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Studying the Signaling Role of 2-Oxoglutaric Acid Using Analogs that Mimic the Ketone and Ketal Forms of 2-Oxoglutaric Acid

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Summary

2-Oxoglutaric acid (2-OG), a Krebs cycle intermediate, is a signaling molecule in many organisms. To determine which form of 2-OG, the ketone or the ketal form, is responsible for its signaling function, we have synthesized and characterized various 2-OG analogs. Only 2-methylenepentanedioic acid (2-MPA), which resembles closely the ketone form of 2-OG, is able to elicit cell responses in the cyanobacterium *Anabaena* by inducing nitrogen-fixing cells called heterocysts. None of the analogs mimicking the ketal form of 2-OG are able to induce heterocysts because none of them are able to interact with NtcA, a 2-OG sensor. NtcA interacts with 2-MPA and 2-OG in a similar manner, and it is necessary for heterocyst differentiation induced by 2-MPA. Therefore, it is primarily the ketone form that is responsible for the signaling role of 2-OG in *Anabaena*.

Introduction

The Krebs cycle is a central metabolic pathway conserved in all living organisms [1]. It provides not only the reducing power necessary for the production of respiratory energy, but it also supplies key precursors for the synthesis of important biological molecules such as amino acids, lipids, DNA, pigments, etc. One of its intermediates, 2-oxoglutaric acid (2-OG in Figure 1A), occupies a strategically important position: it provides the carbon skeleton required for ammonium (the reduced form of nitrogen) to be assimilated into amino acids. 2-OG may therefore be the link between carbon metabolism and nitrogen assimilation, and it constitutes a checkpoint for proper balancing of the carbon/nitrogen metabolisms [1]. It has been suggested for a long time that 2-OG might constitute a metabolic signal that regulates the coordination of carbon/nitrogen metabolism in plants and bacteria [2–4]. It has also been established recently that 2-OG can serve as a ligand for an orphan G protein-coupled receptor, GPR99 [5], linking metabolism to blood pressure. However, it is difficult to obtain concrete evidence in vivo to substantiate this hypothesis because 2-OG is rapidly metabolized into a variety of molecules. Nonmetabolizable analogs of 2-OG can help us to distinguish its regulatory functions from its metabolic ones and can provide valuable information about the function of the metabolite. We have explored such a strategy and demonstrated the signaling function of 2-OG in vivo in the cyanobacterium *Anabaena* sp. strain PCC 7120 (which will be referred to hereafter as *Anabaena*) using a nonmetabolizable fluorinated analog, 2,2-difluoropentanedioic acid (DFPA in Figure 1A) [6]. Since 2-OG constitutes the carbon skeleton for ammonium assimilation in *Anabaena*, 2-OG accumulates in cells when exposed to a limitation of a combined nitrogen source in the growth medium [6]. This transient increased level of 2-OG serves as a signal to elicit the differentiation of heterocysts, which can fix atomospheric N_2 to provide a nitrogen source for cell growth. The accumulation of DFPA within cells mimics nitrogen starvation and induces heterocyst differentiation even when ammonium is present [6]. To our knowledge, this study provided the first in vivo evidence that 2-OG serves as a nitrogen status signal to induce the formation of a nitrogen-fixing heterocyst in *Anabaena* [6]. Such studies are important for several reasons. First, they demonstrate in vivo that the Krebs cycle goes beyond its role of metabolism and that the Krebs cycle intermediates are also involved in signal transduction. Second, since the signaling function of 2-oxoglutarate has been suggested in a variety of organisms from bacteria to human [2–6], the 2-OG analog could be very useful in a variety of studies.

2-OG may exist in equilibrium between the ketone form and the hydrated ketal form (Figure 1B), with the ketone form being predominant. The presence of the carboxyl group adjacent to the carbonyl group in 2-OG may favor the formation of the hydrated ketal form. This prompted us to ask which form of 2-OG is responsible for its signaling role: the ketone form or the ketal form. DFPA, which has been used previously as an analog of 2-OG, may resemble both the ketone form and the ketal form: on the one hand, its *gem*-difluoromethylene moiety geometrically resembles the tetrahedral ketal functional group, and on the other hand, its *gem*-difluoromethylene moiety is held as a good bio-isostere

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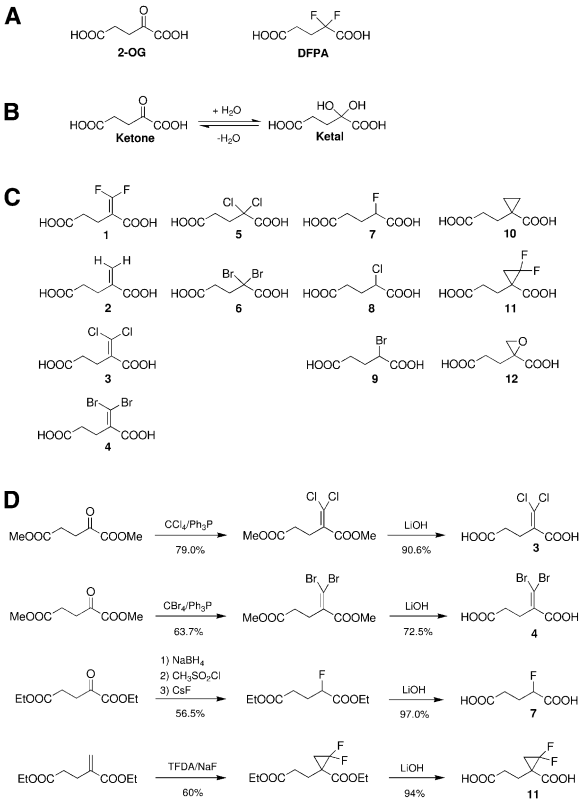

Figure 1. 2-Oxoglutaric Acid and Its Various Analogs

(A) 2-Oxoglutaric acid (2-OG) and its analog, 2,2-difluoropentanedioic acid (DFPA).

