

Fluorescence resonance energy transfer based on interaction of PII and PipX proteins provides a robust and specific biosensor for 2-oxoglutarate, a central metabolite and a signalling molecule

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2-Oxoglutarate is a central metabolite and a signalling molecule in both prokaryotes and eukaryotes. The cellular levels of 2-oxoglutarate vary rapidly in response to environmental changes, but an easy and reliable approach is lacking for the measurement of 2-oxoglutarate. Here we report a biosensor of 2-oxoglutarate based on the 2-oxoglutarate-dependent dissociation of the PII–PipX protein complex from cyanobacteria. Fusions of PII and PipX to either cyan or yellow fluorescent protein can form a complex and their interaction can be detected by fluorescence resonance energy transfer (FRET). Mutations in PII or PipX that affect their interaction strongly decrease the FRET signal. Furthermore, the FRET signal is negatively affected, in a specific and concentration-dependent manner, by the presence of 2-oxoglutarate. This 2-oxoglutarate biosensor responds specifically and rapidly to a large range of 2-oxoglutarate levels and is highly robust under different conditions, including in bacterial cell extracts. We further used this biosensor to study the interaction between PII and its effectors, and our data indicate that excess of Mg^{2+} ions is a key factor for PII to respond efficiently to an increase in 2-oxoglutarate levels. This study paves the way for probing the dynamics of 2-oxoglutarate in various organisms and provides a valuable tool for the understanding of the molecular mechanism in metabolic regulation.

Structured digital abstract

- [PipX binds to PII](#) by [fluorescent resonance energy transfer](#) ([1](#), [2](#), [3](#))

Introduction

The Krebs or tricarboxylic acid (TCA) cycle is a well-conserved central metabolic pathway. It provides important precursors for biosynthesis and the reducing power for ATP production through respiration [1]. Several intermediates of the TCA cycle stand at the junction of different important metabolic pathways,

thus ensuring the balance of different metabolic activities within the cell. Several human diseases are associated with the dysfunctioning of the Krebs cycle or its associated enzymes [2–4]. One of the intermediates is 2-oxoglutarate (α -ketoglutarate, hereafter 2-OG), which serves as a carbon skeleton for nitrogen assimilation.

Abbreviations

2-FPA, 2,2-difluoropentanedioic acid; 2-MPA, 2-methylenepentanedioic acid; 2-OG, 2-oxoglutarate; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; TCA, tricarboxylic acid cycle; YFP, yellow fluorescent protein.

Therefore, 2-OG occupies a strategically important position and constitutes a key element for the proper balancing between nitrogen and carbon assimilation. Numerous studies indicate that 2-OG can be a metabolic signal in bacteria, plants and animals [5–8]. 2-OG serves as a ligand for a G-protein coupled receptor involved in the regulation of blood pressure according to the metabolic state [7]. In both bacteria and plants, 2-OG regulates carbon and nitrogen assimilation, and one receptor of 2-OG conserved in both plants and bacteria is PII [6,8]. The PII protein is a homotrimer and exerts its regulatory function through protein–protein interaction, mostly through the T-loop from each monomer [9]. The core of the PII trimer is a cylindrical and compact structure, with the three T-loops sitting on the core as antenna-like extensions which can adopt flexible or extended conformations. The binding of 2-OG affects the conformation of the T-loops and thus their interaction with PII receptor proteins [10].

Using the cyanobacterium *Anabaena* sp. strain PCC 7120 as a model, we provided important *in vivo* evidence for the signalling function of 2-OG and structural insights into the mechanism of signal transduction [11–14]. In addition to PII, NtcA is another receptor of 2-OG identified from cyanobacteria [15]. NtcA belongs to the CRP/CAP family of transcription factors and acts as a homodimer. The binding of 2-OG produces two effects on NtcA: it stabilizes the NtcA dimer, and it shortens the distance between the two F-helices responsible for DNA binding from 37 Å in the apo form of NtcA to 34 Å in the 2-OG–NtcA complex [12,16]. The latter effect allows the F-helices to fit better into the DNA structure and thus enhances the DNA-binding activity of NtcA.

These studies demonstrate that the 2-OG levels provide an important indicator for the metabolic fluxes in the cells. In addition, the function of 2-OG as a signalling molecule enables cells to sense the metabolic state and triggers a variety of cell responses accordingly. Despite important progress, it is still unknown how the 2-OG level changes in individual cells, and how these changes are affected by different cell activities and in turn regulate cell responses, especially when the changes may occur very rapidly as expected from a very important metabolite such as 2-OG. Even measurement of 2-OG *in vitro* is problematic since it requires either expensive equipment such as LC-MS/MS [17] or relies on an enzymatic assay that gives a high level of noise and little reproducibility (alpha KG Assay Kit; Abcam®). Therefore, we started to design and test a 2-OG sensor system that can quantify the 2-OG levels with the potential of *in vivo* application

for 2-OG analysis. This biosensor system is based on the effect of 2-OG on the interaction of two proteins, PII and PipX, characterized in cyanobacteria [16,18]. The PipX protein was identified as a partner for NtcA [16]. Each NtcA subunit can bind one subunit of PipX in the complex. PipX is required for NtcA-mediated gene expression, and its function has been proposed as a coactivator by stabilizing the active form of NtcA and even helping NtcA to recruit RNA polymerase, although no formal evidence is available [16,18,19].

There exists a complex relationship between 2-OG, PII, PipX and NtcA [16,18,19]. Under nitrogen sufficiency, the level of 2-OG in the cells is low; PipX is sequestered by PII, and NtcA has a low DNA-binding activity. Upon nitrogen limitation, the level of 2-OG increases, leading to the formation of the 2-OG–NtcA complex; 2-OG also binds to PII at the base of the T-loop and affects the binding of PipX negatively. As the concentration of 2-OG increases, the competition between 2-OG and PipX for PII binding frees PipX from PII and enables PipX to switch to NtcA–2-OG, allowing PipX to stabilize the active form of NtcA in complex with 2-OG, and further enhances NtcA-mediated gene expression [19,20]. In summary, the interaction of PipX with either NtcA or PII depends on the intracellular levels of 2-OG, and this observation forms the biochemical basis for the design of our sensor system to monitor the levels of 2-OG. To follow the effect of 2-OG on PII–PipX interaction, we used in this study the approach of fluorescence resonance energy transfer (FRET) [21,22], which is highly sensitive for the detection and quantification of protein–protein interaction. FRET relies on the transfer of the excited state of a donor fluorescent molecule to an acceptor when these two molecules are in close proximity and is therefore strictly correlated to the distance between the two fluorophores [23–26]. We show here that the 2-OG biosensors developed in this study, which we designated PROBS (for protein PII–PipX 2-OG biosensor system), respond quantitatively and specifically to the presence of 2-OG, and this response displays a linear relationship to a wide range of 2-OG concentrations.

Results

Design of 2-oxoglutarate biosensor systems PROBS

The crystal structure of the protein complex between PII and PipX is known in both the unicellular cyanobacterium *Synechococcus elongatus* and the diazotrophic filamentous strain *Anabaena* PCC 7120 [16,18] (Fig. 1B). To design the 2-OG biosensor PROBS, we

