Isolation and characterization of *draT* mutants that have altered regulatory properties of dinitrogenase reductase ADP-ribosyltransferase in *Rhodospirillum rubrum*

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In *Rhodospirillum rubrum*, dinitrogenase reductase ADP-ribosyltransferase (DRAT) is responsible for the ADP-ribosylation of dinitrogenase reductase in response to the addition of NH_4^+ or removal from light, resulting in a decrease in nitrogenase activity. DRAT is itself subject to post-translational regulation; to investigate the mechanism for the regulation of DRAT activity, random PCR mutagenesis of *draT* (encoding DRAT) was performed and mutants with altered DRAT regulation were screened. Two mutants (with substitutions of K103E and N248D) were obtained in which DRAT showed activity under conditions where wild-type DRAT (DRAT-WT) did not. These mutants showed lower nitrogenase activity and a higher degree of ADP-ribosylation of dinitrogenase reductase under N_2 -fixing conditions than was seen in a wild-type control strain. DRAT-K103E was overexpressed and purified. DRAT-K103E displayed a much weaker affinity for an Affi-gel Blue matrix than did DRAT-WT, suggestive of a fairly striking biochemical change. However, there was no significant difference in kinetic constants, such as K_m for NAD and $V_{\max}$, between DRAT-K103E and DRAT-WT. Like DRAT-WT, DRAT-K103E also modified reduced dinitrogenase reductase poorly. The biochemical properties of these variants are rationalized with respect to their behaviour *in vivo*.

Keywords: nitrogen fixation, regulation, ADP-ribosylation, random PCR mutagenesis

INTRODUCTION

In the photosynthetic bacterium *Rhodospirillum rubrum*, the regulation of nitrogenase activity involves the reversible ADP-ribosylation of dinitrogenase reductase (Ludden & Roberts, 1989). Two enzymes that perform this regulation have been characterized. As shown in the model in Fig. 1, dinitrogenase reductase ADP-ribosyltransferase (referred to as DRAT) carries out the transfer of the ADP-ribose from NAD to the Arg-101 residue of one subunit of the dinitrogenase reductase dimer, resulting in inactivation of that enzyme. Dinitrogenase reductase activating glycohydrolase (referred to as DRAG) removes the ADP-ribose from the covalently modified dinitrogenase reductase, restoring its activity.

Abbreviations: DRAG, dinitrogenase reductase activating glycohydrolase; DRAT, dinitrogenase reductase ADP-ribosyltransferase; DRAT-WT, DRAT from wild-type.

The DRAT/DRAG regulatory approach has also been found in other nitrogen-fixing bacteria, such as the phototroph *Rhodobacter capsulatus* (Jouanneau *et al.*, 1989; Masepohl *et al.*, 1993; Pierrard *et al.*, 1993) and the non-photosynthetic *Azospirillum brasilense* and *Azospirillum lipoferum* (Fu *et al.*, 1989; Zhang *et al.*, 1992). In *R. rubrum* and *Rb. capsulatus*, this system responds to fixed nitrogen and to darkness (Kanemoto & Ludden, 1984; Liang *et al.*, 1991; Masepohl *et al.*, 1993; Pierrard *et al.*, 1993); in *A. brasilense* it responds to ammonium and anaerobiosis (Fu *et al.*, 1989; Hartmann & Burris, 1987; Hartmann *et al.*, 1986; Zhang *et al.*, 1992, 1993). The *draTG* genes, which encode DRAT and DRAG, have been cloned and sequenced from each of these bacteria (Fitzmaurice *et al.*, 1989; Fu *et al.*, 1990; Inoue *et al.*, 1996; Ma & Li, 1997; Masepohl *et al.*, 1993; Zhang *et al.*, 1992); the sequence comparisons showed extensive similarity, with some particularly conserved regions (Masepohl *et al.*, 1993).

Fig. 1. Model for the regulation of dinitrogenase reductase in *R. rubrum* by ADP-ribosylation.

Through biochemical and genetic analysis, it has become clear that the activities of DRAT and DRAG are themselves subject to post-translational regulation (Zhang *et al.*, 1997). Under nitrogen-fixing conditions, DRAT appears to be inactive. Following treatment with a stimulus that is 'negative' for nitrogen fixation, such as NH_4^+ or darkness (energy depletion), DRAT is activated, resulting in the modification of dinitrogenase reductase and the loss of nitrogenase activity. However, DRAT activation is only transient and DRAT becomes inactive again even in the continued presence of the negative stimulus (Zhang *et al.*, 1993). In contrast, DRAG is active under nitrogen-fixing conditions and is inactivated by the negative stimulus. Unlike the regulation of DRAT, however, the regulation of DRAG is not transient, but reflects the current physiological status (Kanemoto & Ludden, 1984). Following the negative stimulus, DRAG becomes inactive and remains inactive until the removal of the negative stimulus. DRAG then reactivates dinitrogenase reductase by cleavage of the ADP-ribose group.