(B) 2-OG may involve an equilibrium between the ketone and the hydrated ketal.

(C) Proposed analogs of 2-OG to mimic either the ketone form of 2-OG (probes **1–4**) or the hydrated ketal form of 2-OG (probes **5–12**).

(D) Synthesis of **3**, **4**, **7**, and **11**.

for the ketone functional group [7, 8] due to the special properties contributed by the fluorine atoms. Therefore, DFPA is not suitable to offer information as to which form of 2-OG is responsible for the signaling function. In order to answer this question, we have developed various 2-OG analogs (**1–12** in Figure 1C) as molecular probes to study the signaling role of 2-OG. Vinyl analogs **1–4**, in which the carbonyl group present in 2-OG was replaced by the *gem*-difluorovinyl, vinyl, *gem*-dichlorovinyl, and *gem*-dibromovinyl groups, respectively, were designed to mimic the trigonal ketone form of 2-OG; probes **5–12**, in which the ketal group present in the hydrate form of 2-OG was replaced by monohalogenmethylene, *gem*-dihalogenmethylene, as well as cyclopropyl, *gem*-difluorocyclopropyl, and epoxy groups, were intended to mimic the tetrahedral ketal form of 2-OG. With these analogs, we hoped to probe the structure-function relationship of 2-OG as part of our ongoing project focusing on the 2-OG signaling mechanism.

Results and Discussion

Synthesis

Because the unusually strong electronegativity of the fluorine atom makes the *gem*-difluorovinyl compound **1** highly reactive and notoriously unstable [9], we could not obtain probe **1** in a stable and pure form. We therefore gave up our plan to prepare **1**. Analogs **2**, **5**, **6**, **8–10**, and **12** were prepared according to the literature reports [10–14], while **3**, **4**, **7**, and **11** were synthesized using the methods described in Figure 1D. The synthesis of **3** and **4** was performed via, respectively, dichloromethylenation and dibromomethylenation of dimethyl 2-oxoglutarate with triphenylphosphine and the corresponding tetrahalogenmethane [15], followed by subsequent hydrolysis. Although several methods have been reported for synthesizing **7** [16, 17], we found them to be inefficient and inconvenient. We therefore synthesized **7** by fluorinating diethyl 2-(methylsulfonyloxy)pentanedioate with CsF [18], followed by hydrolyzing the corresponding ester to acid. We attemped to use several methods to synthesize **11**. The most straightforward method consisted of performing difluorocarbene insertion into diethyl 2-methylenepentanedioate [19] and subsequent hydrolysis. All of the synthesized probes were obtained in pure forms and gave satisfactory spectral data. They were soluble in physiological buffers and remained stable for more than 1 week without degradation.

2-MPA, a Vinyl Analog, Induces Heterocyst Formation in *Anabaena*

We then tested the ability of probes **2–12** to mimic the effect of 2-OG in nitrogen metabolism and cell differentiation using the filamentous cyanobacterium *Anabaena*. *Anabaena* is a suitable model for investigating the signaling function of 2-OG and its analogs in nitrogen metabolism because *Anabaena* differentiates a special type of cells called heterocysts in response to combined-nitrogen deprivation. Heterocysts [20, 21] are morphologically identifiable cells that can be easily observed under light microscopy. Heterocyst differentiation is repressed when a combined nitrogen source such as ammonium or nitrate is present in the growth medium. We have shown previously that the intracellular 2-OG levels increased upon deprivation of combined nitrogen in *Anabaena*, and that the nonmetabolizable 2-OG analog DFPA mimics 2-OG and induces heterocyst formation even under repressive conditions, namely, in the presence of ammonium [6, 22].

Among all of the synthesized analogs, only the vinyl analog **2** (2-methylene-pentanedioic acid, 2-MPA) induced heterocyst differentiation in *Anabaena* (Figure 2) in the presence of NH_4^+. None of the tetrahedral analogs, **5–12**, which mimic the ketal from of 2-OG, could elicit a cell response in *Anabaena*. Since 2-MPA is structurally very similar to the ketone form of 2-OG, but is different from the ketal form of 2-OG, the above-described experimental finding suggests that it is the ketone form rather than the ketal form of 2-OG that plays the signaling role in *Anabaena*. The reason vinyl analogs **3** and **4** did not induce heterocyst formation in *Anabaena* is because they do not resemble 2-OG in their structure and size due to the bulky vinyl groups (Figure 3); therefore, they are unable to be recognized by the 2-OG sensor NtcA in *Anabaena*, as described below.

Heterocysts induced by 2-MPA in the presence of 5 mM ammonium were nonfunctional in nitrogen fixation, since NifH, one of the subunits of the nitrogenase complex, was not present, as determined by

