 10X PCR buffer II and 25 mM $MgCl_2$ (AmpliTaq, Applied Biosystems, Foster City, CA).
5. 500 μM $MnCl_2$ in water (sterilized before use).
6. PCR purification kit (QIAquick PCR Purification Kit, QIAGEN, Valencia, CA).
7. Appropriate restriction enzymes with supplied buffers (New England Biolabs, Ipswich, MA).
8. 1% agarose gel containing 0.5 μg/ml ethidium bromide.
9. Gel extraction kit (QIAquick Gel Extraction Kit, QIAGEN, Valencia, CA).
10. Plasmid backbone (digested, gel-purified, and ready for insert ligation).
11. T4 DNA ligase with supplied buffers (New England Biolabs, Ipswich, MA).
12. Electrocompetent BL21(DE3) *E. coli* (*E. coli* EXPRESS Competent Cells, Lucigen Corporation, Middleton, WI).
13. LB agar plates containing 100 μg/ml ampicillin.

2.1.2 Library Expression

1. LB medium containing 100 μg/ml ampicillin.

[1]Choosing a good parent gene is one of the most important steps when engineering proteins by directed evolution. The parent protein should be characterized with the high-throughput screen (section 3.2) and bulk assays (sections 3.3 and 3.4). The parent should also display at least marginal levels of the desired activity, in order to allow the assay to resolve small increases in binding affinity.

2. Sterile toothpicks.
3. Sterile 96 deep-well plates (2 mL volume).
4. Sterile 50% glycerol.
5. Sterile 96-well microtiter plates.
6. TB medium containing 100 μg/ml ampicillin and 200 μM IPTG.

2.2 High-Throughput Screening

2.2.1 Lysate Preparation

1. Lysis buffer: Phosphate buffered saline (PBS) containing 0.75 mg/ml hen egg lysozyme and 5 mg/ml DNAse I (Sigma-Aldrich, St. Louis, MO).
2. 96-well microtiter plates.
3. Phosphate buffered saline (PBS).
4. Neurotransmitters of interest (*see* Note 2).

2.3 Bulk Protein Expression and Purification

2.3.1 Protein Expression

1. LB medium containing 100 μg/ml ampicillin.
2. Sterile culture tubes.
3. TB medium containing 100 μg/ml ampicillin.
4. Sterile culture flask.
5. 1000X IPTG solution (500 mM in water, filter sterilized).

2.3.2 Protein Purification

1. Phosphate buffered saline (PBS).
2. Protein extraction kit (BugBuster Plus Lysonase Kit, EMD Chemicals, San Di-ego, CA).
3. Ni-NTA agarose (QIAGEN, Valencia, CA).
4. Disposable polypropylene column.
5. Ni-NTA wash buffer: 20 mM tris, 100 mM NaCl, 20 mM imidazole, pH 8.0.
6. Ni-NTA elution buffer: 20 mM tris, 100 mM NaCl, 300 mM imidazole, pH 8.0.
7. PD-10 desalting columns (GE Healthcare, Piscataway, NJ).
8. Amicon Ultra-30 centrifugal filter units (Millipore, Billerica, MA)

2.3.3 Measurement of Protein Concentration

1. Sodium hydrosulfite (Sigma-Aldrich, St. Louis, MO).
2. Carbon monoxide tank (CO is a colorless and odorless gas which is highly toxic).

[2]The BM3h spectroscopic binding assay has been performed on numerous neuro-transmitters and other small molecule ligands (6). Neurotransmitters should be chosen with consideration for the neurotransmitter of interest in addition to other small-molecules which may competitively bind. Variants are selected for affinity and specificity toward the neurotransmitter of interest.

2.4 Variant Characterization

2.4.1 Neurotransmitter Titrations

1. Neurotransmitters of interest (*see* Note 2).
2. Cuvettes.

2.4.2 Measurement of MRI Relaxivity

1. Microtiter plates, modified if necessary to fit in an MRI scanner.
2. Neurotransmitters of interest (*see* Note 2).
3. An MRI scanner (*see* Note 3).

3. METHODS

3.1 Preparation of Random Mutagenesis Library

3.1.1 Library Construction

1. Prepare error-prone PCR by combining the following in a thin-walled PCR tube:

   40 μL water

   10 μL 10X PCR buffer II

   5 μL 500 μM $MnCl_2$

   28 μL 25 mM $MgCl_2$

   1 μL parent BM3h plasmid DNA

   5 μL of each primer (forward and reverse)

   5 μL of 20X dNTP mix

   1 μL Amplitaq DNA polymerase

   for a 100 μL total reaction volume (*see* Note 4).