Details of the mechanisms for the regulation of DRAT and DRAG activities themselves are still unknown. Both DRAT and DRAG display substantial activity *in vitro* either when purified or in crude extracts of cells grown under various conditions (Lowery & Ludden, 1989; Triplett *et al.*, 1982). This loss of regulation upon cell breakage suggests that DRAT and DRAG might be regulated by the inhibition of their activities through

loosely binding negative effector(s). Recently, it was found that the redox states of dinitrogenase reductase affect its ability to serve as a substrate for DRAT and DRAG, both *in vitro* and *in vivo* (Halbleib *et al.*, 2000). DRAT can only modify oxidized dinitrogenase reductase and DRAG only removes the ADP-ribosyl group from reduced dinitrogenase reductase. However, whilst the redox state of dinitrogenase reductase probably plays an important role in the regulation of DRAT and DRAG activities, it cannot be the only type of regulation. As mentioned above, DRAT and DRAG are not always regulated in a coordinated way and both proteins can be inactive under some conditions; thus, the regulation of DRAT and DRAG activities must involve additional factors.

To investigate the mechanism of DRAT regulation, we employed random PCR mutagenesis of *draT* from *R. rubrum* and screened for mutants with regulation-altered DRAT. Our success in finding such mutants with relative ease is most easily rationalized on the basis of a model in which these DRAT variants have been damaged at a site where a negative effector binds. This result provides support for the hypothesis of loose-binding negative effectors regulating DRAT activity *in vivo*.

METHODS

Bacterial strains and plasmids. The strains of *R. rubrum* and the plasmids used in this study are listed in Table 1.

Table 1. *Rhodospirillum* strains and plasmids

Strain/plasmid	Relevant genotype and description	Reference
Strains		
UR2	Wild-type	Kanemoto & Ludden (1984)
UR212	<i>draT2::kan</i> mutant	Liang <i>et al.</i> (1991)
UR462	UR212 with pYPZ163 (<i>Pdra-draT</i>)	This study
UR472	Δ <i>draTGB10::kan</i> mutant	This study
UR483	UR472 with pYPZ163 (<i>Pdra-draT</i>)	This study
UR506	UR2 with pYPZ163 (<i>Pdra-draT</i>)	This study
UR508	UR2 with pYPZ170 (<i>Pdra-draT16</i> , K103E)	This study
UR511	UR212 with pYPZ170 (<i>Pdra-draT16</i> , K103E)	This study
UR578	UR472 with pYPZ174 (<i>Pnif-draT16</i> , K103E)	This study
UR581	UR2 with pYPZ179 (<i>Pdra-draT17</i> , N248D)	This study
UR582	UR212 with pYPZ179 (<i>Pdra-draT17</i> , N248D)	This study
UR583	pYPZ173 (<i>draT16</i> , K103E) was integrated into the chromosome of UR472	This study
UR587	UR472 with pYPZ183 (<i>Pnif-draT16GB</i> , K103E)	This study
UR589	pYPZ187 (wild-type <i>draT</i>) was integrated into the chromosome of UR472	This study
UR610	pYPZ190 (<i>draT17</i> , N248D) was integrated into the chromosome of UR472	This study
UR619	pYPZ191 (<i>draT18</i> , N46D) was integrated into the chromosome of UR472	This study
Plasmids		
pUX113	Overexpression plasmid of <i>draT</i> under the control of the <i>nifH</i> promoter on pRK404 (<i>Pnif-draT</i>)	Grunwald <i>et al.</i> (1995)
pYPZ148	3.3 kb <i>draTGB</i> was cloned into pUC19	This study
pYPZ162	2.3 kb <i>draTGB'</i> , together with <i>aacC1</i> gene (encoding Gm ^R) from pUCGM, was cloned into pRK404 (<i>Pdra-draTG</i>)	This study
pYPZ163	0.8 kb <i>EcoRI</i> fragment containing <i>draG'B'</i> was deleted from pYPZ162 (<i>Pdra-draT</i>)	This study
pYPZ165	1 kb <i>BamHI-EcoRV</i> fragment containing <i>draT</i> was cloned into pBSKS(–)	This study
pYPZ170	Similar to pYPZ163, but contains <i>Pdra-draT16</i> (K103E)	This study
pYPZ173	Similar to pYPZ187, but contains <i>Pdra-draT16</i> (K103E)	This study
pYPZ174	Similar to pUX113, but contains <i>Pnif-draT16</i> (K103E)	This study
pYPZ179	Similar to pYPZ163, but contains <i>Pdra-draT17</i> (N248D)	This study
pYPZ183	1.2 kb <i>draG'B</i> was cloned into pYPZ174 (<i>Pnif-draT16GB</i> , K103E)	This study
pYPZ187	<i>Pdra-draT</i> , together with <i>aacC1</i> gene (encoding Gm ^R) from pUCGM, was cloned into pSUP202, a suicide vector for <i>R. rubrum</i>	This study
pYPZ190	Similar to pYPZ187, but contains <i>Pdra-draT17</i> (N248D)	This study
pYPZ191	Similar to pYPZ187, but contains <i>Pdra-draT18</i> (N46D)	This study

Growth conditions and whole-cell nitrogenase activity assay.