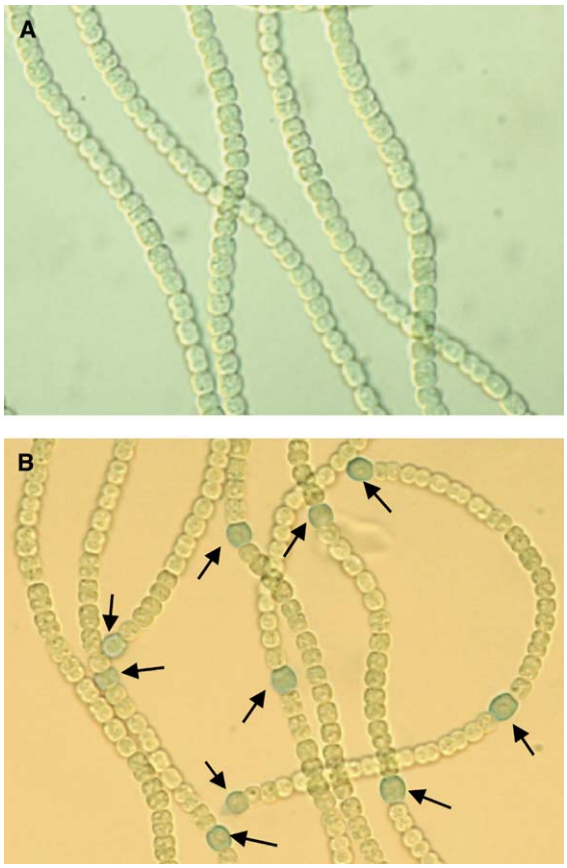

Figure 2. Influence of 2-MPA on Heterocyst Differentiation

(A) The KGTP strain without adding 2-MPA.

(B) Induction of heterocyst differentiation under repressive conditions (in the presence of ammonium) in the KGTP strain incubated with 2-MPA. Filaments were incubated with alcian blue, which specifically stains heterocyst envelope polysaccharides (heterocysts are indicated by arrows).

immunodetection with proteins extracted from cells 48 hr after the addition of 2-MPA (data not shown). This situation is comparable to those reported with heterocysts formed under similar repressive conditions either in the presence of DFPA [6] or in several known developmental mutants [23, 24]. The whole process of heterocyst differentiation, triggered by the accumulation of the 2-OG signal, takes about 24 hr, and the synthesis of the nitrogenase only starts around 18 hr after the induction [20]. It is known that ammonium can switch off *nif* gene expression and nitrogenase activity [25] because it constitutes the preferred nitrogen source for cyanobacteria, whereas molecular nitrogen fixation requires high amounts of ATP and reducing power [26].

2-MPA Resembles 2-OG in Structure and in Biological Recognition

To check whether the vinyl analog 2-MPA is able to mimic the ketone form of 2-OG, we first compared their molecular structures by analyzing the crystal structures. Among the vinyl analogs, 2-MPA has a structure very similar to that of the ketone form of 2-OG (Figure 3): the length of the C=C bond (1.33 Å) in 2-MPA is similar to that of the corresponding C=O bond (1.20 Å) in

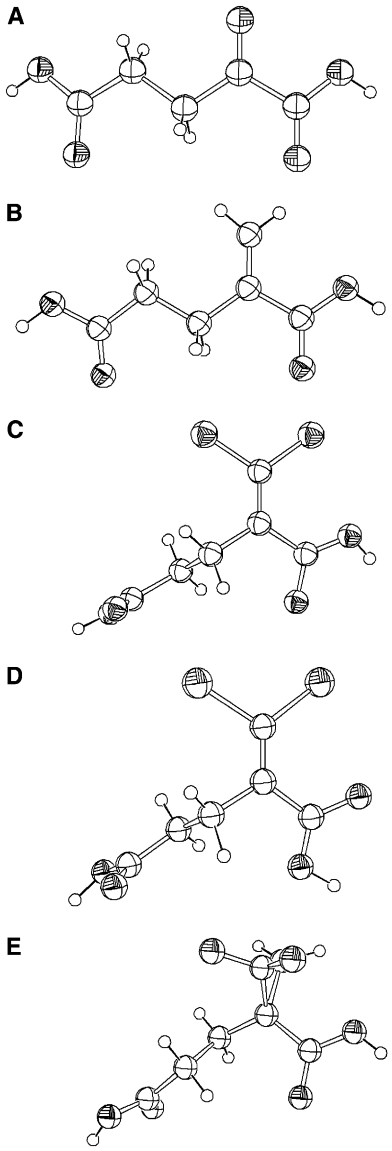

Figure 3. X-Ray Structures of 2-Oxoglutaric Acid and Its Analogs

(A–E) X-ray structures of (A) 2-OG, (B) 2-MPA, (C) **3**, (D) **4**, and (E) **11**.

2-OG, and the two terminal C–H bonds of the vinyl group in 2-MPA are equivalent to the two stereochemically important electron lone pairs of the oxygen atom present in the carbonyl group of 2-OG. Replacing the H atom in the vinyl group present in 2-MPA by Cl and Br significantly increased the size of the vinyl group in both **3** and **4**, resulting in steric hindrance and therefore in structures and conformations that are different from those of 2-OG (Figure 3).

In order to examine the resemblance and the bio-isosteric effects of 2-MPA in relation to 2-OG, we tested the inhibitory effect of 2-MPA on L-glutamic dehydrogenase [27], an enzyme using 2-OG as a substrate. 2-MPA was a competitive inhibitor of L-glutamic dehydrogenase (Figure 4), while **3** showed only weak inhibitory effects with an IC_{50} value of 1160 μM; no inhibitory effects at all were observed with **4** and the other analogs (Table 1).

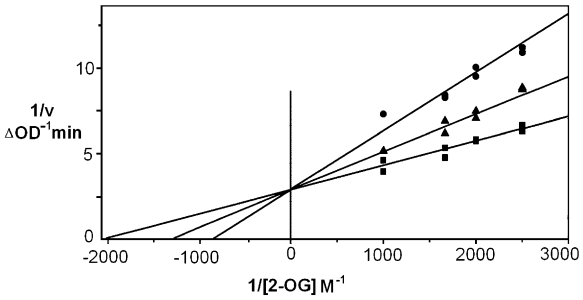

Figure 4. Lineweaver-Burk Plot of the Inhibitory Effects of 2-MPA on the Activity of L-Glutamic Dehydrogenase

The concentrations of 2-MPA were: 0 M (filled square), 2.0×10^{-5} M (filled triangle), and 8.0×10^{-5} M (filled circle).

