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Effect of Nucleotides on the Activity of Dinitrogenase Reductase ADP-Ribosyltransferase from *Rhodospirillum rubrum*†

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ABSTRACT: The mechanism by which MgADP stimulates the activity of dinitrogenase reductase ADP-ribosyltransferase (DRAT) has been examined by using dinitrogenase reductases from *Rhodospirillum rubrum*, *Klebsiella pneumoniae*, and *Azotobacter vinelandii* as acceptor substrates. In the presence of 0.2 mM NAD, maximal rates of ADP-ribosylation of all three acceptors were observed at an ADP concentration of 150 μM; in the absence of added ADP, DRAT activity with the dinitrogenase reductases from *R. rubrum* and *K. pneumoniae* was less than 5% of the maximal rate, but the *A. vinelandii* protein was ADP-ribosylated at 40% of the maximal rate. Of eight dinucleotides tested, only ADP, 2′-deoxy-ADP, and ADP-βS served as activators of the DRAT reaction; ADP, 2′-deoxy-ADP, and ADP-βS were also the only dinucleotides found which inhibited acetylene reduction activity by dinitrogenase reductase. The dinucleotide specificities for both DRAT activation and acetylene reduction inhibition were the same for all three dinitrogenase reductases. In the DRAT reaction with the dinitrogenase reductases from *K. pneumoniae* and *A. vinelandii*, the K_m for NAD was 30-fold higher in the absence of ADP than in its presence; the K_m for NAD with the *R. rubrum* acceptor was not measurable. In the presence of saturating ADP, ADP-ribosylation of dinitrogenase reductase from *R. rubrum* was inhibited 63% by 1.5 mM ATP. It is concluded that MgADP stimulates DRAT activity by lowering the K_m for NAD and that MgADP exerts its effect by binding to dinitrogenase reductase. MgATP inhibits DRAT activity by competing with MgADP for binding to dinitrogenase reductase.

Reduction of dinitrogen to ammonium in biological systems is catalyzed by the nitrogenase enzyme complex. The molybdenum–nitrogenase is composed of two oxygen-labile proteins, dinitrogenase reductase and dinitrogenase. Electrons are transferred from dinitrogenase reductase to dinitrogenase with concomitant hydrolysis of MgATP; substrates, which include protons and acetylene as well as dinitrogen, are reduced at the Fe–Mo cofactor site of dinitrogenase [see Orme-Johnson (1985) for a review of nitrogenase]. Dinitrogenase reductase contains two identical subunits of approximately 30 kDa which share a single 4Fe–4S cluster.

MgATP and MgADP bind to dinitrogenase reductase competitively (Walker & Mortenson, 1973; Thorneley & Cornish-Bowden, 1976). Most studies have indicated that dinitrogenase reductase has two nucleotide binding sites per dimer and that MgADP inhibits nitrogenase activity by com-

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peting with MgATP for at least one of these sites (Thorneley & Cornish-Bowden, 1976; Tso & Burris, 1973). For all of the dinitrogenase reductases for which nucleotide binding affinities have been determined, including the enzymes from *Clostridium pasteurianum*, *Klebsiella pneumoniae*, and *Azotobacter vinelandii* (Cp2, Kp2, and Av2, respectively),[1] MgADP binds more tightly than MgATP (Thorneley & Cornish-Bowden, 1976; Tso & Burris, 1973; Ljones & Burris, 1978; Cordwener et al., 1985). Thus, in vitro nitrogenase activity with Av2 and Kp2 is inhibited 50% when the MgADP:MgATP ratio is 0.1 and 0.2, respectively (Thorneley & Cornish-Bowden, 1976; Weston et al., 1983).

Both MgADP and MgATP cause changes in the conformation of dinitrogenase reductase as evidenced by altered spectroscopic signals for the metal cluster (Orme-Johnson et al., 1972; Zumft et al., 1973; Stephens et al., 1979) and a lowered midpoint potential (Zumft et al., 1974)). However, the conformational changes caused by the two nucleotides are not identical. MgATP causes increased acessibility of the 4Fe–4S cluster to metal chelators; this transition to a more open complex is inhibited by MgADP (Walker & Mortenson, 1973; Ljones & Burris, 1978). Also, MgADP and MgATP cause similar, but not identical, changes in the EPR spectra of the dinitrogenase reductase metal cluster (Lindahl et al., 1987).