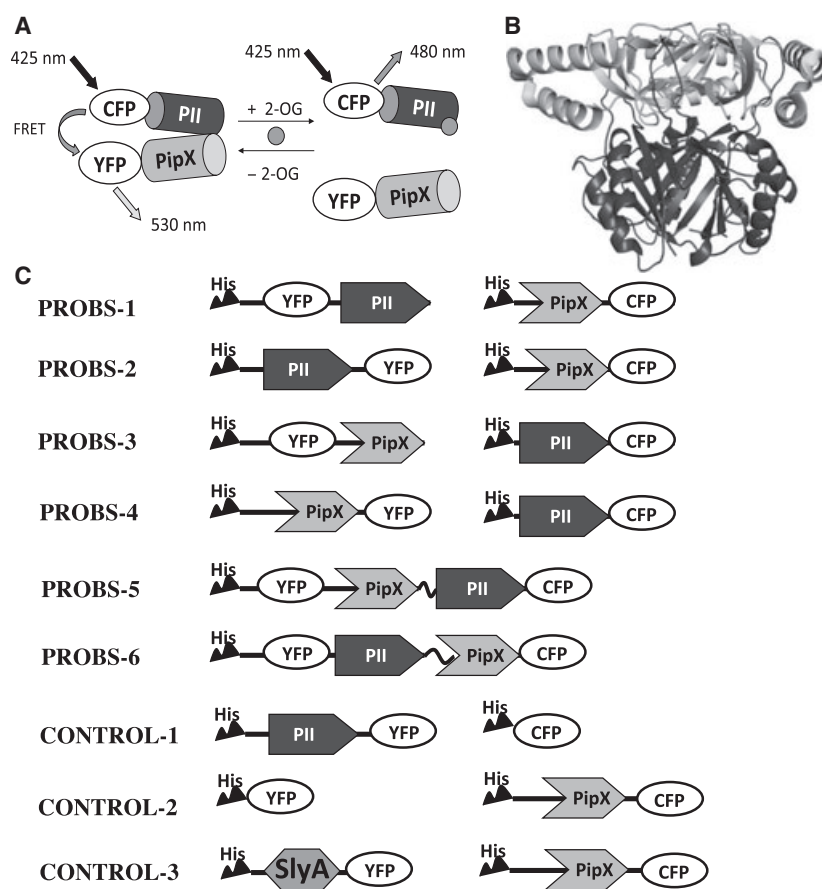


Fig. 1. Concept and design of FRET-based 2-OG biosensor (PROBS). (A) Scheme representing the biochemical basis used for the design of 2-OG biosensors (PROBS). PII (black) and PipX (grey) interact with each other, and the binding of 2-OG to PII disrupts this interaction. CFP and YFP can be fused to each of the interacting partners. In the absence of 2-OG, the two fusions interact together; if the two fluorescent proteins are close enough, CFP emission after excitation at 425 nm can then excite YFP that in turn generates a light signal at 530 nm. In contrast, the binding of 2-OG to PII prevents the formation of the PII–PipX complex; in this case, CFP generates a light signal at 480 nm after excitation, without exciting YFP. (B) Side view of the structure of the PII–PipX complex in *Anabaena* PCC 7120 [18]. PII is shown in black and PipX is shown in grey. (C) List of six PROBS and three CONTROLS. PROBS-1 to PROBS-4 consist of PII and PipX fused to either the N- or the C-terminal ends of YFP or CFP. PROBS-5 and PROBS-6 consist of PII and PipX linked into a single polypeptide by a linker of 21 amino acids with CFP and YFP present at the two ends. CONTROL-1 consists of PII–YFP coupled with CFP alone, while CONTROL-2 consists of PipX–CFP with YFP alone. CONTROL-3 corresponds to a couple formed by PipX–CFP and SlyA–YFP. His represents the Histidine-tag. See also Table 2.

took advantage of the structural information available for PII, PipX and the PII–PipX complexes [16] and of the ability of the FRET approach to quantify protein–protein interaction (Fig. 1A). Cyan and yellow fluorescent protein (CFP and YFP respectively) are an efficient fluorophore pair used in FRET studies [27]. The maximal excitation and emission wavelengths for CFP are 433 nm and 475 nm, respectively, while those of YFP are 514 nm and 528 nm, respectively. As the CFP emission spectrum overlaps the YFP excitation spectrum, CFP can act as a donor and excites YFP as a receptor if the two proteins are close enough to each other (Fig. 1A). We engineered translational fusions in which PipX or PII were fused to CFP or YFP at both

termini (Fig. 1C). These four pairs were named PROBS-1 to PROBS-4.

Additionally, PipX–PII and PII–PipX chimera as a single polypeptide were created in which the two proteins were fused via a flexible linker of 21 amino acids [(GGS) × 3–GS–(GGS) × 3]; the two chimera were further fused to either YFP or CFP at the N- and C-terminal extremities, respectively (PROBS-5 and PROBS-6; Fig. 1C). If properly folded, these two fusions could eventually be used as a positive control for FRET since the two fluorescence proteins are located on the same polypeptide and thus likely to be in proximity to favour energy transfer even without the interaction between PII and PipX. If PII and PipX

could still interact with each other, these single polypeptides could also be used as biosensors for 2-OG, although as we show later this is not the case. Three protein pairs were prepared as negative controls for FRET analysis, CONTROL-1 and CONTROL-2, in which one of the fluorescent proteins was not fused to PII or PipX, and CONTROL-3 in which SlyA, a regulatory protein from *Salmonella typhimurium* [28], was fused to YFP instead of PII (Fig. 1C).

The interaction between PII and PipX could be detected by FRET

To validate our approach, all proteins listed in Fig. 1C were produced in *Escherichia coli* and purified to homogeneity using metal affinity chromatography. The level of purity is >95% for all proteins. To determine whether the fusion proteins with PII and PipX could interact and whether the interaction could be detected by FRET, different protein partners were mixed at an equimolar concentration of 1 μM . Although the maximal excitation wavelength for CFP is 433 nm, CFP was excited at 425 nm in order to reduce the YFP excitation and the emission of fluorescence was recorded between 460 and 600 nm to observe the fluorescence emission of CFP and YFP, at 480 and 530 nm, respectively. For the four pairs, namely PROBS-1 to PROBS-4, as well as the two single polypeptides PROBS-5 and PROBS-6, peak emissions from both CFP and YFP were observed, indicating that energy transfer occurred from CFP to YFP after the excitation of CFP (Fig. 2). As negative controls, when only a CFP fusion was present only a CFP emission was detected after excitation at 425 nm; similarly, when only a YFP fusion was present no emission peak was found after excitation at 425 nm (Fig. S1), but if excited at 480 nm an emission peak from YFP could be recorded at 530 nm (data not shown). For CONTROL-1 and CONTROL-2, for which either PII or PipX is lacking in one of the pairs, no FRET occurred. Furthermore, no FRET was observed when PipX–CFP fusion was incubated with the fusion SlyA–YFP (CONTROL-3), SlyA being a regulatory protein from *S. typhimurium*. From these experiments, we conclude that PII and PipX interaction could take place after fusions to either CFP or YFP. PROBS-2 and PROBS-3 give the best results of energy transfer in FRET analysis, since YFP emission is much stronger than CFP emission. As for PROBS-5 and PROBS-6, FRET could again be detected, and the energy transfer from CFP to YFP is highly efficient for PROBS-5 with a large YFP emission peak compared with that of CFP. Since both CFP and YFP were present on the

same polypeptide, the FRET events recorded for PROBS-5 and PROBS-6 could be explained by either interaction between PII and PipX or just the proximity of the two fluorescent peptides. As shown below, the second hypothesis was confirmed.

PROBS-1 to PROBS-4 respond to 2-OG, a physiological effector of PII

If FRET detected in PROBS-1 to PROBS-6 was caused by the interaction between PII and PipX, in the presence of 2-OG, the physiological effector of PII, the FRET signal should be negatively affected. We therefore tested the effect of 2-OG on the FRET signal. As shown for PROBS-2 (Fig. 3A), as the concentration of 2-OG increases, the emission from CFP increases with concomitant decrease of YFP, indicating that 2-OG weakens fluorescence energy transfer from CFP to YFP. We thus conclude that 2-OG dissociates the two proteins in PROBS-2. Similar results are obtained for PROBS-1, PROBS-3 and PROBS-4 (Fig. 3B). The decrease of the relative emission ratio (ref. emission ratio), defined from the ratio between the fluorescence emission from YFP over that of CFP with the fluorescence emission from YFP over that of CFP obtained without addition of 2-OG as 1, reflects the dissociation of the PII–PipX complex. This dissociation displays a concentration dependence on 2-OG, within the range of 10 μM to 10 mM (Fig. 3B).

However, 2-OG-dependent FRET variation was hardly observed for PROBS-5 and PROBS-6 (Fig. 3B), suggesting that no distance change occurred between CFP and YFP in the presence of 2-OG. From these results, we conclude that FRET observed for these two polypeptides was caused by the fact that both CFP and YFP are located on the same proteins and could be in close proximity after folding for fluorescence energy transfer to occur. Such a fluorescence energy transfer is independent of the interaction between PII and PipX, thus being insensitive to the presence of 2-OG. Therefore, PROBS-5 and PROBS-6 are not suitable for use as a 2-OG sensor.