These results indicate that 2-MPA, although its vinyl group is apolar, constitutes a suitable bio-isostere for the keto function, contrary to the polar but rather bulky dichlorovinyl and dibromovinyl groups in **3** and **4**. Moreover, the similarity between the K_M value (0.56 mM) obtained with 2-OG and the K_i value (0.34 mM) obtained with 2-MPA on the L-glutamic dehydrogenase suggests that 2-MPA and 2-OG have a similar binding affinity with this enzyme and confirms that they indeed resemble each other in many respects.

The structural resemblance between 2-MPA and 2-OG was further confirmed by the fact that they are recognized in a similar way by the 2-OG permease KgtP [28]. Both 2-MPA and 2-OG are negatively charged molecules at physiological conditions, and they cannot be taken up efficiently by *Anabaena* [22]. A recombinant strain of *Anabaena* (strain KGTP) expressing KgtP from *E. coli* was therefore used [28]. This strain can efficiently take up both 2-OG and DFPA [6, 22]. Like 2-OG and DFPA, 2-MPA can be taken up by the KGTP strain, as shown by the results of whole-cell NMR recordings with ^{1}H-HRMAS NMR (High Resolution Magic Angle Spinning NMR) (Figures 5A and 5B), an excellent nondestructive method for in vivo studies on the metabolic profiles and kinetics in whole cells/tissues [29]. 2-MPA has characteristic ^{1}H-NMR signals associated with the vinyl group at 5.3 and 5.7 ppm. Since the 5.3 ppm signal was submerged in other NMR signals, we used the 5.7 ppm signal to monitor the uptake of 2-MPA in KGTP by performing ^{1}H-HRMAS NMR (Figures 5A and 5B). Neither the wild-type strain nor the KGTP strain untreated with 2-MPA produced this NMR vinyl signal (data not shown). When the KGTP strain was incubated with 2-MPA, a clear cut NMR vinyl signal was observed at 5.7 ppm with increasing incubation time (Figure 5B), while only a weak signal was recorded in the wild-type strain treated under similar conditions (Figure 5A). These results confirm that 2-MPA enters *Anabaena* via the

Table 1. The Effect of IC_{50} Values of the 2-OG Analogs on the Activity of the L-Glutamic Dehydrogenase and the pKa Values of the 2-OG Analogs in Comparison with Those of 2-OG

Entry	IC_{50} (mM)	pKa_1	pKa_2
2-OG	—	2.35 (2.47)[a]	4.85 (4.68)[a]
2-MPA	0.12	4.01	4.99
3	1.16	2.80	4.69
4	>10.0	2.78	4.52
5	>10.0	2.54	4.81
6	>10.0	2.62	4.00
7	>10.0	2.98	5.19
8	>10.0	2.29	4.78
9	5.00	3.40	4.55
10	>10.0	4.53	5.14
11	>10.0	3.35	4.87
12	>10.0	2.59	4.72

[a] The data in brackets are reported values for 2-OG.

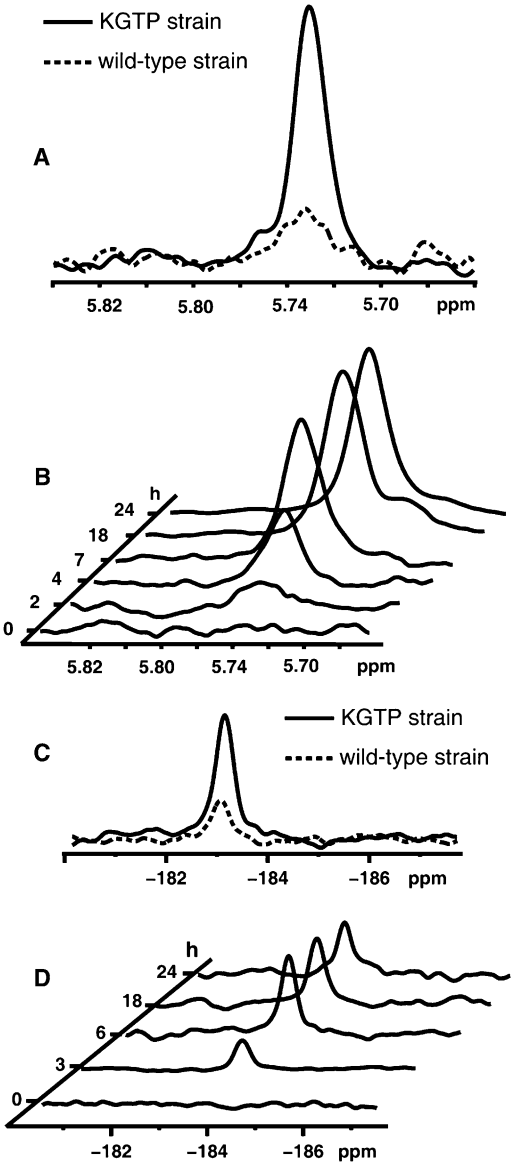

Figure 5. HRMAS NMR Study on the Uptake of 2-MPA and **7** in the KGTP Strain

(A–D) (A and B) ^{1}H-HRMAS NMR study on the uptake of 2-MPA in the KGTP strain and (C and D) ^{19}F-HRMAS NMR study on the uptake of **7** in the KGTP strain. (A) ^{1}H-NMR spectra recorded with the KGTP strain and the wild-type strain in the presence of 2-MPA. (B) ^{1}H-NMR spectra recorded with the KGTP strain in the presence of 2-MPA at the incubation time indicated. (C) ^{19}F-NMR spectra recorded with the KGTP strain and the wild-type strain in the presence of **7**. (D) ^{19}F-NMR spectra recorded with the KGTP strain in the presence of **7** at the incubation time indicated.

