Substitution of Histidine for Arginine-101 of Dinitrogenase Reductase Disrupts Electron Transfer to Dinitrogenase†

Robert G. Lowery,‡ Christina L. Chang,§ Lawrence C. Davis,§ Marie-Claire McKenna,∥ Philip J. Stephens,∥ and Paul W. Ludden*,‡

Department of Biochemistry, University of Wisconsin—Madison, 420 Henry Mall, Madison, Wisconsin 53706, Department of Biochemistry, Kansas State University, Willard Hall, Manhattan, Kansas 66506, and Department of Chemistry, University of Southern California, Los Angeles, California 90089

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ABSTRACT: Dinitrogenase reductase from *Klebsiella pneumoniae* strain UN1041 has a histidine residue substituted for arginine at position 101. The mutant dinitrogenase reductase was purified and characterized in order to determine the importance of arginine-101 in the interaction between dinitrogenase and dinitrogenase reductase during electron transfer. Purified dinitrogenase reductase from UN1041 is a dimer of 67 kDa, contains a functional 4Fe–4S cluster, undergoes a MgATP-dependent conformational change, and is competent for ATP hydrolysis uncoupled from substrate reduction in the presence of dinitrogenase. However, the mutant protein is unable to support the reduction of protons or acetylene by dinitrogenase. A 100-fold molar excess of Kp2 from UN1041 does not inhibit electron transfer from wild-type dinitrogenase reductase to dinitrogenase. It is concluded that the interaction of dinitrogenase reductase with dinitrogenase during reductant-independent ATP hydrolysis is different than the interaction between the two proteins during electron transfer; the substitution of histidine for arginine at position 101 disrupts only the latter interaction. The same conclusions are reached using wild-type dinitrogenase reductase which has been ADP-ribosylated at arginine-101.

Nitrogenase, the enzyme which catalyzes the reduction of dinitrogen to ammonia in nitrogen-fixing organisms, is composed of two oxygen-labile proteins, dinitrogenase reductase (Fe protein, component II) and dinitrogenase (MoFe protein, component I). Dinitrogenase reductase from all known sources contains two identical subunits of approximately 30 kDa which share a single 4Fe–4S cluster. Dinitrogenase, an $\alpha_2\beta_2$ tetramer, contains 4Fe–4S clusters and a Mo-containing cofactor (FeMoco) [see Orme-Johnson (1985) for a review of nitrogenase]. Reducing equivalents are passed, in single electron steps, from dinitrogenase reductase to dinitrogenase with concomitant hydrolysis of 2 ATP/e^- (Ljones & Burris, 1972, 1978a,b; Kennedy et al., 1968). Kinetic studies are consistent with dissociation of oxidized dinitrogenase reductase from dinitrogenase between each electron-transfer event (Hageman & Burris, 1978); dissociation of the complex is believed to be the rate-limiting step in the overall reaction (Thorneley & Lowe, 1983). Substrate molecules are reduced by dinitrogenase, probably at the Fe–Mo cofactor. Nitrogenase catalyzes the reduction of protons to H_2 and acetylene to ethylene in addition to the reduction of N_2 to ammonia (Orme-Johnson, 1985).

Nitrogenase activity is regulated in *Rhodospirillum rubrum* by reversible ADP-ribosylation of dinitrogenase reductase at arginine-101 (Pope et al., 1985). Since there is extensive structural and functional homology between the dinitrogenase reductases from different species of bacteria (Pretorious et al., 1987; Emerich & Burris, 1978), it is not surprising that the regulatory ADP-ribosyltransferase from *R. rubrum* (dinitrogenase reductase ADP-ribosyltransferase, DRAT)[1] will modify dinitrogenase reductases from *Klebsiella pneumoniae*, *Azotobacter vinelandii*, and *Clostridium pasteurianum* (Kp2, Av2, and Cp2 respectively) in addition to the *R. rubrum* protein (Lowery & Ludden, 1988). Using the heterologous Cp2/Av1 inactive complex (Emerich & Burris, 1976), we have previously provided evidence which suggests that ADP-ribosylation of dinitrogenase reductase prevents formation of a complex with dinitrogenase and thereby prevents electron transfer (Murrell et al., 1988). The data also suggest that arginine-101 is buried between the two components of the nitrogenase complex.