By its action, 2-OG can be regarded as an inhibitor of the PII–PipX complex. Using the 2-OG concentration effect on the FRET ratio, we calculated the half maximal inhibitory concentration of 2-OG (IC_{50}) for the PROBS pairs (PROBS-1 to PROBS-4). First, the IC_{50} of 2-OG on the four PROBS-1 to PROBS-4 at 1 μM of protein concentration was calculated using a four-parameter equation with a variable slope. Then, as the Hill constant was close to -1 , we used the PRISM equation $\log(\text{inhibitor})$ versus response without variable slope for all experiments. The IC_{50} was esti-

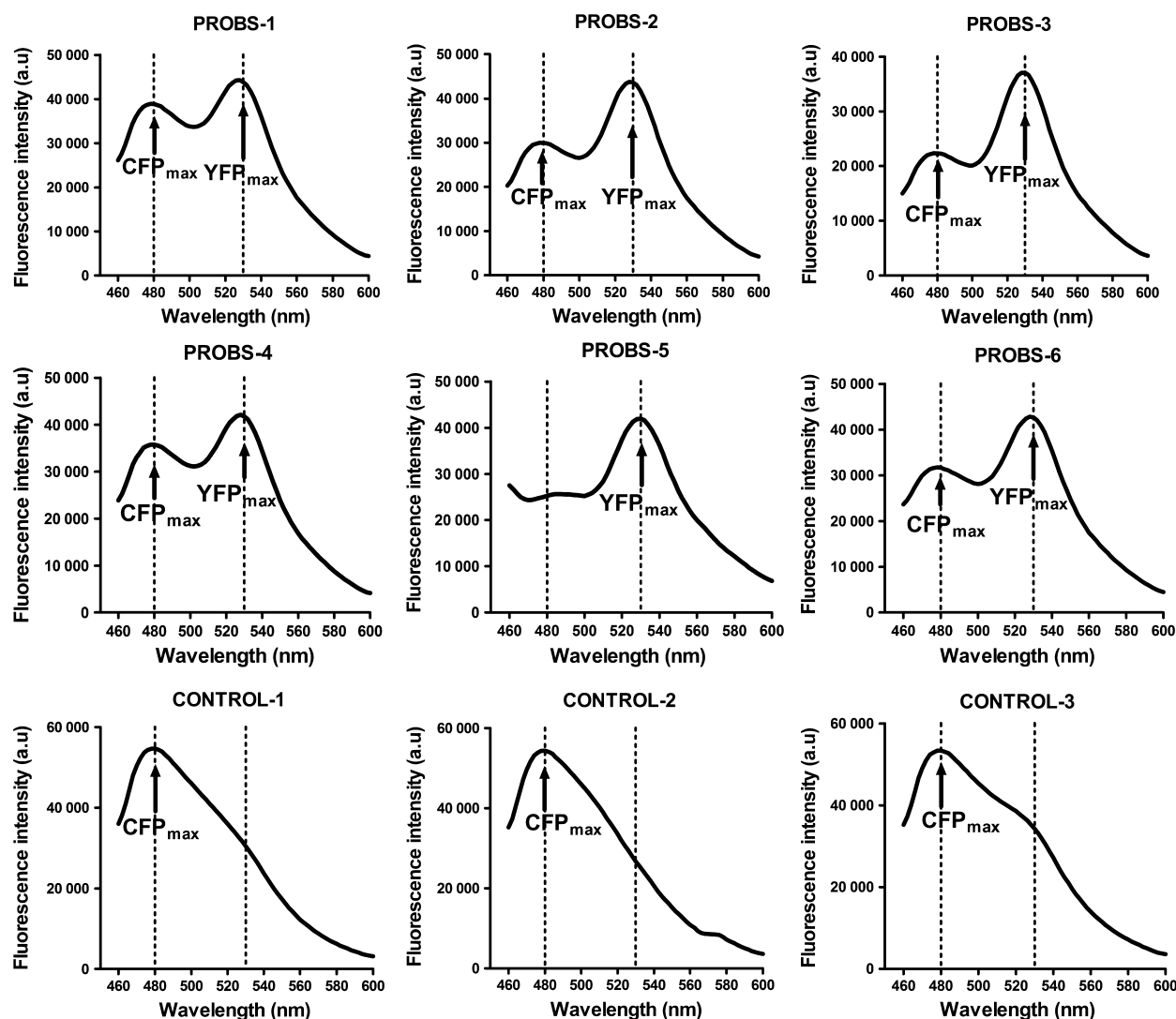


Fig. 2. FRET analysis of PROBS-1 to PROBS-4 and CONTROL-1 to CONTROL-3. For FRET analysis, each sample was excited at 425 nm and the emission intensities were recorded at wavelengths from 460 to 600 nm. Cyan and yellow fluorescence peaks, at 480 nm and 530 nm, corresponding to the peak emission of CFP and YFP, respectively, are indicated by arrows. Results were obtained with 1 μ M of protein at 30 °C from three independent measurements. See Fig. 1 for details of each sample.

mated to be 0.6 mM for PROBS-1, 0.8 mM for PROBS-2, 0.3 mM for PROBS-3 and 0.15 mM for PROBS-4 (Table 1). These results are in good agreement with the inhibitory effect of 2-OG on the PII–NAGK complex (IC_{50} = 0.9 mM), the NAGK protein being an *N*-acetylglutamate kinase that catalyses the committed step in arginine biosynthesis in *S. elongatus* [10].

To further confirm that the interaction between PII and PipX was responsible for the events of FRET observed in PROBS-1 to PROBS-4, we took PROBS-2 as an example and engineered two amino acid substitutions that affect the interaction between PII and PipX. As observed in the crystal structure of *Anabaena*

PCC 7120, the PII–PipX complex is stabilized through two intermolecular hydrogen bonds between residues Thr52 and Glu54 of PII and Tyr35 and Gln37 of PipX [18]. We thus substituted Tyr35 and Gln37 residues of PipX as well as Thr52 and Glu54 of PII by proline residues and studied the effect of these mutations on FRET. As shown in Fig. 3C, both mutant alleles weakened the fluorescence energy transfer from CFP to YFP significantly without the addition of 2-OG, and these values were close to that of the wild-type PROBS-2 in the presence of 10 mM of 2-OG that normally dissociates the PII–PipX complex (Fig. 3C). Furthermore, when 2-OG was added for the two