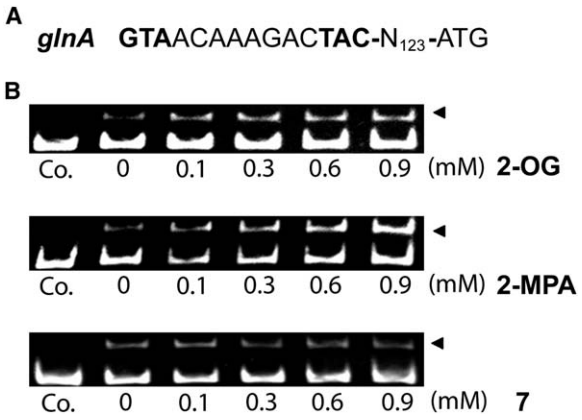

Figure 6. Effects of 2-OG and Its Analogs on the DNA Binding Activity of NtcA

(A) Organization of the promoter region for *glnA*; the putative NtcA binding site is highlighted with bold characters.

(B) DNA binding activity of NtcA toward the promoter region of *glnA* in the presence of various concentrations of 2-OG, 2-MPA, and **7**. Control (Co): DNA fragment without NtcA. DNA/protein complexes are indicated by arrows.

2-OG permease, which provides further evidence that 2-MPA resembles 2-OG.

2-MPA Mimics the Signaling Role of 2-OG

As shown above, 2-MPA can be efficiently taken up through the 2-OG permease and can induce heterocyst formation in *Anabaena*. Furthermore, the in vivo NMR data obtained with cells incubated with 2-MPA showed that this molecule can accumulate in the cells, reaching the maximum level within 7 hr of incubation (Figure 5B), which corresponds to the level required to trigger heterocyst formation in *Anabaena*, as previously observed with 2-OG and DFPA [6, 22]. Moreover, the steady accumulation of 2-MPA in cells further suggests that 2-MPA is stable and resistant to metabolization in *Anabaena*, although we cannot exclude the possibility of minor degradation of 2-MPA in vivo, which cannot be detected within the limit of the NMR analytical method used here.

To further confirm that 2-MPA mimics the signaling role of 2-OG in *Anabaena*, we studied the effects of 2-MPA on the DNA binding activity of NtcA, a 2-OG sensor [26, 30, 31]. NtcA belongs to the CRP protein family of transcription factors and is a general regulator of nitrogen metabolism in cyanobacteria including *Anabaena* [26, 32, 33]. NtcA is also necessary for the initiation of heterocyst differentiation [26, 31, 32]. NtcA recognizes the consensus sequence $GTAN_8ATC$ in promoter regions of its target genes and activates their expression, depending on nitrogen availability [26]. We previously established that the presence of 2-OG and DFPA enhanced the DNA binding affinity of NtcA [6]. The effects of 2-MPA on the DNA binding activity of NtcA from *Anabaena* were examined by performing DNA mobility shift assays with the promoter region of *glnA* encoding the glutamine synthetase, which is known to be under the positive control of NtcA [26]. As shown in Figure 6, the binding of NtcA to DNA was enhanced with increasing concentrations of 2-MPA, as previously observed with 2-OG and DFPA [6]. However, none of the other analogs, **3–12**, had an effect on the DNA binding activity of NtcA to DNA, as illustrated by the experiment carried out with **7** (Figure 6B and data not shown). This provides a further indication that close resemblance between 2-MPA and 2-OG is required for 2-MPA to bind to the 2-OG sensor and exercise its signaling function as 2-OG.

The formation of heterocysts induced by the vinyl analog 2-MPA was found to require NtcA in vivo, as in the case of 2-OG and DFPA. The gene *ntcA* is involved in the initiation of heterocyst development [20, 26, 32, 34]. The plasmid bearing the *kgtP* gene encoding the 2-OG permease [28] was transferred by conjugation into a Δ*ntcA* mutant. As with the KGTP strain, the mutant thus obtained was then incubated with 2-MPA in the presence of ammonium, but no heterocyst differentiation was observed (data not shown). These data show that the differentiation process triggered by 2-MPA in *Anabaena* requires NtcA, which is consistent with the results obtained with the DNA mobility shift assay in vitro (Figure 6).

Structural Requirement for Mimicking the Signaling Role of 2-OG

As we have described above, various analogs have been designed and synthesized in order to determine which form of 2-OG (Figure 1B) is responsible for its signaling role. Among these analogs, only the vinyl analog 2-MPA (**2**), which resembles closely the ketone form of 2-OG in structure and in biological recoginition, is able to elicit cell responses in the cyanobacterium *Anabaena* by inducing heterocysts. This suggests that it is the ketone form rather than the ketal form of 2-OG that plays the signaling role in *Anabaena*. Due to the bulky dichlorovinyl and dibromovinyl groups at C2, vinyl analogs **3** and **4** do not closely resemble 2-OG in structure, and they are not able to interact with NtcA or to induce heterocyst formation in *Anabaena* (data not shown).

The tetrahedral analogs, **5–12**, having various moieties at C2, are intended to mimic the tetrahedral ketal form of 2-OG. They are structurally different from the ketone form of 2-OG (as shown by **11** in Figure 3E). Indeed, they are very poor inhibitors of the L-glutamic dehydrogenase (Table 1), and they do not affect the binding of NtcA to DNA (**7** in Figure 6B and data not shown). They are therefore poorly recognized by both the 2-OG-related enzyme L-glutamic dehydrogenase and the 2-OG sensor NtcA due to the structural difference between them and 2-OG.