The method of derepression for nitrogenase in *R. rubrum* has been described previously (Kanemoto & Ludden, 1984), but has been modified for simplicity. *R. rubrum* was first grown in rich (SMN) medium (Fitzmaurice *et al.*, 1989), then directly inoculated into malate-glutamate (MG) medium (Lehman & Roberts, 1991) with 60-fold dilution. After evacuation of the gas space and flushing with argon, cultures were derepressed for nitrogenase under light for 2 d. The whole-cell nitrogenase activity assay and the darkness/NH₄Cl treatments have been described previously (Zhang *et al.*, 1995a). For purification of DRAT and dinitrogenase reductase, *R. rubrum* was grown in 22-litre carboys. The MG medium was supplemented with fructose (5 g l⁻¹) to increase cell yields.

Construction of *R. rubrum draTG* deletion mutant. To construct a *draTG* deletion mutant, a 3.3 kb *PstI* fragment containing *draTGB* (Fitzmaurice *et al.*, 1989) was subcloned into pUC19, yielding pYPZ148. *draB* is an open reading frame (ORF) immediately downstream of *draG* in *R. rubrum*, *A. brasilense* and *A. lipoferum* (Fitzmaurice *et al.*, 1989; Inoue *et al.*, 1996; Ma & Li, 1997; Zhang *et al.*, 1992) and loss-of-function mutations in this gene cause a small decrease in DRAG activity in *R. rubrum* (Liang *et al.*, 1991). In this report, we have decided to give this ORF a *dra* gene designation (*draB*) both because of the mutation phenotype and because of the apparent co-transcription of *draB* with *draTG* (D. P. Lies & G. P. Roberts, unpublished data). pYPZ148 was digested with *AflIII* and *BstBI* and this deleted part of *draT*, all of *draG* and part of *draB*. After treatment with mung bean nuclease to create blunt ends, this fragment was ligated with a 1.4 kb *HincII* fragment containing the Km^R cassette gene from pUC4K (Vieira & Messing, 1982), so that the deleted region of *draT'draGdraB'* was replaced by the Km^R cassette. The deletion/insertion region was subcloned into pSUP202 (Simon *et al.*, 1983) and conjugated into the *R. rubrum* wild-type, as described previously (Liang *et al.*, 1991). pSUP202 is unable to replicate in *R. rubrum* and Sm^R Km^R colonies were selected and replica-printed to screen for Cm^S (Cm^R is encoded by the vector sequence) colonies resulting from a double-crossover recombination event. The mutation eliminates functional

DRAT and DRAG and was designated $\Delta draTGB10::kan$. The strain was designated UR472 and was used thereafter as a host strain to screen and analyse *draT* mutations.

Integration of *draT* alleles into the chromosome of UR472 ($\Delta draTGB10::kan$). The various *draT* alleles were cloned into pSUP202, then transferred into *R. rubrum* UR472. Transconjugants with a single crossover were selected ($Sm^R Tc^R$), so that a single copy of *draT* was integrated into the chromosome, but no functional allele of *draG* was present.

Random PCR mutagenesis. The protocol for random PCR mutagenesis was based on the methods described previously (Leung *et al.*, 1989; Vogel & Das, 1994). To increase the mutation frequency, 0.5 mM $MnCl_2$ and high concentrations of dGTP, dCTP and dTTP (1 mM), as well as a low concentration of dATP (0.2 mM), were used. The cycle profile was as follows: 45 s at 94 °C, 1 min at 42 °C and 2.5 min at 72 °C, for 30 cycles. *Taq* DNA polymerase from *Thermus aquaticus* (Fisher) was used in the reaction, as it supports a high mutation frequency (Keohavong & Thilly, 1989; Lundberg *et al.*, 1991).

Three plasmids, pYPZ162, pYPZ163 and pYPZ165, were constructed for random PCR mutagenesis and screening. A 1 kb *Bam*HI–*Eco*RI fragment of *R. rubrum draT* was subcloned into pBSKS(–) (Stratagene), yielding pYPZ165. This plasmid was used as a template for the PCR reaction. A 2.3 kb fragment containing *R. rubrum nifH'draTGB'* was cloned into pRK404 (Ditta *et al.*, 1985), and an *aacC1* gene from pUCGM encoding gentamicin acetyltransferase-3-1 (Gm^R) (Schweizer, 1993) was inserted at the 3'-end of the *nifH'* gene, yielding pYPZ162. A 0.8 kb *Eco*RI fragment containing *draG'B'* was deleted from pYPZ162, yielding pYPZ163. After PCR amplification with pYPZ165 as a template, DNA was precipitated with ethanol and digested with *Bam*HI and *Eco*RI, then ligated with pYPZ163 which had been digested with the same restriction enzymes to delete wild-type *draT*. The ligation mixture was transformed into *Escherichia coli* DH5 α to generate a *draT* mutant library.

Screening of *draT* mutants. Plasmids from the *draT* mutant library were transferred into *R. rubrum* strain UR212 or strain UR472 as described previously (Grunwald *et al.*, 1995). $Sm^R Tc^R Gm^R$ transconjugants of *R. rubrum* were grown in SMN, then inoculated into nitrogen-free liquid medium at a 200-fold dilution or diluted and plated on nitrogen-free medium. This medium was similar to MN medium (Lehman & Roberts, 1991), except that NH_4Cl was omitted. Cells were grown anaerobically in nitrogen-free medium containing 10 μg gentamicin ml^{-1} under a dark/light regimen, with 90 min light and 30 min dark. This increased the difference in the growth rate between the mutants and the wild-type.