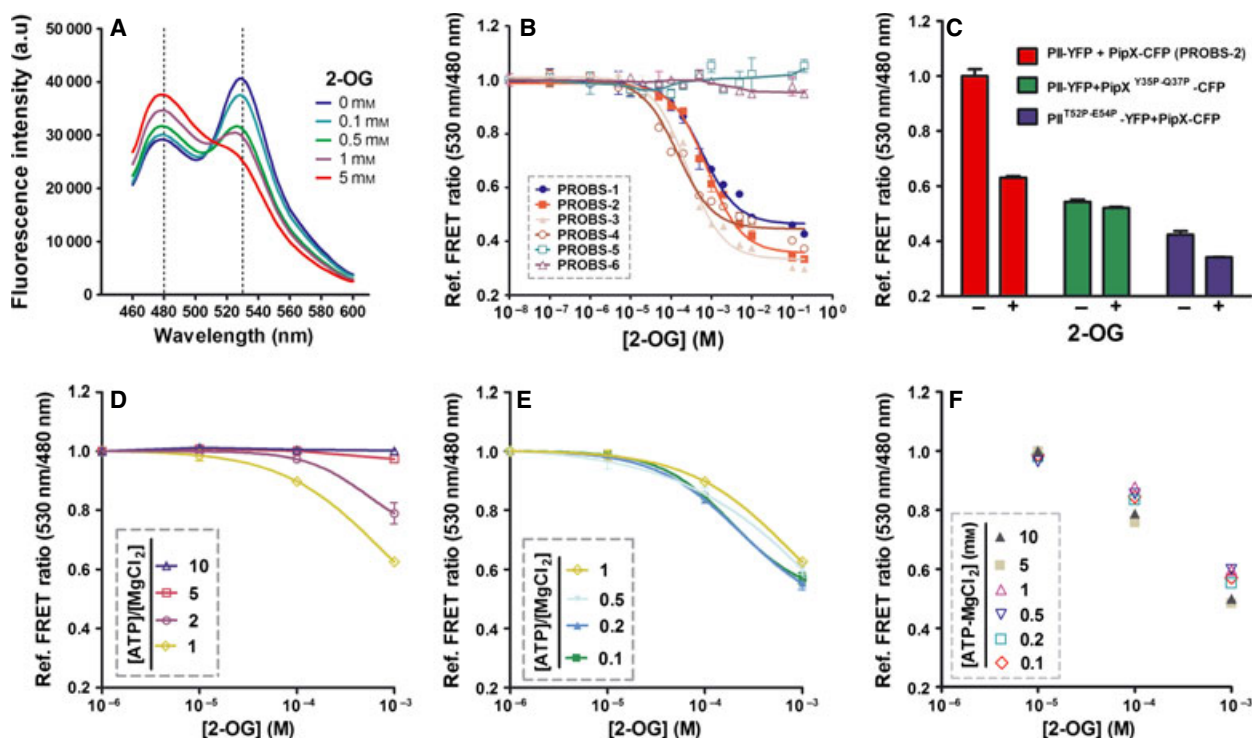


Fig. 3. Characterization of PROBS. (A) Effect of 2-OG on FRET from CFP to YFP. Fluorescence emission spectra of PROBS-2 at 1 μM, excited at 425 nm, with different 2-OG concentrations. Cyan and yellow fluorescence peaks, at 480 nm and 530 nm respectively, are indicated by the vertical dashed lines. (B) 2-OG titration curves of the six PROBS. The relative FRET ratio (YFP emission at 530 nm/CFP emission at 480 nm) is presented as a function of the 2-OG concentration. See also Table 1. (C) Effect of mutations in PII or PipX on the FRET ratio without 2-OG (–) or in the presence of 10 mM of 2-OG (+). PII-YFP + PipX-CFP corresponds to PROBS-2; PII-YFP + PipX^{Y35P-Q37P}-CFP corresponds to PROBS-2 in which Tyr35 and Gln37 of PipX were both substituted by a Pro residue; PII^{T52P-E54P}-YFP + PipX-CFP corresponds to PROBS-2 in which Thr52 and Glu54 were each replaced by a Pro residue. Results were obtained with 1 μM of protein at 30 °C from three independent measurements. (D), (E) 2-OG titration curve (1 μM to 1 mM) obtained with 1 μM PROBS-2 at pH 7.4 incubated in the presence of 1 mM MgCl₂ and different concentrations of ATP. In (D), the ATP concentrations are 1–10 times (1–10 mM) higher than the MgCl₂ concentration. In (E), the ATP concentration is 1–10 times (0.1–1 mM) lower than the MgCl₂ concentration. (F) Change of FRET ratio with 1 μM PROBS-2 with different concentrations of 2-OG in the presence of equimolar ATP and MgCl₂. ATP and MgCl₂ were maintained at the same concentration, from 0.1 mM to 10 mM.

mutant forms of the biosensors, the intensity of the FRET still decreased especially for PII^{T52P-E54P}-YFP+PipX-CFP, but to a much lesser extent compared with the wild-type form of PROBS-2. These results show that the interaction between PII and PipX, although not completely abolished, was severely affected by the mutations introduced to either PII or PipX. The residual interaction between PII and PipX with the mutant forms of the biosensor still remained sensitive to the addition of 2-OG, albeit weakly, as expected.

Mg-ATP is required for 2-OG-mediated decrease of FRET in the biosensor

Several studies demonstrated that both ATP and Mg²⁺ are essential for efficient 2-OG binding to the PII protein [29–31]. 2-OG binds to PII in the vicinity

of Mg-ATP, with the Mg²⁺ coordinating 2-OG and ATP. For this reason, the buffer used in the FRET experiments described above was supplemented with ATP and MgCl₂. Our experiments showed indeed that neither ATP nor Mg²⁺ is required for the formation of the PII–PipX complex since FRET was detected in their absence (data not shown), but they are strictly necessary for 2-OG-dependent dissociation of the complex (Fig. 3D–F). This result is in agreement with the observation that Mg-ATP binding on PII is indispensable for the binding of 2-OG. In Mg²⁺ excess, when ATP was added at concentrations between 0.1 and 1 mM while Mg²⁺ ions (at 1 mM) and proteins (at 1 μM) remained constant, the behaviour of 2-OG sensing of PROBS-2 was very similar (Fig. 3E). However, we observed little change in the FRET signal with excess of ATP compared with Mg²⁺ (Fig. 3D); when

Table 1. Characteristics of the PROBS based on the 2-OG titration curves. ND, not determined.

	PROBS 1	PROBS 2	PROBS 3	PROBS 4	PROBS 5	PROBS 6	CONTROL 1	CONTROL 2	CONTROL 3
Molecular weight of the YPF fusion prot (kDa)	42.2	42.2	40.7	40.7	81.2	81.2	42.2	30	45.8
Molecular weight of the CFP fusion prot (kDa)	40.5	40.5	42	42			31.2	40.5	40.5
IC ₅₀ , inhibitory concentration 50 (μM)	600 ± 60	782 ± 54	256 ± 20	149 ± 18	ND	ND	ND	ND	ND
FRET ratio change for 10 mM 2-OG (%)	53 ± 1	63 ± 1	68 ± 1	56 ± 1	4 ± 3	4 ± 2	ND	ND	ND
R ²	0.9815	0.9908	0.9875	0.9774	0.5849	0.9776	ND	ND	ND

the concentration of ATP increased to 5 or 10 mM, 2-OG was unable to dissociate the protein complex, while a concentration of ATP of 2 mM gave an intermediate response of PROBS-2 to 2-OG. Both ATP and Mg-ATP can bind to PII, but only the latter molecule allowed subsequent binding of 2-OG [10,31]. Thus, if the concentration of ATP is too high relative to that of Mg²⁺, such conditions favour the formation of PII bound with ATP rather than Mg-ATP. In such a case, 2-OG will be unable to bind to PII and thus to dissociate the PII–PipX complex. Indeed, when we kept ATP and Mg²⁺ at equimolar concentrations, PROBS-2 became sensitive again to 2-OG variation even at high ATP concentrations (2–10 mM) (Fig. 3F).

PROBS-2 is highly robust and responds specifically to 2-OG

Since a number of metabolites are structurally similar to 2-OG, we sought to determine whether PROBS-1 to PROBS-4 developed here could be specific enough to be used as 2-OG biosensors. As 2-OG is an intermediate of the TCA cycle, we compared the effect of 2-OG with that of several intermediates of the TCA cycle using PROBS-2 as an example. As shown in Fig. 4A, PROBS-2 responds specifically to 2-OG since isocitrate, succinate, oxaloacetate or fumarate had no effect on the PII–PipX interaction as shown by the absence of detectable changes in the FRET ratio in the presence of up to 10 mM of these compounds. PROBS-2 is slightly sensitive to citrate and L-glutamate when their concentration is above 1 mM. The physiological level of citrate known in *E. coli* is about 2 mM [17]; the interference from citrate on the FRET signal compared with the strong effect induced by 2-OG is thus rather weak. The intracellular glutamate concentration is about 96 mM in *E. coli* [17], which may potentially interfere with the measurement of 2-OG; however, this interference could be minimized since the changes in

the levels of 2-OG and glutamate within the cells are inversely related as 2-OG is a carbon skeleton for amino acid synthesis [33]. Furthermore, as we show later, 2-OG could be measured quantitatively using cell extracts. Glutaric, α-ketoadipic, lactic and malic acids had little effect on the FRET signal. We further introduced in our test two synthetic analogues of 2-OG, 2-methylenepentanedioic acid (2-MPA) and 2,2-difluoropentanedioic acid (2-FPA). In the 2-MPA and 2-FPA structures, the 2-OG carbonyl group is replaced by a vinyl group or a fluorine atom, respectively [13]. 2-MPA – but not 2-FPA – could interact with NtcA [32]; however, none of them could interact with PII, since PII and NtcA do not share a structurally similar binding pocket for 2-OG (unpublished data) [12,18,32]. The FRET analyses showed that neither 2-MPA nor 2-FPA had the ability to disrupt the PII–PipX complex as no difference in the FRET ratio was observed in the presence of up to 10 mM of these analogues (Fig. 4A). All these results demonstrate that the biosensor PROBS-2 is highly specific for 2-OG.