Furthermore, we have examined whether the tetrahedral analogs could be recognized by the 2-OG permease, which would allow them to enter into cells. We therefore studied the uptake of **7** and **11** in the KGTP strain by ^{19}F-HRMAS NMR thanks to their characteristic ^{19}F-NMR signals. For the other analogs, we were not able to follow their uptake process due to the lack of the convenient and reliable NMR signals. Both **7** and **11** are tetrahedral analogs of 2-OG. Considering the C2 position in these analogs, **7** is less bulky than DFPA, while **11** is more bulky than DFPA [6]. Results from ^{19}F-HRMAS NMR analysis showed that there was no detectable **11** in either the KGTP strain or the wild-type strain (data not shown), whereas **7** could be taken up by the KGTP strain (Figure 5D). A less efficient uptake of **7** could also be observed in the wild-type strain (Figure 5C). By comparing the ^{19}F-NMR spectra (data not

shown), we see that the uptake of **7** in KGTP is, however, less efficient than that of DFPA. These results indicated that **7** could be poorly recognized by the 2-OG permease, while **11** could not be recognized at all by the 2-OG permease. Together with our previous results obtained with DFPA, we can conclude that 2-OG analogs that closely resemble 2-OG (such as 2-MPA and DFPA) can be recognized efficiently by the 2-OG permease, whereas those that do not resemble 2-OG or poorly resemble 2-OG, because of the presence of either a too big (such as **11**) or a too small (such as **7**) moiety at the C2 position, are not recognized or poorly recognized by the 2-OG permease. The fact that **7** could enter into *Anabaena* but not induce the heterocyst can be explained by the absence of an interaction between **7** and NtcA (Figure 6), and the low level of **7** in cells due to its inefficient uptake may also be a reason for this lack of induction.

To know whether the pKa value of the carboxylic group at C1 is also important for mimicking the signaling role of 2-OG, we have measured the pKa values of all of the synthesized analogs. As shown in Table 1, the replacement of the carbonyl group at C2 by nonpolar moieties (such as in 2-MPA and **10**) increases the pKa value of the carboxylic group at C1, leading to similar pKa values for the two carboxylic groups at C1 and C5, whereas the introduction of polar moieties at C2 keeps their pKa values in line with that of 2-OG. However, 2-MPA is able to mimic 2-OG even though it has a pKa value of the carboxylic group at C1 that is very different from that of 2-OG. Therefore, the pKa value of the carboxylic group at C1 may not be very important for 2-OG to exercise its signaling role.

Significance

Various analogs of 2-OG were synthesized and characterized in this study in order to identify the form of 2-OG responsible for its signaling role in vivo. Only the vinyl analog 2-MPA, which mimics the ketone form of 2-OG and resembles 2-OG in many respects, is able to mimic nitrogen starvation and induce heterocyst differentiation in *Anabaena*. These results lead us to conclude that it is primarily the ketone form of 2-OG that is responsible for the signaling function of 2-OG.

Among the 2-OG analogs described in this work, the chemical modification at the C2 position leads to significant structural and biochemical differences between 2-MPA and the other analogs: 2-MPA closely resembles 2-OG, while all of the other analogs neither induce heterocyst differentiation in *Anabaena* nor resemble 2-OG due to the presence of either a too big (such as 11) or a too small (such as 7) moiety at the C2 position. Therefore, the structural requirment at C2 is very important for the analogs to mimic the signaling role of 2-OG.

DFPA, the first analog of 2-OG developed in our laboratories, may mimic both the ketone form and the ketal form of 2-OG due to its Janus-faced features contributed by the fluorine atoms. The fact that the vinyl analog 2-MPA was able to play a similar signaling role in *Anabaena* as the *gem*-difluoromethylene analog DFPA [6] suggests that both 2-MPA and DFPA mimic the ketone form of 2-OG. Therefore, both 2-MPA and DFPA should provide useful means for further investigations of the signaling role of 2-OG and the underlying signaling pathways.

Experimental Procedures

General

Probes 2-MPA, **5**, **6**, **8**–**10**, and **12** were prepared according to the literature reports [10–14]. ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 300 MHz and 75 MHz, respectively, on a Varian Mercury-VX300 spectrometer. ^{19}F-NMR spectra were recorded at 564.5 MHz on a Varian Mercury-VX600 spectrometer with CF_3COOH as an external standard. ^{1}H-HRMAS NMR spectra were recorded at 400 MHz on a Bruker Avance spectrometer. Chemical shifts are reported in parts per million (ppm) with TMS as an internal reference. FAB-MS was determined using a ZAB-HF-3F mass spectrometer. L-Glutamic dehydrogenase was purchased from Sigma.

Synthesis of 3, 4, 7, and 11

Synthesis of 3

Carbon tetrachloride (0.300 ml, 3.15 mmol) was added in a solution of dimethyl 2-oxoglutarate (0.182 g, 1.05 mmol) and triphenylphosphine (1.65 g, 6.29 mmol) in 10.0 ml anhydrous acetonitrile under Ar at 0°C. Then, the reaction mixture was allowed to warm to 25°C; the color of the solution changed to deep red. After stirring for 40 min at 25°C, the reaction mixture was extracted with ethyl ether (20.0 ml), and then the solvent was removed. The obtained residue was purified by column chromatography (petrol ether/ethyl ether, 20/1) to give the ester product as an oil (0.200 g, 79.0%). A solution of 1 N LiOH (7.36 ml, 7.36 mmol) at 0°C was added slowly to a solution of this product (0.148 g, 0.610 mmol) in acetone (4.00 ml). After stirring 30 min at 0°C, the reaction mixture was washed with ethyl ether to get rid of impurities. Then, the aqueous phase was acidified to pH 1.0 and extracted with ethyl acetate. The organic phases were combined, evaporated under reduced pressure, and dried in vacuo, giving **3** as a pale-yellow solid (0.120 g, 90.6%). Crystals were grown by slowly evaporating a solution of **3** in ethyl ether/hexane. ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm) 2.78 (t, J = 7.7 Hz, 2H), 2.51 (t, J = 7.9 Hz, 2H); ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm) 174.21, 166.81, 132.99, 125.49, 31.45, 28.05; FAB-MS: 213.0 (M, 100.0), 215.0 (M+2, 63.6), 217.0 (M+4, 9.09).