2. Perform PCR with the following program:
   1. 120 second initial melting at 95 °C
   2. 30 second melting at 95 °C
   3. 30 second annealing at 57 °C
   4. 90 second extension at 72 °C
   5. repeat steps 2–4 for 14 cycles
3. Purify the PCR product using the PCR purification kit.
4. Perform a double digest on the PCR product for 2 hours at 37 °C with the appropriate restriction enzymes.

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[3]This protocol was developed on a 40-cm-bore Bruker Avance 4.7 T MRI scanner, equipped with 12-cm-inner-diameter 26 G cm^{-1} triple-axis gradients and a 10-cm- inner-diameter birdcage resonator radiofrequency coil. A wide variety of MRI systems could be used for relaxivity determination; the principle requirements are that magnetic and radiofrequency fields, and gradient field linearity, be constant across the sample. A nuclear magnetic resonance (NMR) spectrometer could be used in place of an MRI scanner, though measurements would then generally need to be performed with one sample at a time.

[4]Mutations are randomly introduced by the addition of $MnCl_2$ to the PCR. The mutation rate (average number of nucleotide substitutions per gene) can be tuned by altering the concentration of $MnCl_2$ in the reaction mixture. For most applications 1–2 nucleotide mutations per gene is ideal, which can be achieved by using a final concentration of 25 μM $MnCl_2$. Different mutation rates can be screened by varying [$MnCl_2$] from 5–200 μM, and assessing the fraction of functional variants.

5. Run the restriction digest product on a 1% agarose gel, and excise the appropriate DNA fragment. Use the gel extraction kit to separate the DNA from the agarose.
6. Ligate the digested fragment into the cut plasmid backbone using T4 DNA ligase. Allow the ligation reaction to at 16 °C for at least 12 hours.
7. Transform the ligation product into electrocompetent BL21(DE3) cells using a 0.1 cm electroporation cuvette. After electroporation, quickly add 1 ml of recovery media to the cells and incubate at 37 °C for 1 hour.
8. Plate the transformed cells on LB agar plates and incubate at 37 °C overnight (*see* Note 5).

3.1.2 Library Expression

1. Fill sterile 96 deep-well plates with 400 μL of LB containing 100 μg/ml ampicil-lin per well.
2. Using sterile toothpicks, pick individual *E. coli* colonies from the plated transformation into each well of the 96 deep-well plates. Be sure to inoculate a few wells with the parent (as a positive control) and keep a few wells empty (as a negative control and to detect contamination).
3. Grow 96 well starter cultures shaking at 300 RPM and 37 °C overnight.
4. Fill sterile 96 deep-well plates with 1.2 mL of TB containing 100 μg/ml ampicil-lin and 200 μM IPTG per well. Using a pipetting robot or multi-channel pipette, transfer 100 μL/well of each LB starter culture to each TB plate.
5. Fill sterile microtiter plates with 50 μL of 50% glycerol per well. Using a pipet-ting robot or multi-channel pipettor, transfer 50 μL/well of each LB starter culture to each microtiter plate. Store glycerol stocks at −80 °C for later use.
6. Grow 96 well TB cultures shaking at 300 RPM and 30 °C overnight.
7. Pellet TB cultures by centrifugation at 3500 RCF for 15 minutes. Pour off supernatant and freeze cell pellets.

3.2 High-Throughput Screening

3.2.1 Lysate Preparation

1. Thaw frozen cell pellets containing expressed BM3h variants (or controls) at room temperature, add 650 μL/well of lysis buffer, and resuspend the pellets using a pipetting robot (*see* Note 6).
2. Incubate the lysate at 37 °C for 30 minutes, again resuspend, and then incubate the lysate at 37 °C for another 30 minutes.
3. Clarify the lysate by centrifugation at 5500 RCF for 15 minutes.
4. Using a pipetting robot, transfer 200 μL/well clarified lysate to a microtiter plate. The assay requires one microtiter plate per neurotransmitter screened.

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[5]The volume of transformed *E. coli* to be plated should be determined by the transformation efficiency of the competent cells and the number of variants to be screened. There must be enough colonies for the high-throughput screens, yet they need to be sparse enough to be individually picked. If unsure of the transformation efficiency, multiple transformation volumes can be plated on different plates. We typically screened 400–800 variants per generation of directed evolution.

[6]We typically only screen one to two plates at a time because performing titrations on multiple neurotransmitters can be quite laborious. This means that each round of directed evolution requires screening across multiple days.

