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A FRET Enzyme-Based Probe for Monitoring Hydrogen Sulfide

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Supporting Information

ABSTRACT: Fluorescently labeled cobalt peptide deformylase (Co-PDF) can be efficiently used as a fluorescence-resonance-energy-transfer-based sensing device for hydrogen sulfide (H_2S). The proof of concept of our sensor system is substantiated by spectroscopic, structural, and theoretical results. Monohydrogen sulfide coordination to Co-PDF and Ni-PDF was verified by X-ray crystallography. Density functional theory calculations were performed to gain insight into the characteristics of the coordination adduct between H_2S and the cobalt cofactor in Co-PDF.

Hydrogen sulfide (H_2S) is among the oldest and simplest of molecules whose reaction chemistry has very recently attracted attention from several research groups.[1] For hundreds of years, it has been known solely as a harmful gas.[2] More recently, H_2S has emerged as the "third gaseous transmitter" in biology, along with nitric oxide and carbon monoxide.[3,4]

Selective tracking of this small molecule in physiological conditions is particularly relevant to elucidate its complex contributions to both healthy and disease states. The challenge calls for a new generation of biocompatible sensing devices to assess endogenous concentrations of H_2S.[5]

Very recently, fluorescence-based systems for H_2S detection have been proposed as effective probes for biological applications.[5−8] These probes can detect H_2S in aqueous solution with high sensitivity and selectivity.[5−7] In all of these systems, the molecule acting as the recognition element has an abiotic origin. Aiming to develop more biocompatible devices, we focused on the design of fluorescence-enzyme-based biosensors. It is well-known that H_2S can bind to heme proteins, inducing different responses that, in turn, modulate its cytotoxic and cytoprotective activities.[9] Thus, the first system that we devised as a fluorescent H_2S sensor makes use of a heme protein. In particular, we employed myoglobin from horse skeletal muscle (Mb).[10]

A limitation of our Mb monitoring system is the low amplitude of the fluorescence signals. Furthermore, by subsequent additions of H_2S, Mb(Fe^{3+}) is reduced to the ferrous form: the cuvette sample resulted in a mixture of Mb(Fe^{3+})−H_2S, Mb(Fe^{2+})−H_2S, and Mb(Fe^{2+}). A similar reduction has been observed by Scheidt et al. for monohydrogen sulfide (HS^-) coordination in iron porphyrinates.[11]

To improve the Mb-based H_2S sensor, we had to overcome some of its limitations. In the current work, we extended the same approach to cobalt-containing peptide deformylase from *Escherichia coli* (Co-PDF). PDF's native metal cofactor is Fe^{II}, but it can easily be replaced by other thiophilic metal ions. Whereas PDF with Fe^{II} (Fe-PDF) is very sensitive to molecular oxygen, the variants Co-PDF, Ni-PDF, and Zn-PDF are stable.[12,13] PDF has the same overall structure with any of the four metals.[14] The active site metal is coordinated by the imidazole atoms His132Nε2 and H136Nε2 and the thiolate C90Sγ with trigonal-pyramidal geometry. In the resting state, one water molecule (possibly two water molecules at low pH) completes the metal coordination polyhedron. The Glu133 side chain polarizes or deprotonates one aqua ligand during formyl peptide hydrolysis. Cobalt is known to have a strong affinity for sulfur-containing ligands, as does nickel.[15,16] In the first instance, we focused on Co-PDF for our experiments.