Synthesis of 4

Carbon tetrabromide (1.57 g, 4.74 mmol) was added in a solution of triphenylphosphine (2.49 g, 9.48 mmol) in 15 ml anhydrous CH_2Cl_2 at 0°C. The color of the reaction solution changed immediately to yellow and finally to orange. After stirring for 30 min at 0°C, a solution of dimethyl 2-oxoglutarate (0.550 g, 3.16 mmol) in 5.0 ml anhydrous CH_2Cl_2 was added dropwise, and the reaction solution was stirred at 0°C for 150 min. The reaction mixture was diluted with 20 ml petrol ether, and white precipitation appeared immediately. The precipitate was filtrated and washed with ethyl ether. The filtration was combined and evaporated under reduced pressure. Purification of the crude residue by chromatography (petrol ether/ethyl ether, 30/1) gave the ester product (0.660 g, 63.7%) as an oil. A solution of 1 N LiOH (24.0 ml, 24.0 mmol) at 0°C was added slowly to a solution of this product (0.660 g, 2.01 mmol) in 20.0 ml acetone. After stirring for 30 min at 0°C, the reaction mixture was washed with ethyl ether to get rid of impurities. Then, the aqueous layer was acidified to pH 1.0 and extracted with ethyl acetate. The organic phases were combined, evaporated under reduced pressure, and dried in vacuo, giving **4** as a yellow solid (0.440 g, 72.5%). Crystals were grown by slowly evaporating a solution of **4** in ethyl ether/hexane. ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm) 2.73 (t, J = 7.7 Hz, 2H), 2.51 (t, J = 7.7 Hz, 2H); ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm) 175.32, 169.16, 141.61, 94.35, 32.08, 31.93; FAB-MS: 301.0 (M, 46.67), 303.0 (M+2, 100.0), 305.0 (M+4, 51.67).

Synthesis of 7

Anhydrous CsF (0.228 g, 1.5 mmol) was dissolved in 5 ml dry N-methylformamide at 80°C under Ar. A solution of diethyl 2-(methylsulfonyloxy)pentanedioat (0.141 g, 0.5 mmol) in 2 ml dry N-methylformamide was added to the mixture slowly at this temperature. After 15 hr at 80°C, the solution was extracted with 20 ml ethyl ether, and the obtained residue was purified by column chromatography

(petrol ether/ethyl ether, 4/1) to give the ester product as an oil (0.058 g, 56.5%). A total of 3 ml 2.5 N LiOH solution at 0°C was added slowly to a solution of this product (0.084g, 0.4 mmol) in THF (4.00 ml). After stirring for 3 hr at 0°C, the reaction mixture was washed with ethyl ether to get rid of impurities. Then, the aqueous phase was acidified to pH 1.0 and extracted with ethyl acetate. The organic phases were combined, evaporated under reduced pressure, and dried in vacuo, giving **7** as a pale-yellow solid (0.058 g, 97.0%). ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm) 4.97 (ddd, $^2J_{FH}$ = 45.9 Hz, $^3J_{HH}$ = 3.9 Hz, $^3J_{HH}$ = 3.9 Hz, 1H), 2.48 (m, 2H), 2.10 (m, 2H); ^{19}F-NMR (564.6 MHz, CD_3OD) δ (ppm) 194.5 (ddd, $^2J_{HF}$ = 50.2 Hz, $^3J_{HF}$ = 20.3 Hz, $^3J_{HF}$ = 22.6 Hz).

Synthesis of 11

Diethyl 2-methyleneglutarate (0.100 g, 0.740 mmol) was mixed with NaF (0.400 mg, 0.009 mmol) and methyl benzoate (0.130 ml). The temperature was raised to 110°C. $FSO_2CF_2COOTMS$ (TFDA) (0.555 g, 2.22 mmol, 0.8 eq/h) was added dropwise, and the reaction was then stirred at 110°C for 7 hr. The obtained residue was purified by column chromatography (petrol ether/ethyl ether, 30/1 ~ 10/1) to give the ester product as a yellowish oil (0.111 g, 60.0%). A solution of 2.5 N LiOH (3.40 ml, 8.50 mmol) at 0°C was added slowly to a solution of this compound (0.121 g, 0.500 mmol) in THF (5.00 ml). The reaction was warmed to room temperature and was strirred for 2 hr. The reaction mixture was washed with ethyl ether to get rid of impurities. Then, the aqueous phase was acidified to pH 1.0 and extracted with ethyl acetate. The organic phases were combined, evaporated under reduced pressure, and dried in vacuo, giving **11** as a pale-yellow solid (0.091 g, 94.0%), which can be further purified by crystalization in ethyl ether/hexane. ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm) 2.47 (m, 2H), 2.28 (m, 1H), 2.18 (m, 1H), 1.80 (m, 1H), 1.56 (m, 1H); ^{13}C-NMR(150 MHz, CD_3OD) δ (ppm) 175.2, 169.5, 113.3 (t, 1C, $^1J_{CF}$ = 285.6 Hz), 34.3 (t, 1C, $^2J_{CF}$ = 10.0 Hz), 31.0, 23.6, 20.2 (t, 1C, $^2J_{CF}$ = 9.1 Hz); ^{19}F-NMR (564.6 MHz, CD_3OD) δ (ppm) −137.2 (ddd, $^2J_{FF}$ = 150.0 Hz, $^3J_{HF}$ = 6.5 Hz, $^3J_{HF}$ = 7.1 Hz), −137.5 (ddd, $^2J_{FF}$ = 154.0 Hz, $^3J_{HF}$ = 10.2 Hz, $^3J_{HF}$ = 9.0 Hz); MS (ESI): 193.0 $(M-H)^-$.

