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Characterization of altered regulation variants of dinitrogenase reductase-activating glycohydrolase from *Rhodospirillum rubrum*

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Abstract In *Rhodospirillum rubrum*, nitrogenase activity is subject to posttranslational regulation through the adenosine diphosphate (ADP)-ribosylation of dinitrogenase reductase by dinitrogenase reductase ADP-ribosyltransferase (DRAT) and dinitrogenase reductase-activating glycohydrolase (DRAG). To study the posttranslational regulation of DRAG, its gene was mutagenized and colonies screened for altered DRAG regulation. Three different mutants were found and the DRAG variants displayed different biochemical properties including an altered affinity for divalent metal ions. Taken together, the results suggest that the site involved in regulation is physically near the metal binding site of DRAG.

Key words: Nitrogen fixation; Posttranslational regulation; Adenosine diphosphate-ribosylation; Dinitrogenase reductase-activating glycohydrolase; *Rhodospirillum rubrum*

1. Introduction

Adenosine diphosphate (ADP)-ribosylation is a reversible posttranslational modification of proteins involving the addition of the ADP ribose moiety of nicotinamide adenine dinucleotide (NAD) to an acceptor protein or amino acid [1,2]. This ADP-ribosylation is particularly well described in the bacterium *Rhodospirillum rubrum*, in which it regulates nitrogen fixation through the reversible modification of dinitrogenase reductase [3,4]. Nitrogenase is a protein complex of two components: dinitrogenase is a tetramer of the *nifD* and *nifK* products and contains the active site of dinitrogen reduction, and dinitrogenase reductase is a dimer of the *nifH* product and transfers electrons to dinitrogenase [5].

Two enzymes have been found to perform this reversible regulation of nitrogenase activity in *R. rubrum*. Under certain conditions, dinitrogenase reductase ADP-ribosyltransferase (DRAT, the product of *draT*) transfers an ADP-ribosyl group from NAD to one subunit of dinitrogenase reductase and the ADP-ribosylated dinitrogenase reductase is no longer competent to transfer electrons to dinitrogenase [6]. The ADP-ribosyl group on the inactivated dinitrogenase reductase can be removed by the dinitrogenase reductase-activating glycohydrolase (DRAG; the product of *draG*) thus recovering dinitrogenase reductase activity [2,3,7].

This reversible regulation by the activity of both DRAT and DRAG is subject to tight regulation to avoid the possibility of futile cycling of NAD. Under conditions appropriate for nitrogen fixation (energy sufficiency and a deficiency in fixed nitrogen) DRAT is inactive, but DRAG is active and maintains the active form of dinitrogenase reductase. However, after an environmental shift that renders nitrogen fixation undesirable, DRAG loses its activity and DRAT becomes active, causing the ADP-ribosylation of dinitrogenase reductase. Unlike DRAG, DRAT is only transiently activated by the environmental stimuli, so that after a period of ADP-ribosylation, both DRAT and DRAG are inactive [8]. This results in a plateau of nitrogenase activity reflecting the amount of dinitrogenase reductase that was not ADP-ribosylated during DRAT's active period. When conditions change again to those favorable to nitrogenase activity, DRAG recovers its activity and restores nitrogenase activity by removing the ADP-ribosyl group from dinitrogenase reductase [9].

The tight regulation of nitrogenase by the DRAT–DRAG system reflects at least two levels of control. A significant portion of this regulation depends on the oxidation state of dinitrogenase reductase: oxidized dinitrogenase reductase is a good substrate for DRAG and a poor substrate for DRAT, whereas the reduced protein has the opposite properties [10]. This can, however, not account for all of the regulation of DRAT and DRAG that is seen in vivo, and recently another aspect of the regulatory process has been uncovered. The P_{II} protein is a regulatory protein found in almost all studied prokaryotes and some plants that appears to be involved in regulation of central nitrogen metabolism or the balance of nitrogen and carbon metabolism in all systems where it has been studied [11]. *R. rubrum* has been shown to have three homologs of this protein of which two, termed GlnB and GlnJ, are involved in regulation of DRAT and DRAG [12]. In the absence of both of these P_{II} homologs, the DRAT/G system no longer ADP-ribosylates dinitrogenase reductase in response to either fixed nitrogen sufficiency or energy deficiency. The absence of detectable covalent modification of either DRAT or DRAG, coupled with their apparent high activity in all examined extracts suggests that the regulation of their activity is through inhibitory factors that are lost or diluted upon cell breakage. It is our working hypothesis that these inhibitory factors might be the GlnB or GlnJ proteins themselves or other molecules that they affect.