DNA sequencing analysis. DNA sequences were determined using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). Sequencing data were analysed with DNASTAR software programs (DNASTAR).

DRAT overexpression. The overexpression plasmid (pUX113) for wild-type *draT* was constructed previously (Grunwald *et al.*, 1995). For overexpression of DRAT-K103E, the *Bbs*I–*Bsa*I fragment, containing mutated *draT16* (K103E) from pYPZ170, was cloned into pUX113 to replace the *draT* region, yielding pYPZ174.

Protein purification. The purification of dinitrogenase reductases and DRAT has been reported previously (Grunwald & Ludden, 1997; Lowery & Ludden, 1988). On the Affi-gel column, DRAT-K103E showed substantially weaker binding

than did DRAT-WT, eluting with 150 mM NaCl and 1 mM ADP. After the Affi-gel Blue column, DRAT-K103E was bound to a Phenyl Sepharose column (Pharmacia Biotech) and eluted with 50 % ethylene glycol.

Protein immunoblotting. A trichloroacetic acid precipitation method and the protein immunoblotting procedure have been described previously (Zhang *et al.*, 1993). ECL Western blotting detection reagents (Amersham) were used for detection.

In vitro DRAT activity assay. NAD labelled with ^{32}P was used as a donor of ADP-ribose for the *in vitro* DRAT activity assay, as described previously (Grunwald *et al.*, 1995; Grunwald & Ludden, 1997; Lowery & Ludden, 1988). DRAT activity is expressed as nmol ADP-ribose transferred per min per mg protein.

RESULTS

Random PCR mutagenesis and screening of *draT* mutants from *R. rubrum*

It has been our working hypothesis that DRAT activity is regulated through dissociable negative effector(s). We tested this hypothesis by seeking DRAT variants that have lost the site at which such effectors would bind; this should result in strains with 'constitutive' DRAT activity. Because of the lack of easy selection/screening methods for such regulatory mutants, it was important to mutagenize *draT* efficiently so that the desired mutants would exist at a detectable frequency. We therefore employed random PCR mutagenesis on *draT*.

First, we developed a method of screening for the desired *draT* mutants, based on the expected difference between wild-type and *draT* mutants in terms of their nitrogenase activity and growth rates in nitrogen-free medium (Table 2). Strain UR212 (*draT::kan*) was used as the host strain for screening the plasmid-borne mutants. Because of the *kan* insertion in *draT* and its polar effect on *draG*, UR212 has no DRAT and has a low DRAG activity (less than 5 % of wild-type DRAG activity) (Liang *et al.*, 1991). This low DRAG activity has three advantages in the screen. First, unlike wild-type DRAG levels, this low DRAG activity should provide less competition for the desired 'constitutively' active DRAT, so nitrogenase activity in these mutants should be very low. Second, this low DRAG activity supported high initial nitrogenase activity in the presence of multicopy *draT*⁺. Although most of the DRAT from this multicopy plasmid was still regulated normally and was in an inactive form under N_2 -fixing conditions, a low background level of DRAT activity was present, which resulted in the dramatic decrease in nitrogenase activity and the modification of dinitrogenase reductase when no DRAG was present. Such a low nitrogenase activity with plasmid-borne DRAT-WT would make it difficult to screen for 'constitutively' active DRAT mutants that cause even lower nitrogenase activity. However, the low DRAG activity in UR212 compensated for the leaky DRAT-WT activity and supported a higher level of nitrogenase activity. Third, this low DRAG activity supported the recovery of

Table 2. Summary of the screening of *draT* mutants and their expected phenotypes in nitrogen-free medium with a light/dark-cycle regimen

Strain UR212 (*draT::kan*) was used as the host strain for screening the plasmid-borne mutants. Because of the *kan* insertion in *draT* and its polar effect on *draG*, UR212 has no DRAT and has low DRAG activity (less than 5 % of wild-type DRAG activity).

Type of DRAT mutant on the plasmid in the UR212 background	Nitrogenase activity (modification of dinitrogenase reductase) under light	Nitrogenase activity under light/dark regimen	Growth rate in N-free medium	No. of mutants found
No mutation or silent mutation (wild-type DRAT)	Medium activity (some modification)	Loses activity and recovers	Medium	NA
‘Constitutively’ active DRAT	Low activity (complete modification)	Loses activity, but does not recover	Slow	2
Loss-of-function DRAT	High activity (no modification)	Remains active	Fast	> 50

NA, Not applicable.

nitrogenase activity after cells were shifted from dark to light. This increased the difference in growth rate between the mutants with ‘constitutively’ active DRAT and the wild-type under the light/dark cycle regimen.

Different *draT* mutants were expected to show different growth rates under these conditions (Table 2). Strain UR212 with wild-type *draT* on the plasmid (strain UR462) shows a medium level of nitrogenase activity and shows moderate growth in nitrogen-free medium under dark/light shift conditions, because even the low DRAG activity of this strain supports moderate initial nitrogenase activity and allows the recovery of nitrogenase activity after each shift from darkness to light. *draT* mutants that have lost catalytic activity (referred to as the loss-of-function mutants) have a faster rate of growth, since they have higher initial nitrogenase activity and retain their nitrogenase reductase in an active form during each shift to darkness. In contrast, if the UR212 derivative contained a ‘constitutively’ active DRAT, this strain would have a very poor growth rate, because this ‘constitutively’ active DRAT could compete with the low DRAG activity, resulting in a low nitrogenase activity regardless of illumination conditions.