To better characterize the biosensors developed in this study, we used PROBS-2 as an example and analysed the performance of the sensor under different conditions corresponding to those that might be encountered for *in vitro* or *in vivo* studies. We first tested the impact of a change in the concentration of the protein fusions on the FRET signal. We thus measured the FRET ratio with seven different concentrations of PROBS-2 (Fig. 4B). The IC₅₀ values of 2-OG deduced from these analyses were 10 ± 1.7, 8.5 ± 1.2, 282 ± 54, 441 ± 114, 1200 ± 132, 1000 ± 35 and 1300 ± 34 μM in the presence of 0.01, 0.1, 0.5, 0.8, 1, 1.5 and 2 μM of purified proteins, respectively. A lower biosensor concentration (0.01 or 0.1 μM) is sensitive to a lower level of 2-OG (in the 1–100 μM range) but becomes saturated when the 2-OG level increases over 100 μM, whereas the biosensor at concentrations of 0.5 or 1 μM has the ability to measure variation of the

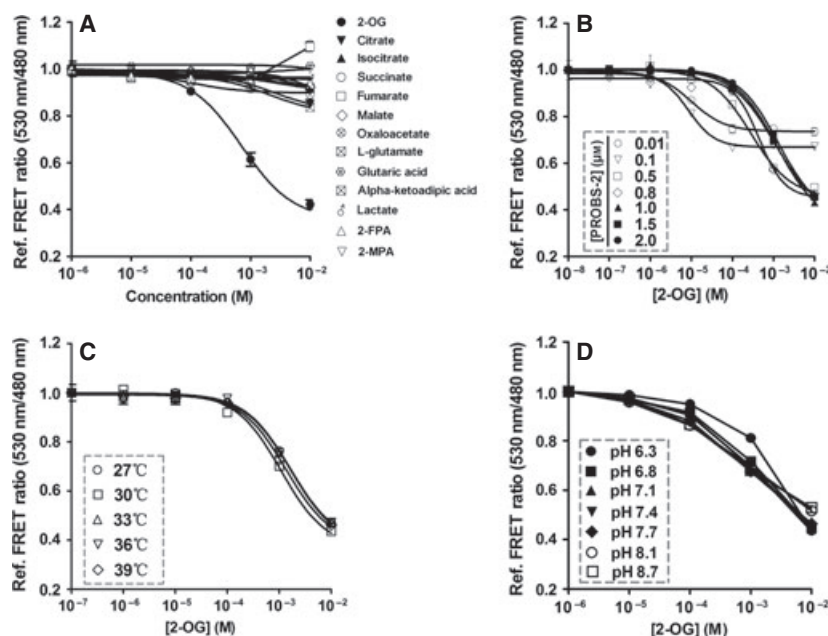


Fig. 4. Specificity of the PROBS-2 biosensor. (A) Specificity of PROBS-2. The 2-OG titration curve of PROBS-2 was obtained in the presence of different metabolites at different concentrations. Citrate, isocitrate, succinate, fumarate and oxaloacetate are intermediates of the TCA cycle. Other compounds tested include the 4-C compound lactate, the 5-C compound glutamic acid, the 6-C compound α -ketoadipic acid, glutamate, and 2-FPA and 2-MPA that are two synthetic analogues of 2-OG. Results were obtained from three independent measurements (1 μ M PROBS-2 at 30 °C, pH 7.4). (B) 2-OG titration curve at different 2-OG concentrations from 0.01 μ M to 10 mM in the presence of 0.1 μ M, 0.5 μ M, 0.8 μ M, 1 μ M, 1.5 μ M and 2 μ M PROBS-2 protein (30 °C, pH 7.4). (C) 2-OG titration curve of PROBS-2 as a function of temperature from 27 °C to 39 °C with 2-OG concentration from 0.1 μ M to 10 mM (1 μ M protein at pH 7.4). (D) 2-OG titration curve of PROBS-2 as a function of different pH values from 6.3 to 8.7 with 2-OG concentration changing from 1 μ M to 10 mM (1 μ M protein at 30 °C).

2-OG levels in the range 0.01–10 mM. A higher concentration of the biosensor (1.5 or 2 μ M) did not show better sensitivity. The biosensor at a concentration of 0.5–1 μ M is therefore suitable to sense and respond to 2-OG variations in a cell or cell extracts since 2-OG levels vary between 0.5 and 10 mM in *E. coli* and 0.1 and 0.3 mM in *Anabaena* PCC7120 [13,17].

The optimal growth temperature for *E. coli* is 37 °C while that of *Anabaena* is 28 °C. We therefore exposed the PROBS-2 biosensor to temperatures between 27 and 39 °C. The fusion proteins were incubated for 20 min at the desired temperature prior to 2-OG addition. The 2-OG titration curves did not show any significant difference at the tested temperatures (Fig. 4C). The intracellular pH of *E. coli* varies between 7.2 and 7.8 [34] while that of *Anabaena* is maintained at 7.5 [35]. The tolerance to pH of the PROBS-2 biosensor was tested between pH 6.3 and 8.7 (Fig. 4D). When the concentration of 2-OG is 1 mM, we observed that the decrease of the FRET ratio was slightly less important at pH 6.3, which can be explained by the fact that lower pH is known to affect negatively the formation of Mg-ATP [36], which in turn is necessary for 2-OG

binding to PII (see also Fig. 3). However, this effect was no longer visible when the concentration of 2-OG increased to 10 mM. Otherwise, no significant differences were detected at pH values ranging from 6.8 to 8.7. These results show that the PROBS-2 biosensor is robust and sensitive at different conditions tested.

2-OG biosensor responds rapidly to variations of 2-OG levels

The levels of 2-OG *in vivo* can vary rapidly in response to environmental changes. We thus sought to determine whether our biosensor system could respond reliably and fast enough to an increase or decrease of the 2-OG levels. We first determined, for a given concentration of 2-OG, the time necessary for the biosensor to reach equilibrium. As shown in Fig. 5A, the addition of 2-OG at 0.5 or 1 mM induced a FRET ratio decrease within minutes, which reached equilibrium in $\sim$ 5 min and remained stable for at least 50 min (Fig. 5A). To monitor the response of the sensor to variations of 2-OG, the 2-OG concentration was either decreased by dilution or increased by addition of