In the photosynthetic bacterium *Rhodospirillum rubrum*, nitrogenase activity is modulated in response to light intensity and ammonia concentration by ADP-ribosylation of dinitrogenase reductase (Kanemoto & Ludden, 1984; Pope et al., 1985). Dinitrogenase reductase ADP-ribosyltransferase (DRAT) catalyzes the inactivation of dinitrogenase reductase by ADP-ribosylation of arginine-101 on a single subunit of the dinitrogenase reductase homodimer (Pope et al., 1985; Lowery et al., 1986). Covalent attachment on an ADP-ribose moiety to dinitrogenase reductase apparently prevents formation of a complex with dinitrogenase (Murrell et al., 1988). Activation of dinitrogenase reductase by removal of the ADP-ribose moiety is catalyzed by dinitrogenase reductase activating glycohydrolase (DRAG) (Ludden & Burris, 1976; Saari et al., 1986). DRAG, which is irreversibly inactivated by oxygen, has been purified to homogeneity (Saari et al., 1984), and DRAT, which is not oxygen sensitive, has recently been purified to near-homogeneity (Lowery & Ludden, 1988). DRAT uses NAD (but not NADH) as a donor molecule and native dinitrogenase reductase as substrate (Lowery & Ludden, 1988) and has maximal activity in the presence of free divalent metal (Lowery et al., 1986).[2]

The mechanisms by which DRAT and DRAG are regulated in vivo are not known. MeATP is required for activation of dinitrogenase reductase by DRAG in vitro (Ludden & Burris, 1979; Saari et al., 1986). It has been suggested that the conformational transition caused by MeATP binding to native dinitrogenase reductase exposes the bound ADP-ribose moiety to DRAG because both oxygen-denatured dinitrogenase reductase and ADP-ribosylated guanidino compounds are effective as DRAG substrates in the absence of ATP (Ludden & Burris, 1979; Saari et al., 1986; Pope et al., 1986). DRAT activity is stimulated by MeADP in vitro; ATP and AMP are ineffective (Lowery et al., 1986). The regulation of DRAG and DRAT by adenine nucleotides in vivo is an attractive possibility.

Unlike other arginine-specific ADP-ribosyltransferases (Hayaishi & Ueda, 1985), DRAT is extremely specific with respect to acceptor substrates. The only DRAT substrates which have been found are dinitrogenase reductases from other organisms (Lowery & Ludden, 1988), all of which share extensive structural and functional homology with the *R. rubrum* protein[3] (Pretorious et al., 1987; Emerich & Burris, 1978). In order to elucidate the mechanism of DRAT activation by MgADP, we have examined the effect of MgADP and a number of other dinucleotides on the DRAT-catalyzed ADP-ribosylation of dinitrogenase reductases from *R. rubrum*, *Azotobacter vinelandii*, and *Klebsiella pneumoniae*.

Experimental Procedures

Growth of Bacteria and Purification of Enzymes. Photoheterotrophically grown *R. rubrum* ATCC 11170 was used as a source of DRAT and of Rr2 (Ormerod et al., 1961; Lowery & Ludden, 1988). In order to obtain highly active DRAT fractions that contained no ADP (ADP stabilizes DRAT during purification), the following modifications of the DRAT purification protocol (Lowery & Ludden, 1988) were used: ADP was omitted from the buffer in the gel filtration step (Sephacryl S-200 HR), and the final purification step (FPLC on a Pharmacia Mono Q column) was omitted. BSA was added to the S-200 fractions to a final concentration of 1 mg mL^{-1} in order to increase the stability of DRAT to freeze–thawing in the absence of ADP. The DRAT obtained with this procedure had a specific activity of 4–15 nmol of ADP-ribose transferred per minute per milligram with Av2 as acceptor.