There is extensive homology in the amino acid sequence in the region of the DRAT-target arginine of Kp2, Av2, Cp2, and Rr2 (Pretorious et al., 1987). A conserved cysteine residue three residues to the amino-terminal side of the target arginine has been proposed to be ligated to the 4Fe–4S cluster in Av2 (Hausinger & Howard, 1983). The proximity of the target arginine to the 4Fe–4S cluster is consistent with the idea that this region of dinitrogenase reductase may interact with dinitrogenase during electron transfer.

In order to elucidate the role, if any, of the DRAT-target arginine in the interaction between dinitrogenase reductase

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* To whom correspondence should be addressed.
‡ University of Wisconsin—Madison.
§ Kansas State University.
∥ University of Southern California.

[1] Abbreviations: Av1, dinitrogenase from *Azotobacter vinelandii*; Av2, dinitrogenase reductase from *A. vinelandii*; Cp2, dinitrogenase reductase from *Clostridium pasteurianum*; DRAG, dinitrogenase reductase activating glycohydrolase; DRAT, dinitrogenase reductase ADP-ribosyltransferase; Kp1, dinitrogenase from *Klebsiella pneumoniae*; Kp2(UN900) and Kp2(UN1041), dinitrogenase reductase from *K. pneumoniae* strain UN900 and UN1041, respectively; MOPS, 3-(*N*-morphlino)propanesulfonic acid; Rr2, dinitrogenase reductase from *Rhodospirillum rubrum*; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; WT, wild type.

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and dinitrogenase, we have characterized the mutant dinitrogenase reductase from *K. pneumoniae* strain UN1041 which has a single amino acid altered: a histidine residue is substituted for the DRAT-target arginine at position 101 (Chang & Davis, 1988).

Experimental Procedures

Bacterial Strains and Growth Conditions. Wild-type *K. pneumoniae* (UN900) was grown anaerobically in a 300-L fermentor in modified minimal medium (Nieva-Gomez et al., 1980; Hill & Kavanaugh, 1980) at 30 °C. The mutant strain (UN1041), which has been described previously (Roberts et al., 1978), was grown in smaller (8-L) batches. Growth and derepression of *nif* genes were as previously described (Shah et al., 1983). Cells were harvested by centrifugation, frozen in liquid N_2, and stored at −80 °C.

Purification of Nitrogenase Proteins. The cell breakage and initial chromatographic step used for purification of Kp2(UN900) and Kp2(UN1041) were identical; both were modifications of previously published methods (Shah, 1986). All of the steps of the purification were performed anaerobically, under an N_2 atmosphere. Buffers were evacuated and flushed 3× with N_2 and contained 2 mM sodium dithionite. Purification of nitrogenase proteins from UN900 was monitored by acetylene reduction assays of column fractions. Kp2 from UN1041 was assayed by an immunoblot procedure. Both assays are described below. Protein concentration was determined by using the biuret method.

Frozen cell paste (200–400 g) was thawed in 3 volumes of 50 mM Tris-HCl, pH 7.7, containing DNase at a concentration of 10 μg/mL (Sigma, DN-25), and the cells were broken anaerobically with a French pressure cell at 16000 psi. The broken cell preparation was centrifuged at 50000*g*, for 1 h, at 4 °C. The supernatant was applied to a 5 × 20 cm DE52–cellulose column which had been equilibrated with 50 mM Tris-HCl, pH 7.7, containing 50 mM NaCl. Kp1 and Kp2 were eluted with a 2-L linear gradient of NaCl (0.1–0.5 M).

For Kp2(UN1041) purification, DE52 fractions containing dinitrogenase reductase cross-reacting material were diluted and applied to a 1 × 10 cm Mono Q FPLC column (Pharmacia, Piscataway, NJ) which was equilibrated with 100 mM MOPS, pH 7.5, containing 200 mM NaCl. The column was washed with 12 mL of equilibration buffer before Kp2(UN1041) was eluted with a 150-mL linear gradient of NaCl (0.2–0.6 M). Kp2(UN1041) was eluted in a 7.5-mL fraction between 420 and 440 mM NaCl. Following dilution, the protein was concentrated 10-fold (to approximately 40 mg/mL) on a small DE52–cellulose column.