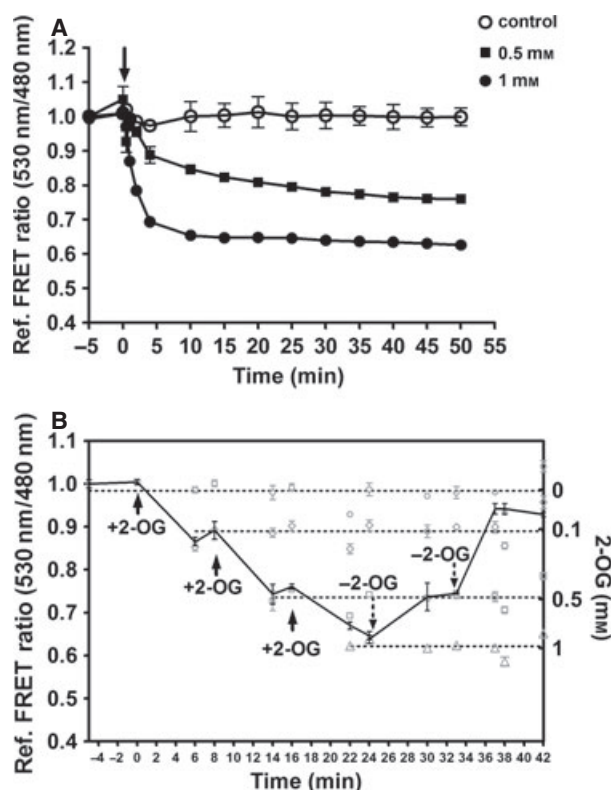


Fig. 5. Dynamic response of PROBS-2 to rapid changes of 2-OG levels. (A) The FRET ratio changes after injection (time 0) of 0.5 mM, 1 mM 2-OG or control buffer (control). Emission data were recorded before (time minus) and after the injection every minute during the first 5 min and then every 5 min for the next 45 min. Results were obtained with three independent measurements ($1 \mu\text{M}$ PROBS-2 at 30°C , pH 7.4). (B) FRET ratio curves during continuous changes of 2-OG concentration. The experiment starts without 2-OG. Then five consecutive changes in 2-OG concentration in the same sample were achieved by adding 2-OG (solid arrow) at time 0 min to a final concentration of 0.1 mM, at 8 min to 0.5 mM and 16 min to 1 mM, or by dilution of 2-OG while other components were maintained constant (dashed arrow): decrease of 2-OG to 0.5 mM at 24 min and to 0.1 mM at 33 min. As control, an aliquot was kept before each change in 2-OG level, and the variation of FRET was recorded until the end of the entire experiments (dashed line; open circle for 0 mM, open diamond for 0.1 mM, open square for 0.5 mM and open triangle for 1 mM). Three independent experiments were performed ($1 \mu\text{M}$ PROBS-2 at 30°C , pH 7.4).

2-OG while all other components were kept constant (Fig. 5B). The experiment was first initiated without 2-OG, and then with 0.1 mM of 2-OG (time 0); the 2-OG concentration was further increased to 0.5 and 1 mM after 8 and 16 min, respectively, and was then decreased by dilution to 0.5 and 0.1 mM after 24 and 33 min (Fig. 5B). This experiment demonstrates that the biosensor responds rapidly to increase or decrease of 2-OG levels, reaching equilibrium within 5–6 min.

In addition, when the 2-OG reached similar levels after dilution or re-addition of 2-OG, the FRET ratios obtained were comparable. For example, the FRET ratios at equilibrium at times 8 and 38 min (both with 0.1 mM of 2-OG) were similar, as well as those at times 16 and 33 min (both with 0.5 mM of 2-OG). As a control, each time that the 2-OG level was increased or decreased, an aliquot of the sample was kept without further modification and the FRET was examined until the end of the experiments; as expected, the FRET signal remained constant under such conditions (Fig. 5B). These results demonstrate that PROBS-2 is stable and responds rapidly to variations of 2-OG levels and can be used in real-time experiments.

2-OG can be quantified *ex vivo* from cell extracts of *E. coli* using the PROBS system

To assess the performance of our biosensor, we used PROBS-2 to measure 2-OG levels in cell extracts of *E. coli*. *E. coli* were cultured in a Gutnick minimal salts medium with 0.4% mannitol, in the presence of either a low nitrogen availability (2 mM NH_4Cl) or a high nitrogen availability (10 mM). The intracellular 2-OG concentration using our biosensor is 10 times lower ($179 \pm 34 \mu\text{M}$) in *E. coli* cells grown with 10 mM NH_4Cl than with 2 mM NH_4Cl ($1789 \pm 142 \mu\text{M}$) (Fig. 6A). This ratio is in good agreement with data published previously; under similar culture conditions, the level of 2-OG was estimated to vary by a factor of 10 in one report [37] and a factor of 20 [33]. We also followed the changes in the 2-OG levels when nitrogen availability increases in the cell culture with the addition of NH_4^+ . In accordance with the results reported previously [33], the intracellular concentration of 2-OG dropped gradually following the upshift of NH_4Cl from 2 mM to 10 mM, and reached a low level of 2-OG ($186 \pm 25 \mu\text{M}$) after 2 h, as observed in a culture continuously incubated in the presence of 10 mM of NH_4Cl ($179 \pm 34 \mu\text{M}$, Fig. 6B).

Furthermore, we added a determined amount of 2-OG ($500 \mu\text{M}$) to two cell extracts of *E. coli* to determine if 2-OG can be adequately quantified using our biosensor system (Fig. 6C). In cell extract a, the level of 2-OG estimated using PROBS-2 increased from $280 \pm 52 \mu\text{M}$ to $735 \pm 62 \mu\text{M}$; and in cell extract b, from $74 \pm 17 \mu\text{M}$ to $592 \pm 32 \mu\text{M}$. 2-OG could also be measured when added to LB or M9 growth medium, yielding data similar to the standard curve of 2-OG obtained in a buffer without addition of a culture medium (data not shown). These experiments demonstrate the robustness of the PROBS system for the reliable quantification of 2-OG concentrations in a complex solution.

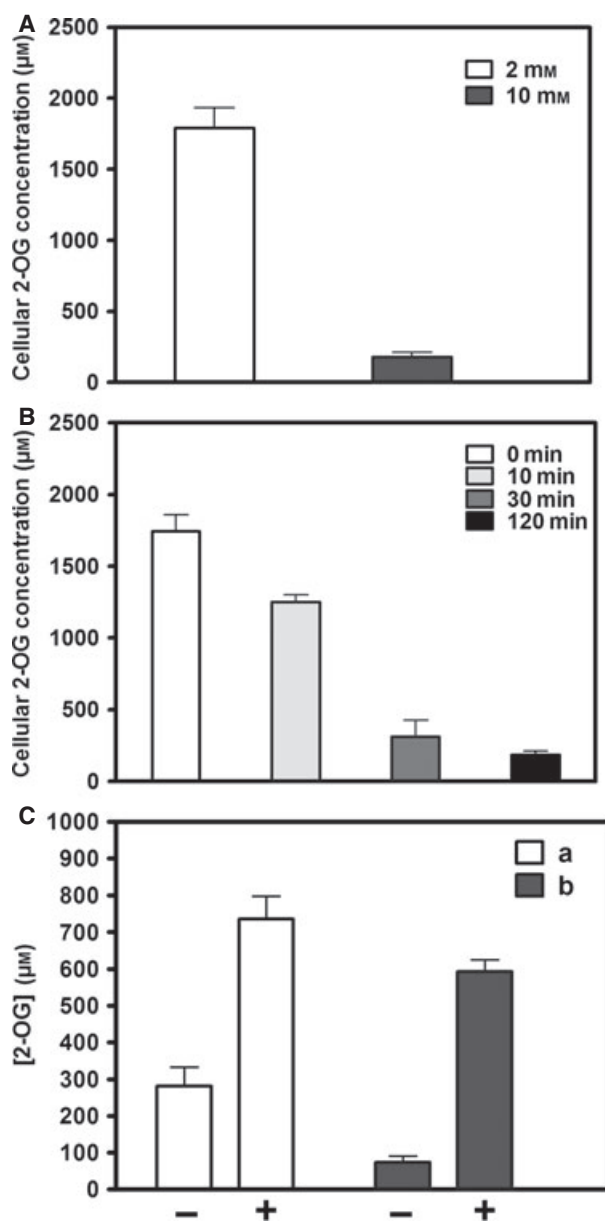


Fig. 6. Quantification of 2-OG using PROBS-2 in bacterial cell extracts. (A) 2-OG concentrations in *E. coli* grown under two nitrogen regimes, with 2 mM or 10 mM of NH₄⁺. The levels of 2-OG were converted to cellular concentration based on the total cell volumes. (B) 2-OG concentrations in *E. coli* cells after upshift from 2 mM to 10 mM NH₄⁺. Samples of *E. coli* culture were collected at 0, 10, 30 and 120 min after the addition of NH₄⁺ to a final concentration of 10 mM. Results were obtained with 0.5 μM of PROBS-2 at 30 °C, with three independent measurements. The value of 2-OG was converted to cellular levels as in (A). (C) Response of PROBS-2 to 2-OG addition in bacterial extracts. 500 μM of 2-OG was added to two bacterial cell extracts (A and B) of *E. coli*.