Dinitrogenase reductases from *R. rubrum* (Ludden & Burris, 1978), *K. pneumoniae* (Shah, 1986), and *A. vinelandii* (Shah & Brill, 1973) were purified by using modifications of previously published procedures. The specific activities of these preparations ranged from 700 to 1400 nmol of C_2H_4 formed min^{-1} mg^{-1} for Av2 and Kp2 and from 250 to 750 nmol of C_2H_4 formed min^{-1} mg^{-1} for Rr2. The reason for the lower specific activity in some Rr2 preparations is that a fraction of the protein was ADP-ribosylated in vivo before isolation.

DRAT Assay. DRAT was assayed by quantitating the incorporation of radioactivity from [α-^{32}P]NAD into dinitrogenase reductase as previously described (Lowery & Ludden, 1988; Moss & Vaughan, 1984). Reactions, buffered with 100 mM MOPS, pH 7.0, were incubated for 20 min at 30 °C, unless indicated otherwise. This assay is linear with time essentially until completion, at which point dinitrogenase reductase becomes the limiting substrate. The dinitrogenase reductase proteins used in these assays were in the reduced form, as confirmed by UV–vis spectroscopy. The DRAT activities reported in this study are initial velocities. [α-^{32}P]NAD was synthesized enzymatically from [α-^{32}P]ATP and NMN.

Acetylene Reduction Assay. Dinitrogenase reductase activity was measured by using a modified acetylene reduction assay (Stewart et al., 1967) in which the ATP regenerating system was omitted so that added dinucleotides would not be phosphorylated by creatine kinase (Weston et al., 1983). Small amounts of nitrogenase proteins (10–40 μg per assay) and short incubation times (2–4 min, at 30 °C) were used so that inhibition from ADP produced by nitrogenase-catalyzed ATP

[1] Abbreviations: Av2, Kp2, and Rr2, dinitrogenase reductase from *Azotobacter vinelandii*, *Klebsiella pneumoniae*, and *Rhodospirillum rubrum*, respectively; DRAG, dinitrogenase reductase ADP-ribosyltransferase; DRAT, dinitrogenase reductase activating glycohydrolase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MMN, nicotinamide mononucleotide.

[2] R. G. Lowery and P. W. Ludden, unpublished data.

[3] L. Lehman and G. P. Roberts, unpublished data.

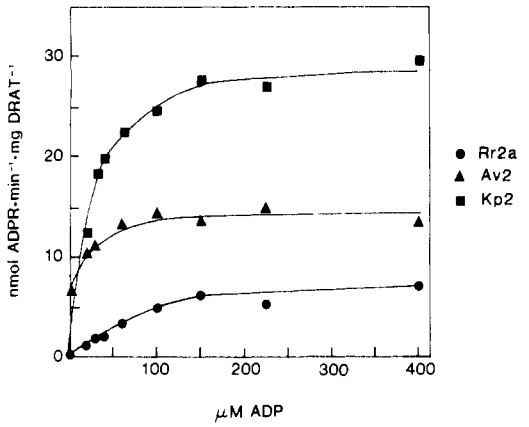

FIGURE 1: Activation of DRAT by MgADP with Rr2 (●), Av2 (▲), and Kp2 (■) as acceptors. DRAT reactions contained 5 mM $MgCl_2$, 0.2 mM [α-^{32}P]NAD, 0.0075 unit of DRAT, and 80 μg of dinitrogenase reductase in a total volume of 25 μL.

hydrolysis would not be significant. The reactions, buffered with 100 mM MOPS, pH 7.5, were carried out in stoppered, 14-mL glass serum vials under an atmosphere of 10% C_2H_2 in N_2. After being quenched with trichloroacetic acid, samples of the gas phase were analyzed for C_2H_4 with a flame ionization gas chromatograph.