3.2.2 Neurotransmitter Titrations

1. Prepare appropriate neurotransmitter serial dilutions in PBS (*see* Note 7).
2. Read the absorbance of the lysate from 350–500 nm (*see* Note 8).
3. Add 10 μL/well of the lowest concentration neurotransmitter and read absorbance from 350–500 nm. Continue to add increasing amounts of neurotransmitter, scanning after each addition. Characteristic spectroscopic results are presented in Fig. 2.
4. Repeat titrations for all desired neurotransmitters.

3.2.3 Data Analysis

1. The data set consists of optical density (OD) readings from 350–500 nm for each BM3h variant in the presence of varying concentrations of each neurotransmitter (*see* Note 9). Binding curves for each variant can be generated by subtracting the baseline absorbance obtained in the absence of neurotransmitter from each spectrum, and then subtracting the minimum from the maximum of each difference spectrum to obtain $\Delta OD_{max\text{-}min}$ values (Fig. 2C).
2. Dissociation constant (K_d) estimates for binding of neurotransmitters to each BM3h variant are obtained by fitting the absorbance data to a ligand-depleting bimolecular association function of the form:

$$\Delta OD_{max-min} = k\left[x + p + K_d - \sqrt{(x + p + K_d)^2 - 4xp}\right]$$

   where k is a constant of proportionality and x and p are the total analyte and BM3h protein concentrations, respectively (*see* Note 10).
3. The best 5–10 variants should be chosen for bulk expression and further characterization (*see* Note 11).

3.3 Bulk Protein Expression and Purification

3.3.1 Protein Expression

1. Using the 96-well glycerol stocks made during step 4 of 3.1.2, inoculate 5 mL LB starter cultures containing 100 μg/ml ampicillin with the top variants chosen from the high-throughput screen. Grow LB starter cultures shaking at 37 °C overnight.
2. Inoculate 50 mL TB cultures with 500 μL of the starter culture and incubate shaking at 37 °C. Once the culture reaches an OD_{600} of 0.6, induce with 50 μL of

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[7] 5–10 neurotransmitter concentrations should be chosen so the final concentrations are approximately logarithmically spaced and centered around the K_d of the parent. Serial dilutions of the neurotransmitter are an effective method to achieve accurately spaced dilutions.

[8] Reading the absorbance from 350–500 nm for each well at each neurotransmitter concentration can be slow, depending on the plate reader. We only read absorbance every 10 nm over the interval from 350 to 500 nm.

[9] Ligand-induced spectral changes may result in shifting of the BM3h absorbance peak (λ_{max}) toward either shorter or longer wavelengths. Blue-shifting of λ_{max} is characteristic of fatty acid binding to BM3h, which is accompanied by a transition of the heme iron from hexacoordinate to pentacoordinate geometry. Red-shifting of λ_{max} is observed with monoamine ligands, and is thought to indicate direct coordination of the heme iron by the ligand.

[10] When analyzing the data, keep track of the increases in volume upon titration. This significantly affects the total neurotransmitter and protein concentrations, which are inputs into the curve fitting. We perform data analysis using the Matlab software package (Mathworks, Natick, MA), but many alternatives are available.

[11] The variants to be selected are those that provide the greatest improvement in affinity and specificity toward the neurotransmitter of interest.

1000X IPTG. Reduce the growth temperature to 30 °C and allow proteins to express for 20–30 hours.

3. After expression, pellet cells by centrifuging at 4000 RCF for 15 minutes and then freeze cell pellets.

3.3.2 Protein Purification

1. Resuspend frozen pellet with protein extraction reagents, 5 mL BugBuster and 10 μL lysonase per gram of protein pellet. Shake for 15–20 minutes at room temperature (*see* Note 12).
2. Clarify lysate by centrifuging at 16,000 RCF for 30 minutes at 4 °C.
3. Load clarified lysate onto a column packed with 5 mL Ni-NTA agarose resin.
4. Wash column with 40 mL of Ni-NTA wash buffer.
5. Elute histidine-tagged BM3h variants with 5 mL of Ni-NTA elution buffer.
6. Run Ni-NTA column elution through at PD-10 column to remove imidazole and to exchange buffer to PBS.
7. Concentrate as needed using Amicon Ultra-30 centrifugal filters.