In this report, DRAG variants have been isolated and characterized that are active under conditions where the normal

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DRAG was inactive. These variants are clearly altered in their affinity for divalent metal ions, but attempts to alter regulation by perturbing metal ion concentration were unsuccessful. The simplest hypothesis is therefore that a site on these variants has been altered that affects their interaction with a negative regulatory molecule and also perturbs the divalent metal binding site.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli DH5α, and *R. rubrum* were cultured as described previously [13,14]. The *R. rubrum* strains used in this study are listed in Table 1.

2.2. Random and localized polymerase chain reaction (PCR) mutagenesis

The random PCR mutagenesis followed a published protocol [15]. Higher concentrations (1 mM) of the deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP) and lower concentrations (0.2 mM) of the deoxyguanosine triphosphate (dGTP), deoxyadenosine triphosphate (dATP), and 0.5 mM $MnCl_2$ and native *Taq* DNA polymerase (Fisher Scientific, USA) were used to increase the mutation frequency in the PCR mixture. The plasmid containing *nifH draTG* with *aacC1* (gentamicin resistance) from pUCGm [16] in pBSKS(-) was used as the template for PCR. The pool of these plasmids was then digested and a 3-kb *Kpn*I–*Pst*I fragment, containing the mutagenized *draG*, was cloned into pRK404 [17], which is stable in *R. rubrum* at a copy number of about 10.

The region immediately around that coding for N100K was further mutagenized using a mixture of 95% of the wild-type nucleotide and 5% of an equimolar mix of all four nucleotides [18] and *Taq* polymerase. A pool of mutagenized clones was digested and a 3-kb *Kpn*I–*Pst*I fragment, containing *draTG* and *aacC1*, was cloned into a pRK404 derivative. This *draG* library was screened in UR215, a strain that is $draT^{+}G^{-}$.

2.3. Nitrogenase assay

Nitrogenase activity was measured by the acetylene reduction method [19].

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting of dinitrogenase reductase

Protein samples were prepared by TCA precipitation [8]. The procedure for modified enzyme-linked immunoblotting [7,20] was used, and visualized using the enhanced chemiluminescence (ECL) detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

2.5. DRAG overexpression, purification, and in vitro DRAG activity assay

The broad host range expression plasmid, pUX115 [21], which contains *draTGB* (*draB* is the open reading frame immediately 3′ of *draG*) under the control of the *nifH* promoter, was used to overexpress the DRAG variants. An *Afl*II site in the middle of *draT* and a *Pst*I site on the vector were used to exchange the 2.3-kb wild-type *draTGB* on the plasmid with the 1.5-kb mutant *draTG* region. These constructed plasmids were transferred into *R. rubrum* strain UR472, which is deleted for *draTGB*. The cultures were grown in MG medium, which leads to expression from the *nifH* promoter, and the cells harvested and stored as described elsewhere [21]. Purification of dinitrogenase reductase, dinitrogenase, and DRAG from *R. rubrum* were carried out anaerobically following the formerly reported methods [22,23]. In vitro DRAG activity assays were performed by the acetylene reduction method [24].

2.6. Electron paramagnetic resonance (EPR) analysis

The concentration of free Mn^{2+} in a solution containing free and bound Mn^{2+} was determined by EPR at 9.09 GHz with a Varian E-3 EPR spectrometer, as described [25,26]. Metal addition experiments were performed by diluting 100-μl aliquots of the stock DRAG (final concentration 40 μM) with 50 mM MOPS, pH 7.0, 0.2 M NaCl, and 2 mM dithiothreitol (DTT) at 25°C. Mixtures were allowed to react for 20 min.

3. Results and discussion

3.1. Rationale for the screen for 'altered regulation' draG mutants of R. rubrum

In the wild type, DRAG rapidly loses its activity, and DRAT is transiently activated in response to stimuli unfavorable for nitrogen fixation. We sought variants of DRAG that maintained some activity after the removal of light and that would recover nitrogenase activity after DRAT activity was turned off. We therefore assayed the cultures for nitrogenase activity at 60 min after the shift to darkness, reasoning that DRAT is completely inactivated approximately 40 min after the shift, and 20 additional min should be sufficient to significantly restore nitrogenase activity even with relatively low levels of DRAG activity [24].