Discussion

In this study, we developed a series of 2-OG biosensors. This system relies on the interaction between PII and PipX, two well-characterized proteins from cyanobacteria, as well as the ability of the FRET approach to measure and quantify protein–protein interaction. We could detect FRET from CFP to YFP when these fluorescent proteins were fused to either the C-terminal or the N-terminal ends of PII or PipX. For PROBS-1 to PROBS-4, the FRET is strictly dependent on PII–PipX interaction, and this conclusion is supported by several observations. First, the physiological effectors of PII, such as 2-OG, ATP or Mg-ATP, affect FRET, with biochemical kinetics characteristic of PII–PipX interaction or PII–effector interaction (Fig. 3). Second, mutations of either PII or PipX that affect the PII–PipX interaction negatively weaken the fluorescent energy transfer from CFP to YFP (Fig. 3). Finally, no FRET was observed in all the negative controls in which it lacks either one of the fluorescent proteins or one of the two protein-interacting partners (PII or PipX) (Fig. 1). Our biosensors are specific to 2-OG, since other intermediates of the TCA cycle or two structural analogues of 2-OG had little effect on the FRET signal.

The four biosensors, PROBS-1 to PROBS-4, cover a large range of 2-OG concentrations that can be detected, from micromolar to millimolar range, with a linear relationship that can help to establish a standard curve of 2-OG from which 2-OG concentrations can be deduced from various biological samples. As examples, the 2-OG levels were reported to vary within 100–300 μM in the cyanobacterium *Anabaena* PCC 7120 [13] and from 0.5 to 10 mM in *E. coli* [37]. These changes in 2-OG still fall within the effective range that can be detected by our biosensors.

The biosensor system described here provides an easy and reliable assay method for the detection of 2-OG changes in biological samples. We tested the usefulness of our biosensor by using *E. coli* extracts and found that variations of 2-OG could be reliably estimated using one of our biosensors, with results comparable with those reported previously [33], with a change by 10-fold when *E. coli* was cultured in the presence of 2 or 10 mM of NH₄⁺. The values, when converted to cellular concentrations, are somehow different, even though they still fall within the same level of magnitude, compared with those reported previously [33,37]. These differences could be caused by the culture conditions, the way the cell volumes are estimated or the method used to prepare the cell extracts. What is encouraging is that the addition of a

determined amount of 2-OG to a cell extract can be measured reliably using our biosensor system. We also tried to compare our biosensor system with a commercially available kit (alpha KG Assay Kit; Abcam®). Two batches of the commercial kits were tested: one failed to determine a standard curve of 2-OG because the values obtained varied too widely, and another allowed us to determine the variation of 2-OG with a large standard deviation and much smaller differences in 2-OG levels in *E. coli* cell extracts cultured with 2 or 10 mM of NH_4^+ . This poor performance was consistent with our previous experiments using commercial kits to measure 2-OG [13].

The biosensor system could also be used to better understand the molecular mechanism in the interaction between PII and PipX or PII and its effectors. Indeed, our results revealed interesting data on the effect of ATP and Mg-ATP on 2-OG binding to PII. It is well known that Mg^{2+} ions and ATP are necessary for 2-OG binding to PII, because Mg^{2+} is chelated to ATP and ligates to three oxygen atoms of 2-OG in the PII binding pocket [10]. We found that 2-OG cannot effectively dissociate PII from PipX when the concentration of Mg^{2+} ions is two-fold or several fold below that of ATP. ATP, ADP and Mg-ATP can all bind to PII [10,31]. Our observation can thus be explained by a competition between ATP and Mg-ATP for PII binding. Mg^{2+} ion is one of the most abundant divalent cations in cells; in *E. coli*, mostly Mg^{2+} exists in bound forms but 1–2 mM remains free in solution [38]. Such a situation should help the formation of Mg-ATP, thus facilitating 2-OG binding to PII when the cellular concentration of 2-OG increases.

Ultimately, such biosensors have the potential to be used *in vivo* to trace the dynamics of 2-OG in the cells and in real time. We are currently testing various versions of the biosensor system in different bacteria, and the main challenge is to achieve stable expression for the protein couple in cells. Often, the two protein fusions are not expressed at similar levels, making the data highly unreliable. Thus, it is essential to optimize the biosensor system in order to quantify the variations of 2-OG *in vivo*. There are currently only a few biosensor systems for the detection of small signalling molecules; examples include the sensor system for calcium [39] and c-di-GMP [40]. Since 2-OG acts as a metabolic signal in many organisms, the ability to observe spatiotemporal changes in 2-OG levels in the cells will be important to understanding the modulation of metabolic fluxes in response to environmental changes.

Materials and methods

Strains, plasmids, primers and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. Custom oligonucleotides were synthesized by Eurogentec (Anger, France) and are listed in Table 3. *E. coli* K12 strains DH5 α , BL21(DE3) and W3110 were used for construction of recombinant plasmids, protein production and 2-OG extraction respectively. *E. coli* strains were routinely grown in LB medium with antibiotics when required (100 $\mu\text{g}\cdot\text{mL}^{-1}$ for ampicillin, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ for kanamycin).

Plasmid construction

Polymerase chain reactions were performed with a Biorad thermocycler, using the PrimeSTAR DNA polymerase (Ozyme, Saint Quentin en Yvelines, France). *Anabaena* PCC7120 *glnB* (encoding PII) and *pipX* genes were inserted into the pET15b::mCYPet and the pET15b::mY-Pet vectors [41], producing fusions to an N-terminal polyhistidine tag for affinity purification and to monomeric CYPet or YPet. pET-Ypet_PII and pET-Ypet_PipX were constructed by insertion of *glnB* and *pipX* PCR products (amplified using the CSB35/CSB44 and CSB37/CSB45 oligonucleotide couples, respectively) into the *SpeI/BamHI*-digested pYPet plasmid. The PCR products of *glnB* or *pipX* (amplified using the CSB3/CSB47 or CSB5/CSB48 oligonucleotide couples, respectively) were inserted into the *NdeI/KpnI*-digested pCYet to yield pET-PII_CYPet and pET-PipX_CYPet. pET-PII_YPet and pET-PipX_Ypet were constructed by insertion of the mYFP-coding sequence (amplified from pYPet using the H_01 and H_02 oligonucleotides) into pET-PipX_CYPet and pET-PII_CYPet digested with *KpnI* and *BamHI*. pFRET-PipX-PII and pFRET-PII-PipX were constructed as follows: *mYPet-pipX*, *glnB-mCYPet*, *mYPet-glnB* and *pipX-mCYPet* were PCR-amplified from pET-Ypet_PII, pET-Ypet_PipX, pET-PipX_CYPet and pET-PII_CYPet using CSB107/CSB6, CSB13/CSB108, CSB107/CSB4 and CSB15/CSB108, respectively. First, *mYPet-pipX* and *mYPet-glnB* were inserted into the *NdeI/BamHI* sites of the pET28a+ plasmid to yield pET28-mYPet-PipX and pET28-mYPet-PII, respectively. Then, *glnB-mCYPet* and *pipX-mCYPet* were inserted into the *BamHI/HindIII* sites of pET28-mYPet-PipX and pET28-mYPet-PII, respectively, to yield pFRET-PipX-PII and pFRET-PII-PipX.

Site-directed substitutions were introduced by quick-change mutagenesis: complementary pairs of mutagenic oligonucleotides (Table 3) were used to amplify the whole plasmid template and to introduce the mutation at the desired site. All constructs were verified by restriction analyses and DNA sequencing (Millegen, Labège, France).

Table 2. Description of the strains and the plasmids used in this study.

Strain or plasmid	Description	Source or reference
<i>E. coli</i> BL21(DE3)	F [−] <i>ompT gal dcm lon hsdSB(rB[−] mB[−]) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])</i>	Laboratory collection
DH5α	F [−] Φ80 <i>lacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rK [−] , mK ⁺) <i>phoA supE44</i> λ [−] thi-1 <i>gyrA96 relA1</i>	Laboratory collection
W3110	F [−] , lambda-IN(rrnD-rrnE)1 rph-1	Laboratory collection
pET28a(+)		Laboratory collection
pCYPet	pET15b::mCYPet	Ohashi <i>et al.</i> [41]
pYPet	pET15b::mYPet	Ohashi <i>et al.</i> [41]
pET-Ypet_PII	pET15b::mYPet_glnB	This work
pET-PII_YPet	pET15b::glnB_mYPet	This work
pET-Ypet_PipX	pET15b::mYPet_pipX	This work
pET-PipX_Ypet	pET15b::pipX_mYPet	This work
pET-PipX_CYPet	pET15b::pipX_mCYPet	This work
pET-PII_CYPet	pET15b::glnB_mCYPet	This work
pFRET-PipX-PII	pET28a::mYPet_pipX_linker_glnB_mCYPet	This work
pFRET-PII-PipX	pET28a::mYPet_glnB_linker_pipX_mCYPet	This work

Table 3. Oligonucleotides used for mutation engineering and gene cloning in this study. Nucleotides corresponding to restriction sites or mutations are underlined. Nucleotides corresponding to a linker are in bold.