Although mutants with ‘constitutively’ active DRAT are the most interesting ones, loss-of-function mutants were also analysed; they were used to estimate mutation frequency and to determine whether these mutants clustered in specific domains of DRAT. A different *R. rubrum* strain, UR472 (Δ *draTGB*), was used as the host strain for screening this type of mutant. Unlike DRAG activity in UR212 (*draT::kan*), which is low, DRAG activity in UR472 is completely eliminated. When multicopy *draT* was introduced into UR472 (creating UR483; Table 3), the strain showed a very low nitrogenase activity and grew slowly in nitrogen-free medium. A mutant with a loss-of-function mutation in *draT* would grow faster under these conditions.

After random PCR mutagenesis, *draT* mutants were screened in nitrogen-free medium. After a 4–5 d incubation under a dark/light regimen, colonies or liquid cultures with faster or slower growth rates than that of the wild-type control (UR462 or UR483) were picked.

Characterization of *draT* mutants

Approximately 1000 mutagenized plasmids were screened in the background of UR472 (Δ *draTGB::kan*); this indicated that approximately 8 % of the clones represented loss-of-function *draT* mutants, and some were analysed further. Another 1000 mutagenized plasmids were screened in the UR212 (*draT::kan*) background, which allowed detection of mutants with altered DRAT regulation, and two mutants (UR542 and UR543) were found to grow poorly under these selection conditions, suggesting that they had ‘constitutively’ active DRAT. Single colonies were purified from both classes of mutants, and the nitrogenase activity and its regulation were reanalysed. As shown in Table 3, most of the putative loss-of-function mutants showed a higher initial nitrogenase activity than did the control strains (UR483 or UR462), resulting in a fast growth rate in nitrogen-free medium under screening conditions. In these mutants, the regulation of nitrogenase activity in response to darkness was partially or completely abolished. Most of these mutants showed a high residual nitrogenase activity (30–100 % of the initial activity before treatment) after a shift to darkness for 60 min, whereas a very low residual nitrogenase activity (5 %) was seen in wild-type control strains (UR462 and UR483). One mutant (UR537) showed a normal response to dark treatment, but with high initial nitrogenase activity. This mutant is characterized below. Western blotting with antibodies to DRAT revealed that, out of 44 loss-of-function mutants, 30 mutants accumulated little or no DRAT protein, whilst 14 mutants accumulated levels of DRAT protein comparable to those in UR462 (data not shown). Two

Table 3. Summary of *draT* mutants obtained from random PCR mutagenesis

<i>R. rubrum</i> strain	Background strain	Mutations in <i>draT</i> on plasmid	Initial nitrogenase activity*	Darkness response†
UR483	UR472 ($\Delta draTGB::kan$)	Wild-type	30	Normal
UR525	UR472 ($\Delta draTGB::kan$)	M52T	400	None
UR528	UR472 ($\Delta draTGB::kan$)	F74L	300	Partial
UR529	UR472 ($\Delta draTGB::kan$)	Y69C, L263P	600	None
UR530	UR472 ($\Delta draTGB::kan$)	L189P, M201V	600	None
UR531	UR472 ($\Delta draTGB::kan$)	I245V, L276P	600	None
UR532	UR472 ($\Delta draTGB::kan$)	L76P, F230S, S274S	300	None
UR534	UR472 ($\Delta draTGB::kan$)	L250P	250	Partial
UR535	UR472 ($\Delta draTGB::kan$)	W144R	400	Partial
UR537	UR472 ($\Delta draTGB::kan$)	N46D	450	Normal
UR462	UR212 (<i>draT::kan</i>)	Wild-type	350	Normal
UR542	UR212 (<i>draT::kan</i>)	K103E, T237A	40	Normal
UR543	UR212 (<i>draT::kan</i>)	N248D and promoter	100	Normal
UR546	UR212 (<i>draT::kan</i>)	Y169C	700	Partial
UR549	UR212 (<i>draT::kan</i>)	M73V, Y107N, Q164R	900	None
UR550	UR212 (<i>draT::kan</i>)	G19E, F74L, E79E	500	None
UR551	UR212 (<i>draT::kan</i>)	G266S	800	None
UR552	UR212 (<i>draT::kan</i>)	Y190C, Y257stop	800	None

* Nitrogenase activity is expressed as nmol ethylene produced per h per ml of cells at OD₆₀₀ 1.

† To test the response to darkness, cells were shifted from light to darkness for 60 min and then the nitrogenase activity was assayed. The response was scored as follows: none, 80–100 % of the initial nitrogenase activity remained under conditions of darkness; partial, 30–80 % of the initial nitrogenase activity remained; normal, 5–20 % of the initial nitrogenase activity remained.