3.3.3 Measurement of Protein Concentration

1. Dilute BM3h samples to about 10 μM in 100 mM Tris-HCl buffer, pH 8. Transfer to a cuvette and add a few milligrams of sodium hydrosulfite.
2. Read the absorbance spectra from 400–500 nm.
3. Slowly bubble the BM3h solution with CO for 30 seconds.
4. Read the absorbance spectra from 400–500 nm.
5. Calculate the CO-difference spectra by subtracting the pre-CO spectra from the post-CO spectra, and determine the difference between ΔOD values obtained at 450 nm and 490 nm (Fig. 3). The BM3h concentration can be determined using Beer's Law: $\Delta OD_{450-490} = \varepsilon_{450-490}[BM3h]l$, with the extinction coefficient $\varepsilon_{450-490} = 91\ mM^{-1}cm^{-1}$ (8), where l is the light path length.

3.4 Characterization of Purified P450 Variants

3.4.1 Neurotransmitter Titrations

1. Prepare concentrated neurotransmitter solutions (*see* Note 13).
2. Prepare a 1 μM BM3h solution using PBS as a diluent
3. Add the 1 μM protein to a cuvette and read the absorbance from 350–500 nm.
4. Add logarithmically increasing amounts of neurotransmitter and read absorbance from 350–500 nm after each addition.
5. Analyze binding data as described in section 3.2.3.

[12]Cells can also be lysed with an ultrasonic probe or a French press.
[13]For the bulk titrations, we make one concentrated neurotransmitter solution for each ligand concentration to be tested and add small amounts of this solution to the cuvette.

3.4.2 Measurement of Nuclear Magnetic Relaxivity

1. Prepare six to eight BM3h samples with concentrations ranging from 0 to 240 μM using PBS as a diluent.
2. Divide these samples in two, and add saturating amounts of the neurotransmitter of interest to half. Add equivalent amount of buffer to the other half.
3. Fill central wells of a 384-well microtiter plate with 100 μL of each sample (all concentrations with/without neurotransmitter) (*see* Note 14).
4. Fill unused wells within two or more wells of the samples with 100 μL PBS.
5. Image the microtiter plates at 21 °C using a spin echo pulse sequence with short echo time (*TE*) and a range of repetition times (*TR; see* Note 15). Fig. 4A shows a sample image.
6. Calculate longitudinal relaxation rates ($1/T_1$) by curve fitting to the reconstructed image data, using an equation of the form $I = k[1 - \exp(-TR/T_1)]$, where I is the observed MRI signal intensity and k is a constant of proportionality (Fig. 4B). Values of r_1 are subsequently determined by linear fitting to a plot of $1/T_1$ against protein concentration (Fig. 4C; *see* Note 16).

3.5 Directed Evolution

Bulk measurements of ligand binding affinity and relaxivity are used to select BM3h variants with the most desirable properties. The above procedures (sections 3.1 to 3.4) are then repeated over multiple iterations of improvement, until the desired affinity and specificity are achieved (*cf.* Fig. 1). In each generation 400–800 variants are screened, and within 5–10 generations K_d values might be expected to change by 2–3 orders of magnitude (*see* Note 17).

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[14]Given the size of our standard radiofrequency coil, we typically perform experiments in bisected 384-well plates (Fig. 4A); only wells near the center of the plate are used because of field inhomogeneity effects closer to the plate edges. In a different scanner and coil, these requirements could be different.

[15]We have used a *TE* of 10 ms and *TR* values of 73, 116, 186, 298, 477, 763 ms, 1.221, 1.953, 3.125 and 5.000 s. An inversion recovery pulse sequence or an alternative T_1-weighting method could be applied in place of the spin echo sequence (9), provided that a suitable analytical approach is applied to extract relaxation rates from the data. Geometric parameters of the imaging should be chosen to achieve desirable signal-to-noise ratio for the given sample size. We image a single 2-mm slice positioned midway through the sample heights, with a 16 × 8 cm in-plane field of view and a data matrix of 512 × 128 points.

[16]We perform both image reconstruction and relaxation rate determination using custom routines running in Matlab. Equivalent analysis procedures could be implemented with other numerical data processing programs, however, including the software supplied with some MRI scanners.

[17]In generating MRI dopamine sensors (6), we found that incorporation of a ther-mostabilizing mutation (10) improved our ability to continue directed evolution after the first four rounds. By increasing a protein's tolerance to mutagenesis, stability enhancements can facilitate directed evolution in general (11).