ATP-Dependent Fe^{2+} Release. The release of Fe^{2+} from Kp2 was measured as described by Walker and Mortenson (1973), using the chelator bathophenanthrolinedisulfonate (BPS) (Ljones & Burris, 1978). Kp2 was added to 1-mL cuvettes (1-cm path length) containing anaerobic 100 mM MOPS, pH 7.5, 2 mM sodium dithionite, 5 mM $MgCl_2$, and 0.25 mM BPS. The absorbance at 535 nm was allowed to stabilize prior to the addition of ATP alone or simultaneous addition of ATP plus a dinucleotide. The absorbance at 535 nm was monitored continuously for 60 s; the initial rate of Fe^{2+} release was calculated from the change in absorbance between 10 and 30 s by using an extinction coefficient of 22.14 mM^{-1} cm^{-1} for the Fe^{2+}–BPS complex (Blair & Diehl, 1961).

Materials. Apyrase, ATP, NAD, and all dinucleotides except ADP and ADP-βS were from Sigma. ADP-βS was from Boehringer Mannheim, and ADP was from United States Biochemical Corp. [α-^{32}P]ATP was purchased from Amersham.

RESULTS

The effect of MgADP on DRAT activity with reduced Rr2, Av2, and Kp2 in the presence of 0.2 mM NAD is shown in Figure 1. At saturating [ADP], ADP-ribosylation of Rr2 is slower than the reaction with either of the heterologous dinitrogenase reductases. In the absence of ADP, ADP-ribosylation of Rr2 and Kp2 is undetectable under these conditions, but the DRAT activity with Av2 is 40% of that seen with saturating ADP. The sensitivity of DRAT activity to the concentration of ADP is similar for the three dinitrogenase reductases despite the difference in minimum and maximum activities. Half-maximal DRAT activity with Rr2 is reached at an ADP concentration of 55 μM and with Av2 and Kp2 at approximately 25 μM; ADP is saturating at 150 μM in all three cases.

The ADP-ribosylation of Av2 by DRAT in the absence of ADP or any other nucleotide is important because it shows that ADP is not absolutely required for DRAT activity. We confirmed this result with two different approaches because other investigators have recently reported contaminating ADP in purified Av2 preparations (Lindahl et al., 1987). Av2 which had been previously denatured by exposure to air was added to a DRAT reaction with native Kp2. If the Av2 contained tightly bound ADP, it is likely that it would be released upon denaturation of the protein and thus would be available for stimulation of DRAT activity with Kp2; no such stimulation was observed (Table I). As a second means of confirming our results with Av2, we tested the effect of apyrase on DRAT activity with Rr2 and Av2. Other investigators have found that addition of apyrase to preparations of Av2 abolishes the changes in the EPR signal which they report are caused by contaminating ADP (Lindahl et al., 1987). Addition of apyrase to a DRAT reaction with Rr2 as acceptor completely inhibited the activity, presumably because all of the ADP was hydrolyzed to AMP (Table I). However, apyrase had almost no effect on DRAT activity with Av2 either in the presence or in the absence of ADP (Table I). We conclude that our purified Av2 does not contain ADP and that DRAT has catalytic activity in the absence of ADP.

Table I: Demonstration of the Absence of ADP in Preparations of Av2[a]

acceptor	addition	DRAT sp act. (nmol of ADP-ribose min^{-1})
none	O_2-treated Av2	0.5
Kp2	O_2-treated Av2	0.9
Kp2	none	0.6
Kp2	O_2-treated Av2, ADP	15.4
Rr2	none	0.0
Rr2	ADP	1.1
Rr2	ADP, apyrase	0.0
Av2	none	2.0
Av2	ADP	4.2
Av2	ADP, apyrase	2.2
Av2	apyrase	1.7

[a] DRAT assays contained 5 mM $MgCl_2$, 0.2 mM [α-^{32}P]NAD, 100 μg of acceptor protein, DRAT, and 0.25 mM ADP, when present, in a total volume of 30–50 μL. The DRAT preparation used in the experiments with Kp2 was different than that used in the experiment with Rr2 and Av2.