3.2. Random and localized PCR mutagenesis and screening for mutants

After random PCR mutagenesis of the *draG* region, the mutagenized *draG* library was transconjugated into UR215 ($draT^{+}G^{-}$). Nitrogenase activity was determined 60 min after a shift to darkness. The efficiency of the mutagenesis was suggested by two criteria: (i) About 10% of samples displayed little nitrogenase activity in the assay, suggesting loss-of-function mutations in *draG* and (ii) five randomly mutagenized *draG* regions were sequenced and had mutations at a frequency of ~1.4 base changes/target gene. Of 1000 colonies screened, only one had the desired phenotype of relatively high nitrogenase activity 60 min after a shift to darkness. The plasmid *draTG* region of this mutant was sequenced and contained a single mutation, termed *draG21*, causing an N100K substitution.

The region around N100K, the site of the *draG21* mutation, is highly conserved in DRAG from other bacteria. We therefore mutated the 33-bp region (residues 95–105) with localized PCR mutagenesis [18], and repeated the screen. Three mutants

Table 1
Bacterial strains and plasmids

Strains	Relevant genotype and description	Reference
R. rubrum		
UR215	*draG5*	[31]
UR372	*draTGB10*::*kan*	[33]
UR464	*draG5* with *draTG* on a pRK404 derivative	[33]
UR645	like UR464 but expressing DRAG-N100K	this report
UR646	like UR464 but expressing DRAG-V98L	this report
UR647	like UR464 but expressing DRAG-C102S	this report
UR680	like UR464 but with *draT24*::*kan* and DRAG	this report
UR681	like UR464 but with *draT24*::*kan* and DRAG-N100K	this report

A

B

Strains	DRAT type[a]	DRAT level[b]	DRAG type	DRAG level[c]	Nitrogenase activity[d] initial	Nitrogenase activity[d] 60 min dark
UR464	WT	11X	WT	10X	580 +/- 55	36 +/- 11
UR645	WT	11X	N100K	10X	710 +/- 78	661 +/- 46
UR646	WT	11X	V98L	10X	683 +/- 42	618 +/- 43
UR647	WT	11X	C102S	10X	668 +/- 40	652 +/- 75

Fig. 1. ADP-ribosylation of dinitrogenase reductase in the presence of the 'altered regulation' DRAG variants. A: Immunoblot of dinitrogenase reductase. The upper band is ADP-ribosylated dinitrogenase reductase and the lower band is the unmodified subunit of the same protein. The extracts of the samples were prepared from light-grown cultures and 60 min after a shift to darkness. B: Nitrogenase activity assay. [a]WT, wild-type *draT*, as appropriate. [b]'11×' refers to normal levels of DRAT (1×) due to a single gene copy from chromosome and a level approximately 10-fold higher (10×) due to the plasmid location of *draT*. [c]In all cells, the chromosomal copy of *draG* is inactivated, so the cells contained approximately 10 times the normal level DRAG. [d]Nitrogenase activity (nmol ethylene/ml/h) was normalized to an optical density (OD) of 1. The variability of the nitrogenase activity was about 10%. The data are from at least three individual runs.

were detected out of 300 colonies screened and the *dra* region of each mutant was sequenced. In each case a single mutation was found: *draG22* caused V98L, *draG23* caused C102S and *draG21* was reisolated. All three changes are fairly conservative, which suggests that subtle changes in local conformation of this region may be important for this altered regulation.

3.3. *In vivo characterization of 'altered regulation'* draG *mutants*

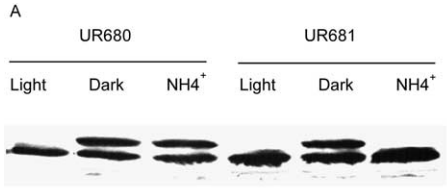
To verify that the detected nitrogenase activity was caused by DRAG variants with altered regulation, the plasmids were moved to a new strain of the same genetic background and shown to confer the same phenotype, verifying causality of the plasmid-borne *draG* mutations.