To test the principal usability of the system as an optical H_2S biosensor, H_2S binding to Co-PDF was assessed via UV−visible spectroscopy. When H_2S was bubbled through a Co-PDF solution, the absorption spectrum significantly changed. Specifically, while the absorption spectrum of Co-PDF exhibits a band at 280 nm and three less intense bands centered at 320, 560, and 660 nm,[17] H_2S addition quenches the 560 and 660 nm bands and leads to the appearance of two new bands at 625 and 665 nm (Figure 1A and Figure S1 in the Supporting Information, SI). Spectroscopic features of Co-PDF make it ideally suited for implementing a fluorescence-resonance-energy-transfer

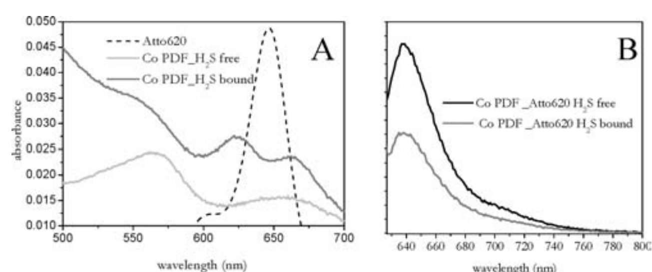

Figure 1. (A) Absorption spectrum of H_2S-free Co-PDF (gray) and the H_2S-bound form (dark gray) and emission spectrum (dotted trace) of Atto620 (λ_{max} = 645 nm). Protein concentration: 125 μM in a 100 mM potassium phosphate buffer (pH = 6.8, room temperature). (B) Fluorescence emission spectrum (λ_{ex} = 620 nm) of Atto620-labeled H_2S-free (black trace) and H_2S-bound (gray trace) Co-PDF. Protein concentration: 100 nM. In this experiment, Co-PDF was labeled on the amines.

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(FRET)-based H_2S biosensor. By covalent attachment of a fluorescent label to the protein whose emission spectrum overlaps with either the 625 or 665 nm band of the protein in its H_2S-bound state, the label fluorescence will be modulated by the difference in the spectral overlap between the H_2S-free and H_2S-bound states of Co-PDF via a FRET mechanism. That is, when the protein is in the H_2S-free state, all of the energy absorbed by the label is emitted as fluorescence. On the other hand, in the H_2S-bound state of Co-PDF, the label fluorescence is (partially) quenched because of FRET to the 625 or 665 nm band. The overlap of the Co-PDF absorption bands (H_2S-free and H_2S-bound forms) with the emission spectrum of Atto620 (λ_{em} = 645 nm), the dye label we selected in this study, can be judged from Figure 1A. In our first experiments, Co-PDF was covalently labeled with the Atto620 dye label on the available amine groups (N-terminus and lysines; for further details on the amine labeling, see section 1.2 in the SI). To test the system, the fluorescence intensity of labeled Co-PDF was monitored during a change from an H_2S-free to an H_2S-saturated environment. Figure 1B shows a typical fluorescence spectrum of a solution containing 100 nM of dye-labeled Co-PDF when excited at the absorption maximum (λ = 620 nm) of the dye (Atto620). The experiment was started by flowing H_2S gas into the fluorescence cuvette (i.e., in saturating conditions). A fluorescence quenching of label emission was clearly observed upon H_2S addition [switching ratio (SR) ~ 50%].[18] Under the experimental conditions tested, the H_2S binding process occurs and results in a clear fluorescence switch.

When argon was bubbled through the solution of the H_2S-bound form of fluorescently labeled Co-PDF, the initial fluorescence intensity value could not be restored (Figure S2 in the SI). This finding suggests that H_2S binding to Co-PDF is irreversible.

To assess whether the amount of FRET-mediated quenching of the label fluorescence varies with the concentration of the analyte, the fluorescence intensity of labeled Co-PDF was monitored after the addition of increasing amounts of KSH solution. Figure S3 in the SI shows that there is a dependence of the fluorescence intensity displayed by the Atto620-labeled Co-PDF on the KSH concentration. When a sample of Atto620-labeled Co-PDF was titrated with fresh buffer, no significant changes in the fluorescence intensity were observed (Figure S4 in the SI).

The detection limit of the proposed system was found to be in the micromolar range (Figure S5 in the SI).