Because the effect of ADP on DRAT activity was dependent on the source of the dinitrogenase reductase used as acceptor, we hypothesized that MgADP was exerting its effect on DRAT activity indirectly, by binding to dinitrogenase reductase. The high specificity of the nucleotide binding sites on dinitrogenase reductase was used as a criterion to test this hypothesis. Eight dinucleotides, including ADP, were tested for binding to dinitrogenase reductase by their ability to inhibit acetylene reduction activity with Rr2, Kp2, and Av2 (Table II). As expected, ADP inhibited acetylene reduction activity by all three dinitrogenase reductases almost completely when present at half the concentration of ATP. ADP-βS and dADP inhibited acetylene reduction activity 80%, 50%, and 40% for Rr2, Av2, and Kp2, respectively. No other dinucleotides caused more than 10% inhibition under these conditions except 8Br-ADP, which inhibited 15% with Rr2. These results differed from those of previous investigators, who reported significant inhibition of Av2 and Kp2 acetylene reduction activity by IDP and etheno-ADP (Weston et al., 1983) under conditions similar to ours. We corroborated our results by using the inhibition of MgATP-dependent Fe^{2+} release from dinitrogenase reductase (Walker & Mortenson, 1973; Ljones & Burris, 1978) as a method to test for dinucleotide binding to Kp2. This method does not depend on the activity of any other protein, as does the inhibition of acetylene reduction activity; thus, it is a more direct indicator of dinucleotide binding to dinitrogenase reductase. ADP, equimolar to ATP, caused a 95% inhibition in the initial rate of Fe^{2+} release from Kp2; dADP and ADP-βS both inhibited greater than 60%; none of the other dinucleotides that were tested inhibited more

Table II: Effect of Various Dinucleotides on DRAT Activity, Nitrogenase Activity, and MgATP-Dependent Fe^{2+} Release from Dinitrogenase Reductase[a]

	% activity						
	DRAT			nitrogenase			Fe^{2+} release
nucleotide	Rr2	Av2	Kp2	Rr2	Av2	Kp2	Kp2
none	<5	39	3	100	100	100	100
ADP	100	100	100	5	6	13	5
ATP	9	36	11				
GDP	<5	41	3	93	100	102	ND
IDP	<5	34	ND	101	113	98	91
etheno-ADP	<5	36	3	91	103	97	85
ADP-βS	99	115	136	19	53	57	39
AMP-CH_2-P	<5	47	13	94	106	100	ND
dADP	123	110	140	20	49	58	37
8Br-ADP	<5	40	7	85	93	92	102

[a] DRAT assays contained 0.1 mM [α-^{32}P]NAD, 5 mM $MgCl_2$, 100 μg of acceptor protein, 0.0075 unit of DRAT, and 1.0 mM dinucleotide, when present, in a total volume of 25 μL. Acetylene reduction assays contained 10 mM $MgCl_2$, 5 mM ATP, 10 mM $Na_2S_2O_4$, 10 μg of dinitrogenase, 10 μg of dinitrogenase reductase, and 2.5 mM dinucleotide, when present, in a total volume of 0.5 mL. In the experiment shown, dinitrogenase from *A. vinelandii* was used for acetylene reduction assays with Rr2 and Av2; the assays with Kp2 were done with the homologous dinitrogenase. Fe^{2+} release from 0.4 mg of Kp2 was initiated by the addition of ATP alone or ATP plus dinucleotide, both to a final concentration of 0.25 mM. $MgCl_2$ was present in excess (5 mM).

Table III: Effect of Av2 Concentration on DRAT Activity in the Presence and in the Absence of ADP[a]

Av2	ADP	DRAT sp act. (nmol of ADP-ribose min^{-1})
100 μg	none	1.9
250 μg	none	1.8
100 μg	0.25 mM	4.1
250 μg	0.25 mM	4.1

[a] DRAT reactions contained 5 mM $MgCl_2$, 0.2 mM [α-^{32}P]NAD, and 0.0075 unit of DRAT in a total volume of 30 μL.

than 15% when present at the same concentration as ATP (Table II).