X-Ray Structural Analysis

Copies of the data can be obtained free of charge upon application to the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Email: deposit@ccdc.cam.ac.uk).

Determination of pKa

A solution of 2 mM 2-OG analog was titrated with 0.1 N NaOH at room temperature. The variation of pH values was recorded by a pH meter (METTLER-TOLEDO Delta 320) across the range of pH values from 2.3 to 12.0. The pKa values for all of the 2-OG analogs were determined by the nonlinear data processing method. The pKa values of 2-OG were also determined by this method and were compared with the reported values.

Inhibition on L-Glutamic Dehydrogenase

The activity of L-glutamic dehydrogenase was monitored spectrophotometrically at 340 nm and 25°C using a modified protocol provided by Sigma. The procedure was as follows: aliquots of L-glutamic dehydrogenase (10 μl) and 2-MPA (10 μl) were added to 980 μl of the assay cocktail, giving the final assay solution containing 88 mM Tris-HCl, 0.94 mM 2-OG, 53 mM NH_4OAc, 0.1 mM NADH, 0.25 mM EDTA, at pH 7.3.

For the determination of IC_{50}, the inhibition of the activity of L-glutamic dehydrogenase by 2-OG analogs was measured at a substrate concentration of 0.9 mM and inhibitor concentrations from 0.02 μM to 2 mM. The data were plotted using the Microcal Origin 6.0 program.

For the determination of K_i, the inhibition of the activity of L-glutamic dehydrogenase by 2-MPA was measured at four substrate concentrations (0.4 mM, 0.5 mM, 0.6 mM, 1 mM) and three inhibitor concentrations (0 μM, 20 μM, 80 μM) using the procedure described above. The Lineweaver-Burk plots were constructed to obtain the values of K_M and K_i.

Culture of the Cyanobacterium *Anabaena* PCC 7120

The KGTP strain of *Anabaena* PCC 7120, expressing the 2-OG permease from *E. coli*, was constructed and cultured as described [28]. Cells were grown in BG11 medium in the presence of 5 mM ammonium as the nitrogen source. During the exponential phase of cell growth, $CuCl_2$ was added to a 0.8 μM final concentration to induce *kgtP* expression. After 18 hr, 1 mM 2-OG analog was added, and heterocysts were observed under an optical microscope 24 hr later.

Immunodetection

Immunodetection with antibodies against NifH was carried out as described in [25]. Total proteins were extracted from cells either exposed to a limitation of combined nitrogen for 24 or 48 hr or treated with 2-MPA in the presence of 5 mM ammonium for 48 hr.

HRMAS NMR

For HRMAS NMR measurement, cells with an OD = 0.4, grown in BG11 medium with ammonium, were incubated with 1 mM each 2-MPA, DFPA, **7**, and **11**, collected at specific times by centrifugation, and washed twice in BG11 with ammonium.

All NMR spectra were recorded on a 400 MHz Bruker Avance spectrometer operating at a ^{1}H and a ^{19}F resonance frequency of 400.1 MHz and 376.8 MHz, respectively. ^{1}H and ^{19}F experiments were performed with a commercial 4 mm HRMAS ^{1}H/^{13}C probe head, modified in house to observe ^{19}F. About 2 mg cell sample was added to 50 μl D_2O, to provide a deuterium lock, and sealed into a 4 mm Zirconia rotor. To improve the resolution, samples were spun at Magic Angle, and the spinning rate was set at 4000 Hz. All experiments were recorded at room temperature.

^{1}H-HRMAS NMR spectra were acquired using a CPMG spin-echo pulse sequence $(90 - [\tau - 180 - \tau]_n -$ Acquisition) as a T_2 filter to remove the effects of lipids and large macromolecules on spectral broadening: n = 150; total spin-spin relaxation delay, $2n\tau$ = 75 ms. A 4.8 μs 90° pulse, with 1024 scans and a recycle time of 3 s were used.

^{19}F-HRMAS MAS NMR spectra were acquired with a single pulse experiment (SPE) with a pulse length of 6 μs and a recycle delay of 3 s. To obtain a good signal-to-noise ratio, 4600 scans were accumulated for each sample. Chemical shifts were referenced to an external standard of 1 M NaF aqueous solution, which has resonance at −120 ppm compared with $CFCl_3$.

DNA Binding Activity of NtcA

A 170 bp DNA fragment corresponding to the *glnA* promoter region was amplified by PCR with the primers 5′-GGATTTTATGTCAAAGTT GACCCC-3′ and 5′-CGAAACAAAGTTGATGAC-3′. The NtcA protein was purified, and the binding shift assay was performed as previously described, using 0.18 μg DNA and 0.14 μg protein in a 20 μl assay system [31]. The DNA bands were visualized after staining with ethidium bromide.

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Accession Numbers

Crystallographic data obtained on the structures of 2-MPA, **3**, **4**, and **11** have been deposited in the Cambridge Crystallographic Data Center with deposition nos. CCDC 261571, 261399, 261400, and 602057, respectively.