Fig. 1 shows the behavior of all three 'altered regulation' DRAG variants when expressed at approximately 10 times normal levels from the plasmid. Fig. 1A shows that UR464, which is the wild-type control, displays relatively normal regulation, with only the active (non-ADP-ribosylated) form of dinitrogenase reductase detectable in the light and with a substantial amount of the ADP-ribosylated form (the upper band) being evident after 60 min in the dark. In contrast, little or no ADP-ribosylated band is detectable under any conditions in any of the three strains with 'altered regulation' DRAG. The left side of Fig. 1B clarifies the relevant proteins and their levels in each strain, while the right side displays the results of a nitrogenase activity assay, which is consistent with the immunoblot analysis.

We were concerned, however, that the elevated levels of the proteins in these experiments might have perturbed the results (although the wild-type control behaved appropriately). The level of expression of the DRAG variants was altered to slightly below the normal levels by moving *draG21* (DRAG-N100K) to a plasmid, pKT163, in which a *kan* cassette had been inserted in *draT*, which is transcriptionally upstream of *draG*. Due to the polarity of the *kan* cassette, expression of DRAG-N100K was reduced to about 3% of normal [24], though the copy number of the plasmid raises the total level of DRAG to about 30% of normal. The results in Fig. 2 with DRAG-N100K show that there was no recovery of nitrogenase activity in this strain (UR681) after the darkness treatment and that the strains behaved as did the wild-type control (UR680). This result is consistent with the hypothesis that these variants have a poorer affinity for the inhibitory factor than does wild-type DRAG. At elevated levels of DRAG, there is a sufficient amount of DRAG that escapes normal regulation as shown in Fig. 1. However, at reduced levels of DRAG, the level of inhibitor would be sufficient to compensate for this poorer affinity and therefore provide effective regulation.

We also tested this strain for its response to NH_4Cl. Although the effect of NH_4Cl addition has been shown to be generally similar to that of darkness, the residual level of nitrogenase activity is typically higher, perhaps due to a shorter or less complete transient activation of DRAT after NH_4Cl [27]. In response to the addition of 5 mM NH_4Cl (Fig. 2), the strain with DRAG-N100K showed no loss of nitrogenase activity under this condition, in contrast to the wild-type control. This result demonstrates that DRAG-N100K has an altered regulatory response to NH_4Cl addition as well. We do not know why the residual levels of nitrogenase activity in UR680 are not higher in response to NH_4Cl, but this minor effect is presumably a complication of altering the DRAT/DRAG ratio. Altering the DRAT/DRAG ratio is certainly also the reason that DRAG-N100K appears normal in response to darkness in Fig. 2, as rationalized in the preceding paragraph. The fact that UR681 shows a much more striking

A



B

Strains	DRAT type[a]	DRAT level[b]	DRAG type	DRAG level[c]	Nitrogenase activity[d] initial	Nitrogenase activity[d] 60 min darkness	Nitrogenase activity[d] 90 min NH_4Cl
UR680	WT	1X	WT	0.3X	719 +/- 45	34 +/- 14	39 +/- 8
UR681	WT	1X	N100K	0.3X	789 +/- 69	32 +/- 2	607 +/- 105

Fig. 2. ADP-ribosylation of dinitrogenase reductase in the presence of a low level of the 'altered regulation' DRAG variants. A: Immunoblot of dinitrogenase reductase. The extracts of the samples were prepared from light-grown cultures, 60 min after a shift to darkness, or 90 min after treatment with 5 mM NH_4Cl. B: Nitrogenase activity assay. [a]WT, wild-type *draT*, as appropriate. [b]'1×' refers to normal levels of DRAT due to a single gene copy. [c]In all cells, the chromosomal copy of *draG* is inactivated, so '0.3×' DRAG levels reflect the combination of the *draT* polar block and the plasmid copy number. [d]Nitrogenase activity (nmol ethylene/ml/h) was normalized to an OD of 1. The variability of the nitrogenase activity was about 10%. The data are from at least three individual runs.

response to darkness than to NH_4Cl is, however, consistent with the generally stronger effect of darkness that is routinely observed.