The same eight dinucleotides were tested for stimulation of DRAT activity with Rr2, Av2, and Kp2 as acceptors; only ADP, ADP-βS, and dADP stimulated DRAT activity (Table II). The correlations between the dinucleotide specificities for inhibition of acetylene reduction activity, inhibition of MgATP-dependent Fe^{2+} release from dinitrogenase reductase, and stimulation of DRAT activity strongly suggest that MgADP exerts its effect on the DRAT reaction by binding to dinitrogenase reductase. The screen for DRAT activators was done with 1 mM dinucleotide; ADP is saturating at 150 μM with Rr2. We also tested the effect of ADP-βS at a concentration of 200 μM and observed maximal stimulation of DRAT activity with Rr2. Thus, it is highly unlikely that the activation we are attributing to ADP-βS is due to contaminating ADP in the ADP-βS.

In order to determine whether MgADP binding to dinitrogenase reductase increased its affinity for DRAT, the effect of Av2 concentration on DRAT activity was examined (Table III). There was no effect of Av2 concentration on DRAT activity in the absence of ADP, and we conclude that MgADP does not activate DRAT by increasing its affinity for dinitrogenase reductase over the range of concentrations tested.

The effect of ADP on the K_m for NAD in the DRAT reaction was examined next. In the presence of MgADP, the K_m for NAD is 2.0 mM with Rr2 (Lowery & Ludden, 1988; Figure 2A, inset), 71 μM with Kp2, and 22 μM with Av2 as acceptor (Figure 2A). In the absence of ADP, the K_m for NAD is 2.5 mM with Kp2 and 0.7 mM with Av2 as acceptor (Figure 2B). Thus, the K_m for NAD in the DRAT reaction is dramatically affected both by MgADP and by the acceptor protein. Although incorporation of radioactivity from [α-^{32}P]NAD into Rr2 has been detected in the absence of added ADP,[2] it has not been possible to obtain an accurate estimate

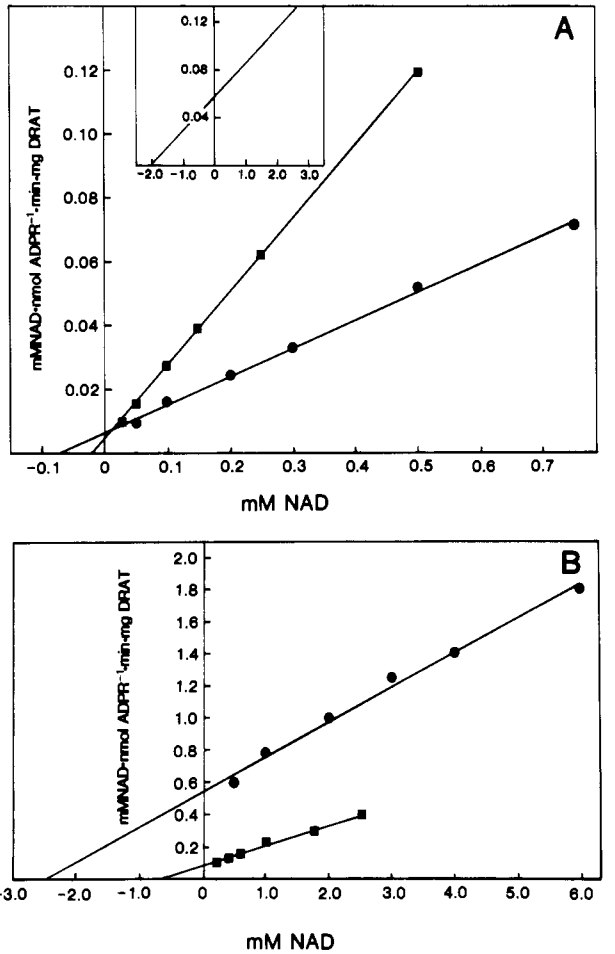

FIGURE 2: Determinations of the K_m for NAD in the DRAT reaction with Rr2, Av2, and Kp2 as acceptors in the presence (A) and in the absence (B) of ADP. DRAT reactions with Av2 (■) and Kp2 (●) contained 5 mM $MgCl_2$, DRAT, and 100 μg of dinitrogenase reductase in a total volume of 25 μL. The experiment with Rr2 (inset), performed under similar conditions, is from Lowery & Ludden (1988).

of the K_m for NAD under these conditions. If the K_m for NAD increases 30-fold in the absence of ADP, as it does when Av2 and Kp2 are used as acceptors for DRAT, a K_m of 60 mM would be expected with Rr2 as acceptor.