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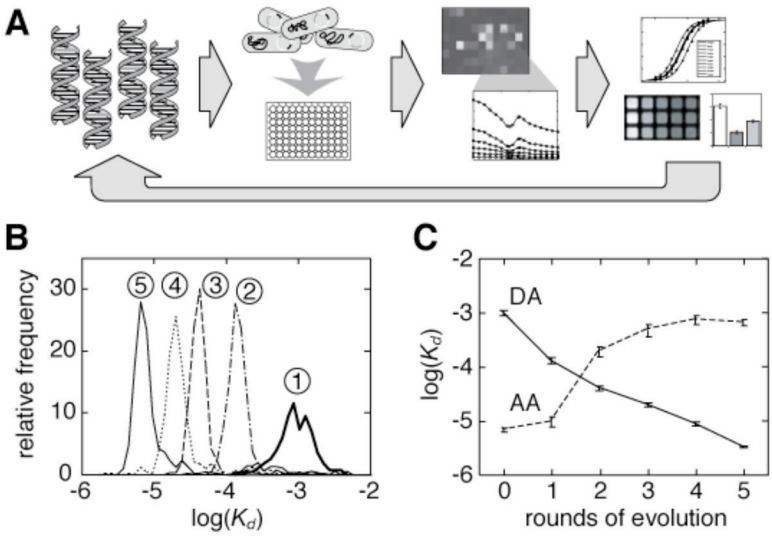

Figure 1. Overview of the directed evolution strategy

(**A**) The parental protein gene is randomly mutated and the resulting variants are transformed into *E. coli*. This library is grown and expressed in 96-well plates. High-throughput neurotransmitter binding assays are then performed in cell lysate. Several of the top variants identified in the high-throughput binding assay are purified and characterized in greater detail. The best variant from each generation is used as the parent in the next generation, and this process is repeated until the desired properties are achieved. (**B**) Distribution of apparent dissociation constants (K_d) for dopamine measured from mutant BM3h proteins generated over five rounds of directed evolution for enhanced dopamine affinity and decreased affinity for the native ligand, arachidonic acid (data from ref. 6). Numbers in circles denote the round of evolution associated with each mutant library. (**C**) K_d values for dopamine (DA, solid line) and arachidonic acid (AA, dashed line), for wild-type BM3h and mutant variants isolated at each round of screening as in B.

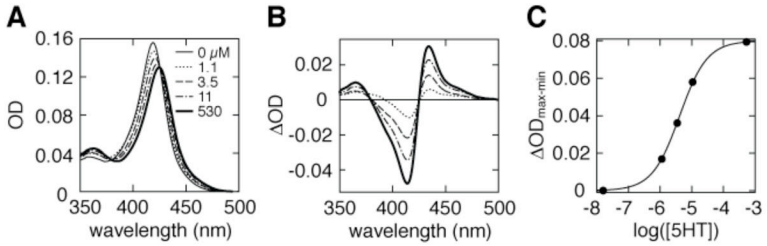

Figure 2. Spectroscopic BM3h binding assay

(**A**) Absorbance spectra (optical density, OD *vs.* wavelength) measured for a BM3h variant in the presence of varying concentrations of a neurotransmitter ligand, in this case serotonin. The spectral change is associated with perturbations to the electronic structure of the BM3h heme, in this case proba-bly resulting from direct coordination of the heme iron by serotonin (12). (**B**) Difference spectra (ΔOD *vs.* wavelength) obtained by subtracting the ligand-free spectrum in panel A from spectra measured at each ligand concentration. (**C**) Maximum and minimum values in the difference spectra are subtracted from one another to yield $\Delta OD_{max\text{-}min}$ values for each serotonin concentration ([5HT]). K_d estimates are obtained by fitting a binding model to these data.