To obtain an indication on the selectivity of the construct, the fluorescence intensity of Atto620-labeled Co-PDF in the presence of biologically relevant and potentially competing thiols [e.g., L-cysteine (L-cys) and glutathione (GSH)] was checked. In the presence of either L-cys or GSH (Figures S6 and S7 in the SI), we observed SRs significantly lower than those found with KSH (Figure S3 in the SI).

We performed several control experiments to exclude the possible influence of other factors on the central FRET experiment (section 1.8 and Figures S8 and S9 in the SI).

The Co-PDF probe closely relates to the Mb-sensing device that we had recently reported.[10] In both cases, when a proper fluorescent label is covalently attached to the protein and FRET between the label and the cofactor binding site of the protein is relied upon, the fluorescent dye acts as a passive "beacon", which is "on" in the H_2S-free state and "off" in the H_2S-bound state of the protein. With Co-PDF as a H_2S-sensing system, we propose herein SRs significantly higher than those obtained with the Mb-based system[10] (50% vs 5%) registered. This finding represents an advantage in the case of real sensing applications.

In order to gain independent evidence for the binding of H_2S to PDF, we performed X-ray diffraction analyses on a Co-PDF single crystal incubated with H_2S (PDB entry code 4AZ4) and a Co-PDF single crystal soaked with β-mercaptoethanol (PDB entry code 4AL3). HS^- replaces water and binds as fourth ligand to the Co^{II} ion (Figure 2A). It, furthermore, weakly hydrogen

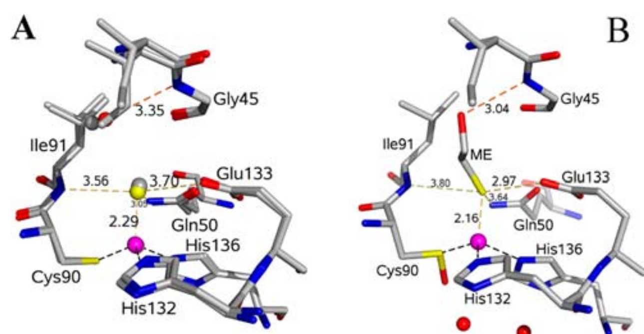

Figure 2. Coordination of thiolates to PDF. (A) Hydrosulfide bound to PDF in Co-PDF (hydrosulfide in yellow and water in red) and Ni-PDF (ligands in gray; distances are omitted). Water molecules alternating with hydrosulfide in the Co-PDF adduct are omitted for clarity. (B) β-Mercaptoethanol (ME) bound in the active site to Co^{II} (magenta). The sulfur (yellow) of hydrosulfide or ME, respectively, coordinates Co^{II}, hydrogen bonds to Glu133, and binds weakly to Gln50 and Ile91. The hydroxyl group of ME displaces a water molecule and clearly hydrogen bonds to Gly45. The observed oxidation of Cys90 to the sulfenic acid does not change the Co^{II} coordination geometry (section 1.6 in the SI).

bonds to Gln50Nε2, Ile91N, and Glu133Oε1. The (hydroxyethyl)sulfide ligand unambiguously binds with its sulfur to the Co^{II} ion. The mercaptoethanol makes an additional hydrogen bond with its hydroxyl group to the main-chain nitrogen atom of Gly45. This interaction is conserved in other structures, e.g., by a water molecule or an ethanol (Figures 2B and Figure S10 in the SI). Not only is binding of thiols determined by the coordinative bond between cobalt and thiolate, but additional interactions allow differently tight binding. Thus, distinguishing different thiols should be possible. To improve the selectivity for H_2S, residues Gly43, Leu91, and Ile44 are candidates for mutations of PDF to change the size of the active site. Even Glu133 and Gln50, which hydrogen bond to HS^-, are amenable because their functionality in the deformylase reaction is not required here.