The K_m for NAD is not the only kinetic parameter of the DRAT reaction which is affected by MgADP. The experiments in Figure 2A,B were all done with the same fraction

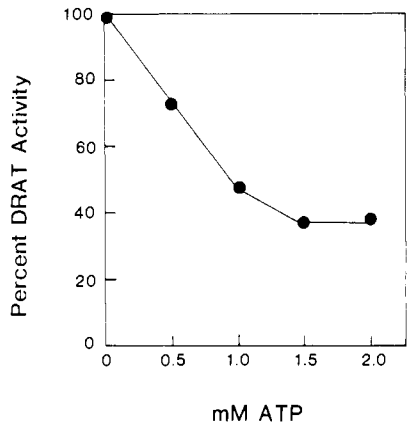

FIGURE 3: Effect of MgATP on DRAT activity with Rr2 as acceptor. DRAT reactions contained 5 mM $MgCl_2$, 0.2 mM [α-^{32}P]NAD, 0.0075 unit of DRAT, 100 μg of Rr2, and 0.25 mM ADP. 100% DRAT activity was 6.8 units mg^{-1}.

of DRAT (except the K_m determination with Rr2); therefore, though the enzyme preparation is not homogeneous, the relative V_{max}'s for the different experiments can be compared. The V_{max} for the DRAT reaction with Av2 in the absence of ADP was 8.1 units mg^{-1} and in the presence of ADP was 4.3 units mg^{-1}. With Kp2 as acceptor, the V_{max}'s were 4.6 (absence of ADP) and 11.5 units mg^{-1} (presence of MgADP). Thus, MgADP lowers the V_{max} for the DRAT reaction with Av2 approximately 2-fold and increases the DRAT V_{max} with Kp2 by about the same factor. These results, and the effects of MgADP on the K_m for NAD, are consistent with the data shown in Figure 1.

MgATP and MgADP compete for binding to dinitrogenase reductase and elicit different conformational changes in the protein. Therefore, it was of interest to determine the effect of MgATP on the ADP-ribosylation of the physiological substrate. At a concentration of MgADP which is saturating for activation of DRAT (0.25 mM), a maximal inhibition of 63% was reached at 1.5 mM ATP (Figure 3). In the absence of ADP, DRAT activity with Av2 is unaffected by 1 mM ATP (Table II). This suggests that MgATP has no direct effect on DRAT activity, that it inhibits in the presence of MgADP by competing for the nucleotide binding sites on dinitrogenase reductase.

Discussion

We have examined the stimulation of DRAT activity by dinucleotides with the dinitrogenase reductases from *R. rubrum*, *A. vinelandii*, and *K. pneumoniae* as ADP-ribose acceptors. The correlation between the dinucleotide specificities for binding to dinitrogenase reductase and stimulation of the DRAT reaction suggests that MgADP exerts its effect on the DRAT reaction by binding to dinitrogenase reductase. The fact that ADP-βS and dADP activate the DRAT reaction at least as well as ADP, but are apparently not as effective as ADP at competing with ATP for binding to dinitrogenase reductase (Table II), is not surprising. Although the ADP analogues might not bind to dinitrogenase reductase as tightly as MgADP, their effect on the K_m for NAD or on the V_{max} of the DRAT reaction could be different than that of MgADP (i.e., lower the K_m for NAD even more than ADP or increase the V_{max}). Also, the dinucleotides were tested for activation of the DRAT reaction at a concentration (1 mM) above saturating for MgADP; thus, differences in binding affinities would not have been detected unless they were fairly large.