Table 4. Comparison of nitrogenase activity from strains with plasmids containing wild-type or altered *draT*

Strain	Chromosomal genotype	Substitution(s) in <i>draT</i> on pRK404	Estimated level of protein accumulation*			<i>In vivo</i> nitrogenase activity
			DRAT ^{wt} †	DRAT ^c ‡	DRAG§	
UR2 (wild-type)	<i>dra</i> ⁺		1	0	1	800
UR462	<i>draT2::kan</i>	Wild-type <i>draT</i>	10	0	< 0.05	360
UR511	<i>draT2::kan</i>	K103E (<i>draT16</i>)	0	10	< 0.05	60
UR582	<i>draT2::kan</i>	N248D (<i>draT17</i>)	0	10	< 0.05	120
UR506	<i>dra</i> ⁺	Wild-type <i>draT</i>	11	0	1	560
UR508	<i>dra</i> ⁺	K103E (<i>draT16</i>)	1	10	1	250
UR581	<i>dra</i> ⁺	N248D (<i>draT17</i>)	1	10	1	600

* The level of DRAT and DRAG is 1 for the wild-type and 10 for strains containing multiple *Pdra-draT* on pRK404, which is at about 10 copies per cell.

† Wild-type DRAT.

‡ Constitutively active DRAT.

§ Low DRAG activity (less than 5 % of the wild-type activity) was seen in the *draT::kan* mutant, because the Km^R insertion has a polar effect on *draG* (Liang *et al.*, 1991).

|| Nitrogenase activity is expressed as nmol ethylene produced per h per ml of cells at OD₆₀₀ 1.

poorly growing strains (UR542 and UR543) showed very low nitrogenase activity under N₂-fixing conditions, and had a level of DRAT protein accumulation comparable to that of UR462, consistent with the presence of 'constitutively' active DRAT variants.

Plasmids were isolated from 16 mutants with a normal level of DRAT: 14 were loss-of-function mutants and two were 'constitutively' active DRAT variants. In each case, the entire mutated *draT* region was sequenced: seven plasmids had a single mutation, six plasmids had

Fig. 2. Immunoblots of dinitrogenase reductase from strains grown under nitrogen-fixing conditions and containing plasmids encoding DRAT-WT (UR462 and UR506), DRAT-K103E (UR511 and UR508) and DRAT-N248D (UR582 and UR581). The parental strain of UR462, UR511 and UR582 is UR212 (*draT2::kan*), which has low DRAG activity. The parental strain of UR506, UR508 and UR581 is wild-type UR2.

two mutations, and three plasmids had three mutations. Most (89%) of the mutations were transitions. These *draT* mutants are summarized in Table 3. DNA sequence analysis showed no clustering of the mutations in the loss-of-function mutants, and no alleles had identical sequences. UR537 has a mutation in *draT* causing an N46D substitution. Both of the mutants with 'constitutively' active DRAT had two mutations in *draT*: UR542 had mutations causing K103E and T237A; UR543 had a mutation causing N248D and another mutation located in the promoter region of *draT*.

Further characterization of the mutants with altered regulation of DRAT activity

To identify which substitutions caused the altered regulation of DRAT activity, a smaller fragment of *draT*, containing only a single substitution of T237A, K103E or N248D, was subcloned into pYPZ163, yielding pYPZ169 (T237A), pYPZ170 (K103E) and pYPZ179 (N248D). These plasmids were transferred into different *R. rubrum* strains for further analysis. For the following experiments, we created strains with different ratios of DRAT and DRAG to verify the presence of 'constitutive' DRAT activity in these DRAT variants.

In the UR212 (*draT2::kan*, with a low DRAG activity) background, strains with plasmid-borne DRAT-T237A (UR544) showed a high level of nitrogenase activity under N_2 -fixing conditions, like the strain with DRAT-WT (data not shown). This indicated that the T237A is not the cause of the 'constitutive' DRAT activity. However, strains with plasmid-borne DRAT-K103E (UR511) or DRAT-N248D (UR582) showed 20–35% of the nitrogenase activity of the wild-type control (UR462) under N_2 -fixing conditions (Table 4). Modification of dinitrogenase reductase was monitored by Western blotting (Fig. 2). Since only one subunit of dinitrogenase reductase is modified and the ADP-ribosylation slows

the migration of that subunit, two bands are seen for the inactive form. Consistent with this, a high degree of modification of dinitrogenase reductase was found in UR511 (DRAT-K103E) and in UR582 (DRAT-N248D), compared with the wild-type control (UR462) under N_2 -fixing conditions. These results indicate that these altered DRAT proteins are significantly active under N_2 -fixing conditions. These DRAT variants, when expressed from the plasmid at levels approximately 10 times that of the wild-type, can compete effectively with the low level of DRAG in this strain background to modify dinitrogenase reductase. However, we have no way of accurately quantifying *in vivo* DRAT activity in these mutants.

When placed in a wild-type (UR2) background with the wild-type level of DRAG, a strain with DRAT-K103E (UR508) again showed lower nitrogenase activity and a higher degree of modification of dinitrogenase reductase than the wild-type control (UR506), but a strain with DRAT-N248D (UR581) showed no significant differences from the wild-type control (Table 4, Fig. 2), indicating that DRAT-N248D has lower 'constitutive' activity than that of DRAT-K103E. These results indicate that DRAT-K103E at this elevated level can compete with the normal level of DRAG to modify dinitrogenase reductase, whereas DRAT-N248D cannot.