Plasmids	Gene, mutation or primer	Additional designation	Oligonucleotide (5' → 3')
pET-YPet_PII	<i>SpeI</i> -PII_Up	CSB35	ACTAGTATGAAAAAAGTAGAAGCTAT
	PII-TAA- <i>Bam</i> HI_Dn	CSB44	GGATCCTTACACGGCTTCTGTATTCTTCTC
pET-YPet_PipX	<i>SpeI</i> -PipX_Up	CSB37	ACTAGTATGAATCCAGAAAACCTCGGAA
	PipX-TAA- <i>Bam</i> HI_Dn	CSB45	GGATCCTTATTGGAAGGTGCGTTGGAAAAC
pET-PipX_CYPet	<i>NdeI</i> -ssATG-PII_Up	CSB3	CATATGAAAAAAGTAGAAGCTATTATC
	PII-ssTAA- <i>KpnI</i> _Dn	CSB47	GGTACCCACGGCTTCTGTATTCTTCTC
pET-PII_CYPet	<i>NdeI</i> -ssATG-PipX_Up	CSB5	CATATGAATCCAGAAAACCTCGGAAACT
	PipX-ssTAA- <i>KpnI</i> _Dn	CSB48	GGTACCTTGAAGGTGCGTTGGAAAAC
pET-PII_YPet and pET-PipX_YPet	<i>KpnI</i> -YFP_Up	H_01	ATATGGTACCATGGTGAGCAAAGGCCA
	YFP- <i>Bam</i> HI_Dn	H_02	TATAGGATCCTTAGCGGCCGCCAC
pFRET-PipX-PII and pFRET-PII-PipX	<i>NdeI</i> -YFP_Up	CSB107	TACATATGGTGAGCAAAGGCCG
	PipX-linker- <i>Bam</i> HI_Dn	CSB6	GGATCC ACTACCACCACTACCTCCAGATCCACCT TGGGAAGGTGCGTTGGAAAAC
	<i>Bam</i> HI-linker-PII_Up	CSB13	GGATCC GGTGGATCTGGAGGTAGTGGTGGTAG TAT GAAAAAAGTAGAAGCTATT
	CYPet- <i>Hind</i> III_Dn	CSB108	TAAAGCTTTTATTGTACAGTTCGTCCAT
	PII-linker- <i>Bam</i> HI_Dn	CSB4	GGATCC ACTACCACCACTACCTCCAGATCCACCC ACGGCTTCTGTATTCTTCTC
pET-PipX ^{Y35P_Q37P} _CYPet	mPipX_Y35P_Q37P_Up	CSB122	GATTTGTTCAACAACACTTCCAGCTCCACGCTTATTC TTTCTAGTA
	mPipX_Y35P_Q37P_Dn	CSB123	TGTTGTGAACAAATCCTGGCTTTCATCAACCATGCA

Protein expression and purification

Protein fusions were purified by immobilized metal ion chromatography. *E. coli* BL21(DE3) cells carrying pET derivative were grown at 37 °C by shaking in LB broth to an A_{600} of 0.5 and then protein fusions were induced by the addition of 0.1 mM isopropyl-β-thiogalactoside for 12 h at 17 °C. Cells were harvested, resuspended in LEW

buffer (Protino Ni-IDA Packed Columns, Macherey-Nagel, Düren, Germany) supplemented with 1 mg·mL^{−1} lysozyme and broken by sonication in ice. Proteins were purified by affinity column according to the manufacturer's instructions. Production level and protein purity were verified by SDS/PAGE. Finally, the buffer was exchanged to 20 mM sodium phosphate buffer pH 7.4, 10% glycerol using PD-10 desalting columns (GE Healthcare, Uppsala, Sweden)

and the protein solutions were stored at -80°C . Protein concentration was determined by the Bradford reaction using the protein assay reagent and the standard curve was performed with BSA (Bio-Rad, Hercules, CA, USA).

Spectroscopy

Spectroscopic analyses were monitored in 96-well plates using a TECAN microplate (Infinite® M200 PRO multi-mode, Hännedorf, Switzerland) reader equipped with a dual injector head for kinetic measurements. The excitation and emission bandwidths of the instrument are 9 nm and 20 nm, respectively. Unless otherwise indicated, 1 μM of each protein was incubated with 1 mM ATP and 1 mM MgCl_2 in 50 μL desalting buffer (20 mM sodium phosphate pH 7.4, 10% glycerol) for 20 min at 30°C prior to addition of 2-OG or of other ligands. Molarity corresponds to the concentration of the PipX or PII protein fusion monomer and the exact mass for each PROBS used for calculating molarity is indicated in Table 1. Fluorescence spectra were recorded 5 min after ligand addition using the fluorescence intensity scan assay (excitation at 425 nm, emission scan from 460 to 600 nm in 2 nm intervals). Kinetics measurements, temperature and pH scans were performed using a fluorescence intensity and interval program to allow simultaneous measurements of YFP (530 nm) and CFP (480 nm) emission signals. FRET ratio was calculated as the 530/480 intensity ratio and reflects the level of excitation of YFP by the CFP, which is directly linked to the distance between the two fluorophores. The relative FRET ratio corresponds to the FRET ratio observed for a specific 2-OG concentration relative to the same ratio without 2-OG. The data were analysed by the PRISM 5 program (GraphPad Software, CA, USA), using the software built-in equations for inhibition of dose-response with or without variable slope.

Determination of 2-OG levels in cell extracts of *E. coli*

Cell extracts were prepared as previously described [13] with minor modifications. Briefly, cultures of *E. coli* W3110 were grown to mid-logarithmic phase ($A_{600} \sim 0.5$) in a Gutnick minimal salts medium supplemented with 0.4% mannitol and 2 mM or 10 mM NH_4Cl . Samples of 100 mL of the culture were filtered through a 0.45- μm membrane (75-mm diameter) and transferred to a 50 mL tube immersed in liquid nitrogen to stop all the reactions. After addition of 850 μL of 0.3 M HClO_4 and 1 mM EDTA, the tube contents were mixed thoroughly and placed on ice for 30 min. The cell extracts were separated by centrifugation and neutralized by the addition of 125 μL of 2 M K_2CO_3 . The resulting KClO_4 precipitate was removed by centrifugation, 50 μL 1 M HEPES was added to the supernatant and the cell extracts were stored at -80°C for further

analysis. For ammonium upshift assay, 400 mL of *E. coli* W3110 cultures in a Gutnick minimal salts medium with 0.4% mannitol and 2 mM NH_4Cl was cultured to mid-logarithmic phase ($A_{600} \sim 0.5$) and NH_4^+ was added to a final concentration of 10 mM. A sample of 100 mL culture was then removed at time 0, 10 min, 30 min and 120 min after the ammonium addition. Cell extracts were prepared as described above. Similar cellular extracts were used to measure the addition of a specific amount of 2-OG.

For the determination of 2-OG concentrations, 10 μL of cell extract was mixed with 90 μL of reaction buffer (20 mM sodium phosphate pH 7.4, glycerol 10%, ATP 1 mM, MgCl_2 1 mM) containing 0.5 μM of the PROBS-2 biosensor. Values of the FRET ratio obtained were compared with a linear range of 2-OG solution standards prepared using the same procedure as the cell extracts. To estimate the 2-OG concentrations within the cells, the absorbance-specific total cell volume of 3.6 $\mu\text{L}\cdot\text{mL}^{-1}\cdot\text{absorbance}^{-1}$ was used for calculations [42].

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Fluorescence intensities of PII-YFP and PROBS-2.