Preliminary experiments on nickel-containing PDF (Ni-PDF) as a possible way to improve the performance of our sensing device (e.g., enhancing its sensitivity and gaining reversibility) are currently underway in our laboratories. We succeeded in obtaining a HS^- Ni-PDF complex in a crystalline form (PDB entry code 4AL2) and performed its crystal structure analysis (Figure 2A and section 1.6 in the SI). The overall fold and Ni^{II} coordination are isomorphous to those of the Co-PDF complex (average rmsd 0.47 Å on C_α). It is worth noting that only a few examples of nonbiological HS^- complexes of nickel are reported.[19]

In order to gain insight into the possible formation of a coordination adduct between H_2S and the active-site metal ion in Co-PDF, density functional calculations (DFT) were undertaken. The active site of Co-PDF was mimicked by a truncated model comprising only the first-shell metal ligands. In particular, our model consists of a divalent cobalt ion coordinated by two

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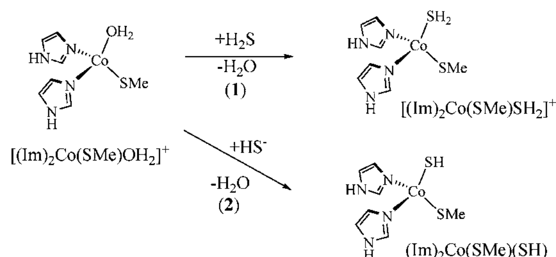
imidazole rings and a CH_3S- group simulating the side chains of His132, His136, and Cys90, respectively. This resembles one model that had been previously used for DFT calculations on the activity of the enzyme.[20] It was previously shown[17,21] that in Co-PDF the metal-bound water molecule is not ionized; hence, in our model, the fourth coordination site is occupied by a water molecule.

The H_2S adduct $[(Im)_2Co(SMe)SH_2]^+$ and the HS^- adduct $(Im)_2Co(SMe)(SH)$ were successfully optimized. In these structures, the coordination polyhedron is best described as distorted tetrahedral with ligand−cobalt bond distances very similar to those observed in Co-PDF (Figure S11 in the SI). In the H_2S adduct $[(Im)_2Co(SMe)SH_2]^+$, the Co−S bond length is 2.57 Å. This is slightly longer than the experimental data of H_2S coordination compounds [ruthenium(II) complexes[22−24]]. In the HS^- adduct $(Im)_2Co(SMe)(SH)$, the HS^- anion is located at 2.32 Å from the metal center. This bond distance is very similar to the M−S bond distance in metal complexes with bridging HS^- groups[25,26] and to that in our structure (Figure 2A). All of our attempts to obtain pentacoordinated HS^- or H_2S adducts in which a water molecule is present in the cobalt coordination sphere were not successful. The H_2S adduct $[(Im)_2Co(SMe)SH_2]^+$ is predicted to be stable in our gas-phase DFT calculations. Nevertheless, its formation starting from the H_2O adduct $[(Im)_2Co(SMe)OH_2]^+$ is not favored. The substitution reaction 1 (Scheme 1) is predicted to be endergonic. The calculated

Scheme 1



Gibbs free-energy change (ΔG) is 11.3 kcal·mol^{-1}. In contrast, the formation of a coordination adduct between HS^- and our model complex is predicted to be exergonic with −24.1 kcal·mol^{-1} (reaction 2, Scheme 1).

In conclusion, fluorescently labeled Co-PDF is suitable as a FRET-based probe for H_2S. The possibility of modifying Co-PDF with a fluorescent label either on the amine groups or on the only cysteine available offers a versatile system and opens the possibility of implementing more advanced sensing devices (e.g., systems carrying two different labels). It should also be mentioned that probably, in view of the presented results, the system proposed here acts as a H_2S dosimeter, which is an irreversible device that progressively accumulates the dose, each time adding up the signal.[27] We believe our findings can be a starting point for possible developments in the fast-growing field of biosensing and, more specifically, of H_2S detection.

■ ASSOCIATED CONTENT

Supporting Information

Experimental part, X-ray diffraction data, and DFT computational details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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