The strongest evidence for the regulation of DRAT-WT activity *in vivo* is that *DraG*⁻ mutants (which have functioning DRAT) showed high nitrogenase activity and had little modification of dinitrogenase reductase under N_2 -fixing conditions, indicating that DRAT must be post-translationally inactivated under these conditions (Liang *et al.*, 1991; Zhang *et al.*, 1992). To compare DRAT variants with DRAT-WT in a *DraG*⁻ background, a single copy of wild-type or 'constitutively' active *draT* was integrated into UR472 (a *draTGB* deletion mutant). The nitrogenase activity in these transconjugants was monitored under N_2 -fixing conditions. In contrast to UR589 (DRAT-WT), in which nitrogenase activity is high (530 nmol ethylene per h per ml of cells at OD₆₀₀ 1), both UR583 (DRAT-K103E) and UR610 (DRAT-N248D) display low levels of nitrogenase activity (50 and 70 nmol ethylene per h per ml of cells at OD₆₀₀ 1, respectively), reflecting the level of DRAT activity that escapes regulation in these mutants. Western blots of dinitrogenase reductase showed that whilst little modification of dinitrogenase reductase was seen in UR589 (*draT*⁺), dinitrogenase reductase in both UR583 and UR610 was completely modified (Fig. 3). This result confirms the altered regulation of DRAT in these strains and this identification of such 'constitutively' active DRAT variants strongly suggests that the model of DRAT regulation through inhibition by negative effectors is correct.

Interestingly, it seems that more than one subunit of dinitrogenase reductase was modified in these mutants, especially after a shift to darkness, as more than 50% of the subunits of dinitrogenase reductase appeared to be

Fig. 3. ADP-ribosylation of dinitrogenase reductase in UR583 (DRAT-K103E), UR589 (DRAT-WT) and UR610 (DRAT-N248D). Each of these strains has a single copy of various *draT* alleles in the chromosome, without functional *draG*. Crude extracts were prepared from cells before and after a shift of the cultures to darkness for 60 min.

ADP-ribosylated. The reason for this apparent over-modification is unknown.

In vitro characterization of DRAT-K103E

Previous experiments have indicated that more DRAT protein was accumulated in the cell when *draTG* were overexpressed together (Grunwald *et al.*, 1995). However, UR587, overexpressing both DRAT-K103E and DRAG, grew very slowly in MG medium anaerobically under light, which is probably due to a futile cycle of ADP-ribosylation, depleting the NAD pool. Instead, UR578, in which only DRAT-K103E was expressed, was used for overexpression of DRAT-K103E, and it grew only slightly more slowly than did UR2 (wild-type). DRAT-K103E was purified according to the published protocol for DRAT-WT, with some modifications (as described in Methods), since DRAT-K103E showed substantially weaker binding to Affi-gel. The difference in affinity to Affi-gel is interesting, since Affi-gel Blue is usually used to purify proteins containing a dinucleotide fold (Thompson *et al.*, 1975); it suggests that DRAT-WT and DRAT-K103E might have different abilities to bind dinucleotides.

The K_m for NAD and the V_{max} for DRAT-WT and DRAT-K103E in the presence of ADP were determined. When dinitrogenase reductase from *Azotobacter vinelandii* was used as an acceptor of ADP-ribose in an *in vitro* assay, the V_{max} (nmol ADP-ribose transferred per min per mg of protein) values of DRAT-WT and DRAT-K103E were 89 ± 3 and 71 ± 2 , respectively, and the K_m values for NAD of DRAT-WT and DRAT-K103E were $268 \pm 31 \mu\text{M}$ and $187 \pm 20 \mu\text{M}$, respectively. When dinitrogenase reductase from *R. rubrum* was used as the substrate, DRAT-WT again displayed a K_m value for NAD that was about 30% higher than that of DRAT-K103E (data not shown). These results indicate that any differences in the K_m for NAD or V_{max} do not seem sufficient to explain the altered regulation in DRAT-K103E.

In vivo modification of dinitrogenase reductase in a *nifD* mutant by DRAT-WT and DRAT-K103E

Halbleib *et al.* (2000) reported recently that DRAT can modify only oxidized dinitrogenase reductase and that DRAG only removes an ADP-ribosyl group from reduced dinitrogenase reductase. In an *R. rubrum nifD* mutant, most dinitrogenase reductase is reduced, because it lacks dinitrogenase to accept electrons from it; the rate of modification of dinitrogenase reductase is very slow in this mutant (Halbleib *et al.*, 2000). To test if DRAT-K103E responds differently to the redox states of dinitrogenase reductase, both pYPZ173 (DRAT-K103E) and pYPZ187 (DRAT-WT) were transferred separately into the *R. rubrum nifD* mutant and integrated into the chromosome. The rate of modification of dinitrogenase reductase was similar in both strains (data not shown), indicating that DRAT-K103E is similar to DRAT-WT in its ability to modify reduced dinitrogenase reductase.