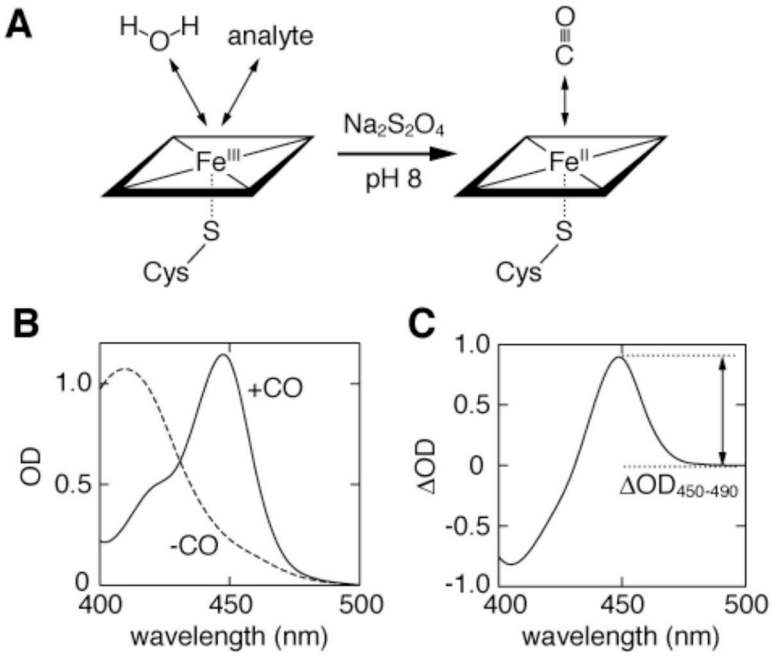

Figure 3. Quantification of BM3h concentration

(**A**) Binding of analytes near the ferric heme of stably oxidized BM3h (Cys400 ligand shown) modulates relaxivity probably by competing with water molecules (left), but also complicates quantification of BM3h concentration by affecting the protein's absorbance spectrum. Robust, analyte-independent concentration measurement is possible by reducing the protein with sodium hydrosulfite and measuring spectra in the presence and absence of carbon monoxide (CO, right). (**B**) Sample BM3h spectra obtained before (−CO, dashed line) and after (+CO, solid line) CO binding. (**C**) The difference spectrum obtained by subtracting the -CO spectrum from the +CO spectrum. Quantification of BM3h concentration is performed by measuring the difference between ΔOD values recorded at 450 nm and 490 nm ($\Delta OD_{450–490}$), and then applying Beer's law with an extinction coefficient of 91 $mM^{-1}cm^{-1}$ (8).

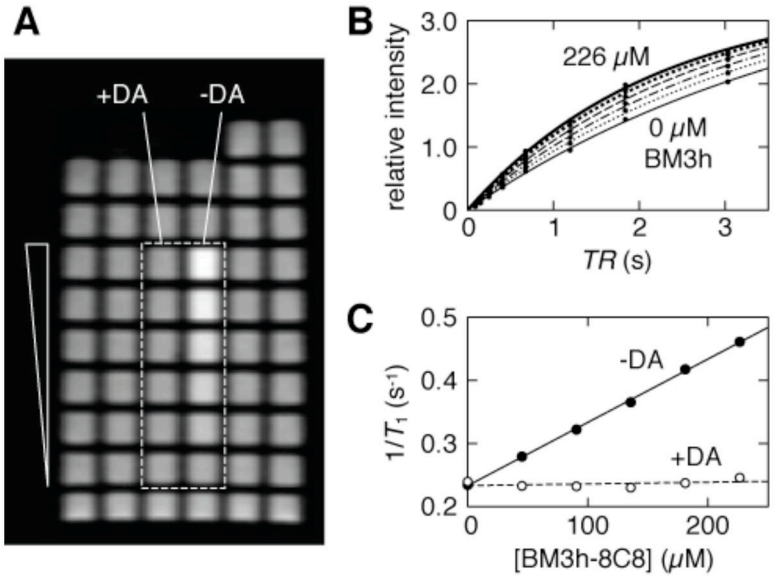

Figure 4. Measurement of relaxivity by MRI

(**A**) Wells of microtiter plate are filled with protein solutions (inside dashed rectangle) or buffer (outside). The buffer wells limit magnetic susceptibility-related artifacts in images; extra buffer wells (top right) are used to identify the plate orientation. A T_1-weighted spin echo image obtained at 4.7 T is shown here (*TE/TR* = 10/477 ms), containing two columns of BM3h solutions ranging from 0–256 μM (increasing [BM3h] indicated by wedge), in the presence (+DA, left column) or absence (−DA, right column) of 1 mM dopamine. The BM3h variant used here (BM3h-8C8) was selected for dopamine binding, and intensity differences between the +DA and −DA conditions are readily apparent. (**B**) Quantification of MRI signal in images obtained at multiple *TR* values enables estimation of the relaxation rate ($1/T_1$) for each condition. An exponential decay curve with time constant T_1 (lines) is fitted to each plot of relative MRI intensity *vs. TR* (circles); superimposed plots with fitted curves are shown here for 0–266 μM BM3h-8C8 in the absence of dopamine (*cf.* righthand column in panel A). (**C**) Values of $1/T_1$ in the presence (open circles) or absence (filled circles) of dopamine are plotted as a function of protein concentration ([BM3h-8C8]). Longitudinal relaxivity (r_1) values are given by the slopes of linear fits to the data.