3.4. Purification of 'altered regulation' DRAG variants

In order to examine the biochemical basis for the altered regulation seen in whole cells, all three DRAG variants were purified according to the procedures described for the wild type [21] with the following exceptions. Normal DRAG has a strong affinity to the membrane pellet in cell extracts and can be eluted with 500 mM NaCl [28]. Indeed, it has been postulated that a critical element of DRAG regulation might be through its membrane association, with membrane binding causing the inactivation of DRAG [29]. While DRAG-N100K showed significantly lower affinity for the membrane (99% eluted under these conditions) than did normal DRAG (1% eluted) when both were overexpressed, DRAG-V98L (11% eluted), and DRAG-C102S (1% eluted) behaved much more like normal DRAG. While there was not a consistent correlation between altered regulation and membrane association, the fact that two DRAG variants were clearly altered in this property does suggest that membrane localization might indeed be one mechanism for DRAG regulation.

The other purification difference with the DRAG variants occurred on the hydroxylapatite column. Normal DRAG binds to this column and is eluted with 250 mM phosphate in the elution buffer, and few proteins of any sort fail to elute with 300 mM of phosphate. However, all three altered regulation DRAG variants required at least 600 mM phosphate for elution. Hydroxylapatite has two different adsorbing sites on its crystal surface: calcium sites that bind acidic groups, carboxyls and phosphates, and phosphate sites that bind basic protein groups. Elution from the former is achieved by anions (usually phosphates), while elution from the latter is effected by cations such as Na^+. Neither the normal DRAG nor the variants were eluted from the column with 1 M NaCl. Therefore, while both normal DRAG and the DRAG variants have a high affinity for the phosphate sites, all three 'altered regulation' DRAG variants have a much higher affinity for the calcium site of the hydroxylapatite column.

3.5. DRAG-N100K and DRAG-C102S have altered affinities for Mn^{2+} and Mg^{2+}

The above purification suggested that the DRAG variants have an elevated affinity for the calcium sites on the hydroxylapatite column. It is known that calcium is not required for the DRAG regulation in vivo or in vitro [30], although an efflux of calcium has been reported to coincide with the ADP-ribosylation of dinitrogenase reductase and therefore with the inactivation of DRAG [31]. However, some divalent cations clearly play an important role in the activity of DRAG, since a binuclear Mn^{2+} center has been demonstrated in the purified DRAG [1,32] and Fe^{2+} is also able to support DRAG activity, although Mg^{2+} does not.

DRAG and DRAG-N100K were both similarly inhibited by the presence of 0.5 mM $CaCl_2$ in terms of their ability to remove the ADP-ribose group from dinitrogenase reductase in the presence of 25 mM $MgCl_2$ and 0.5 mM of $MnCl_2$ (data not shown). The requirement of normal DRAG and DRAG-N100K for Mn^{2+} was examined in the presence of 10 mM of $MgCl_2$ and in the ATP-regenerating system described in Section 2. Again, the two proteins showed similar activity at various concentrations of $MnCl_2$, with maximum activity at 1 mM of $MnCl_2$ (data not shown).

The affinities of normal DRAG, DRAG-N100K and DRAG-C102S for Mn^{2+} were compared by detection of free Mn^{2+} using EPR spectrometry. DRAG-N100K and DRAG-C102S both display a significantly poorer affinity (K_D of 185 and 950 μM, respectively) for Mn^{2+} than does normal DRAG (K_D of 50 μM in this assay, which is slightly higher than the 27 μM value reported by Antharavally et al. [32]). Because of these results, we tested the idea that metal ion binding might be the basis for the altered regulatory properties of these variants in vivo by comparing the behavior of wild-type DRAG and DRAG-N100K in different genetic backgrounds and under a variety of metal ion levels in the medium. Although suggestive differences between strains with wild-type DRAG and those with the DRAG variant were occasionally detected, no striking and reproducible results were observed (data not shown). We know little about the availability of divalent cations in vivo and it is quite possible that our alteration of external levels did not perturb the intracellular levels, but the results suggest that differential metal ion binding is not the basis for the altered regulation in these DRAG variants. The notion of a direct linkage between these properties is also inconsistent with the result that DRAG-N100K, with a lower affinity for metals in vitro, displays at least as much activity in vivo as does the normal DRAG.

It is therefore our working hypothesis that the substitutions alter DRAG regulation, presumably by perturbing a surface of the protein that interacts with an inhibitory factor. The simultaneous alteration of metal binding in these variants might therefore be merely coincidental, consistent with the metal binding site being immediately adjacent to the critical surface for regulation.

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