Further characterization of the mutant with DRAT-N46D (UR537)

In preliminary screening, UR537 showed an interesting phenotype. It had substantial nitrogenase activity under derepression conditions, but responded normally to darkness, indicating that DRAT-N46D could still be activated. Western blotting of DRAT revealed that this mutant accumulated normal amounts of DRAT protein (data not shown). When a single copy of altered *draT* was integrated in a *DraG*⁻ background, UR619 showed substantial nitrogenase activity (650 nmol ethylene per h per ml of cells at OD₆₀₀ 1) and a normal darkness response (data not shown). However, in response to NH₄⁺, UR619 responded poorly and 80% residual nitrogenase activity remained after 10 mM NH₄Cl was added for 60 min, compared to only 10% residual nitrogenase activity remaining in the wild-type control (UR589) (data not shown). In crude extracts, UR619 had about 8% of the DRAT activity of UR589 (data not shown), indicating that N46D substitution substantially reduced (but did not completely eliminate) DRAT activity. Previous data suggested that darkness generated a stronger signal for the activation of DRAT, since a faster rate of modification of dinitrogenase reductase and a lower residual nitrogenase activity were seen in response to darkness than in response to NH₄⁺ (Zhang *et al.*, 1995a). It is our hypothesis that the signal from darkness is strong enough to activate DRAT-N46D, but that the signal from NH₄⁺ is too weak to do so.

DISCUSSION

Although there is substantial evidence that both DRAT and DRAG activities themselves are regulated, the mechanism for this regulation is unknown. Two general hypotheses have been proposed for the nature of DRAT and DRAG regulation: (1) regulation through interaction with the substrate, dinitrogenase reductase, and (2) regulation through interaction with either small molecules or other protein effectors.

It is clear that dinitrogenase reductase is involved in some portion of the regulation of DRAT and DRAG activities. The most striking case is the recent demonstration that the different redox states of dinitrogenase reductase are required for DRAT and DRAG (Halbleib *et al.*, 2000). DRAT can modify only oxidized dinitrogenase reductase and DRAG removes the ADP-ribosyl group only from reduced dinitrogenase reductase. The second indication that substrate recognition affects DRAT activity comes from the results of Kim *et al.* (1999), who employed a completely different genetic hunt for DRAT variants that were able to modify an E112K variant of dinitrogenase reductase. This E112K variant of dinitrogenase reductase is a poor substrate for wild-type DRAT. Surprisingly, this different search also identified DRAT-K103E, as well as another DRAT-Q81R variant. Further analysis showed that DRAT-Q81R had a lower level of 'constitutive' DRAT activity than DRAT-K103E and DRAT-N248D. In a complementary study, DRAT-N248D, identified in the present screen for altered regulation, was also able to modify the E112K variant of dinitrogenase reductase (Kim *et al.*, 1999). These results suggest that there is a correlation between the regulation of DRAT activity and its substrate recognition.

Despite the importance of dinitrogenase reductase in the regulation of DRAT/DRAG activities, this cannot be the entire basis of the regulation, for four reasons. (1) As demonstrated in this work, the 'constitutively' active DRAT (DRAT-K103E) is not altered in its ability to ADP-ribosylate reduced dinitrogenase reductase. (2) The degree of the redox effect on DRAT/DRAG does not seem to be sufficient to account for the extremely tight regulation of DRAT/DRAG seen *in vivo*. Little DRAT activity was found under nitrogen-limiting conditions, but the redox states of dinitrogenase reductase change continuously during substrate reduction. (3) DRAT and DRAG are not always regulated in a coordinated way and both proteins can be inactive in some conditions, so the regulation of DRAT and DRAG must involve more complicated or totally different mechanisms. Consistent with this, only the regulation of DRAG appears to be altered in *R. rubrum* and *A. brasilense ntrBC* mutants, but DRAT regulation is altered in an *R. rubrum glnB* mutant (Zhang *et al.*, 1994, 1995b, 2000). (4) Effects of *ntrBC* mutations on the regulation of DRAG activity in *R. rubrum* are extremely difficult to trace to effects on the substrate itself, since it has no significant effect on *nif* expression (Zhang *et al.*, 1994, 1995b). It is more likely that this mutation perturbs either small molecules or protein effectors which interact with DRAG *in vivo*.

The different affinity to the Affi-gel Blue column of DRAT-WT and DRAT-K103E is extremely interesting, as it suggests that the basis for the altered regulation of this DRAT variant might be due to a different affinity for a dinucleotide or a similar molecule. The regulation of DRAT activity through its accessibility to NAD would be a very attractive hypothesis, based on the fact that NAD is required for the interaction between DRAT and

dinitrogenase reductase *in vitro* (Grunwald & Ludden, 1997) and on reports showing that exogenous NAD can perturb the modification of dinitrogenase reductase (Norén *et al.*, 1997; Soliman & Nordlund, 1992). However, our *in vitro* analysis of the affinity for NAD of DRAT-WT and DRAT-K103E would seem to preclude this as the basis for the altered regulation by DRAT variants, suggesting that NAD alone is not the direct effector for regulation of DRAT *in vivo*. Nevertheless, the dramatically altered affinity of DRAT-K103E to the Affi-gel Blue column, compared to that of DRAT-WT, suggests a significant alteration in the surface of this DRAT variant and is consistent with altered binding either to some small molecules or to protein effectors.

In summary, the data in this report strongly suggest that DRAT activity is regulated *in vivo*, probably via a small molecule effector such as a nucleotide or a dinucleotide. Whilst identification of such an effector might be difficult, we believe that the DRAT variants described here will prove to be a highly valuable control in screening for these effectors with the *in vitro* DRAT assay.

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