MgATP is required for activation of ADP-ribosylated dinitrogenase reductase by DRAG, but not for N-glycohydrolysis of ADP-ribose from oxygen-denatured Rr2 or from arginine analogues (Saari et al., 1986; Ludden & Burris, 1979; Pope et al., 1986). Thus, both enzymes of the ADP-ribosylation regulatory cycle are indirectly activated by nucleotides, apparently through their interaction with the nucleotide binding sites on dinitrogenase reductase. Consistent with this hypothesis, DRAG activity is inhibited by MgADP (Saari et al., 1986), and DRAT activity is inhibited by MgATP (Figure 3).

With Av2 or Kp2 as acceptor in the DRAT reaction, stimulation of DRAT activity by MgADP results from a decrease in the K_m for NAD. It has not been possible to demonstrate the same effect with the physiological acceptor (Rr2) because measurement of the K_m for NAD in the absence of ADP is not technically feasible. A rapid loss of nitrogenase activity, not correlated with ADP-ribosylation, is observed when Rr2 is incubated in the presence of high concentrations of NAD (20 mM).[2] Furthermore, in the presence of MgADP, the DRAT reaction appears to be inhibited at 25 mM NAD.[2] However, DRAT activity with Rr2 has been detected in the absence of ADP by using sensitive autoradiographic techniques.[2] Given the high degree of structural and functional homology between nitrogenase proteins from different species (Pretorius et al., 1987; Emerich & Burris, 1978), it is likely that MgADP affects the K_m for NAD in the DRAT reaction with Rr2 similarly to the way that it stimulates DRAT activity with the heterologous dinitrogenase reductases. The ability of Av2 to be ADP-ribosylated in the absence of nucleotide may reflect the anomalously low redox potential of this protein in both the nucleotide-free and nucleotide-bound forms (Braaksma et al., 1982).

In effect, this would mean that in vivo, where the NAD concentration is at or below the K_m for NAD in the presence of MgADP, DRAT would be essentially inactive unless MgADP was bound to Rr2. Similarly, removal of the ADP-ribose moiety by DRAG would not occur unless MgATP was bound to dinitrogenase reductase. The interaction of nucleotides with dinitrogenase reductase is complex; investigators disagree on exactly how the binding of one nucleotide affects the biding of others (Cordwener et al, 1985). However, it is clear, both from kinetic studies of H_2 evolution by nitrogenase (Thorneley & Cornish-Bowden, 1976) and from the effects of nucleotides on the accessibility of the dinitrogenase reductase 4Fe–4S cluster to chelators (Walker & Mortenson, 1973; Ljones & Burris, 1978), that MgATP and MgADP compete with each other for binding to dinitrogenase reductase. Thus, it is tempting to speculate that nucleotide binding to Rr2 plays some role in the regulation of the steady-state level of ADP-ribosylated Rr2 in response to environmental stumuli.

It is not known how the interaction of MgADP with dinitrogenase reductase decreases the K_m for NAD in the DRAT reaction. Kp2, Av2, and Rr2 are very similar proteins, both structurally and functionally. Apparently, even the subtle differences between these three DRAT acceptors are sufficient to cause as much as a 3-fold difference in the K_m for NAD (Figure 2B). Thus, it is reasonable to presume that the conformational changes induced by nucleotide binding to dinitrogenase affect the K_m for NAD in the DRAT reaction. We hope to gain insight into the regulation of nitrogenase activity in vivo by continuing our analysis of the role of nucleotides in the DRAT/DRAG regulatory ADP-ribosylation cycle. Reconstitution of the entire regulatory system in vitro will allow an analysis of the effect of the ATP:ADP ratio on the steady-state level of ADP-ribosylated dinitrogenase reductase.

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Registry No. DRAT, 117590-45-1; ADP, 58-64-0; 2'-deoxy-ADP, 2793-06-8; ADP-βS, 120496-69-7; NAD, 53-84-9; ATP, 56-65-5; MgADP, 7384-99-8; MgATP, 1476-84-2.

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