Because of the large amount of protein, conventional chromatography rather than FPLC was used for further purification of Kp2(UN900) and Kp1. DE52 fractions containing Kp2(UN900) were diluted and applied to a 2.5 × 20 cm column of Q-Sepharose (Pharmacia, Piscataway, NJ), a strong anion-exchange resin similar to the one used in the Mono Q column. Buffers and elution procedure were the same as those used for purification of Kp2(UN1041) on the Mono Q column, except the protocol was scaled up appropriately. The Kp2(UN900) fraction from the Q-Sepharose column contained only a few very minor contaminants; these were removed by gel filtration on a 2.5 × 92 cm column of Sephadex G-100 in 100 mM MOPS, pH 7.5, containing 100 mM NaCl. Kp2(UN900) was concentrated to approximately 40 mg mL^{-1} on a small DE52–cellulose column.

Fractions from the DE52–cellulose column containing Kp1 (from UN900) were diluted and applied to a 2.5 × 20 cm Q-Sepharose column equilibrated with 100 mM MOPS, pH 7.5, containing 100 mM NaCl. The column was washed with 100 mL of the same buffer, and Kp1 was eluted with an 800-mL, linear gradient of NaCl (100–400 mM). This preparation was approximately 85% homogeneous.

Dinitrogenase from *Azotobacter vinelandii* (Av1) was purified as previously described (Shah & Brill, 1973) using the double crystallization procedure.

Substrate Reduction Assays. Acetylene reduction by nitrogenase proteins was assayed as previously described (Stewart et al., 1967). The reactions were carried out in stoppered 14-mL glass serum vials containing 5 mM ATP, 25 mM creatine phosphate, 10 mM $MgCl_2$, 0.075 mg mL^{-1} creatine kinase, and 10 mM sodium dithionite in a total volume of 0.5 mL. The gas phase was 10% C_2H_2 in N_2. C_2H_4 was quantitated on a flame ionization gas chromatograph.

Assays for H_2 evolution by nitrogenase proteins were similar to acetylene reduction assays except that Ar was used as the gas phase. H_2 was quantitated by a thermal conductivity gas chromatograph, with N_2 as the carrier gas.

Chemical Oxidation and Reduction of Kp2. Kp2(UN900) and Kp2(UN1041) were oxidized with Indigo Carmine bound to Dowex as previously described (Pagani et al., 1987) in an anaerobic glovebox containing less than 2 ppm of O_2. The oxidized, desalted protein was diluted with anaerobic 100 mM MOPS, pH 7.5, to a total volume of 1.0 mL in a round-top quartz cuvette (1-cm path length) which was stoppered before removal from the glovebox for spectrophotometric analysis. The oxidized Kp2 was reduced in the cuvette by the addition of a slight excess of dithionite. The absorbance spectra of the reduced and oxidized proteins were scanned from 200 to 600 nm using a Shimadzu UV-160 scanning spectrophotometer with anaerobic MOPS as the reference. Difference spectra were obtained for the 350–500-nm region. The apparent $\Delta\epsilon_{mM}^{430}$(ox-red) for Kp2(UN900) was 5.8 mM^{-1} cm^{-1} and for Kp2(UN1041) was 6.6 mM^{-1} cm^{-1}.

ATP-Dependent Fe Release. The release of Fe^{2+} from Kp2 was measured by using the chelator bathophenanthrolinedisulfonate (BPS) in the Walker and Mortenson assay (1973), as modified by Ljones and Burris (1978a,b). Kp2 was added by syringe to stoppered, 1-mL cuvettes (1-cm path length) containing anaerobic 100 mM MOPS, pH 7.5, sodium dithionite, and BPS as indicated. In some experiments, an ATP-regenerating system was also present. The absorbance at 535 nm was allowed to stabilize prior to the addition of MgATP in volumes from 5 to 25 μL, to final concentrations indicated. Absorbance at 535 nm was monitored and recorded continuously. A value of 22.14 mM^{-1} cm^{-1} was used as the millimolar extinction coefficient for the Fe^{2+}–BPS complex (Blair & Diehl, 1961). The initial velocity of Fe^{2+} release was calculated from the initial slope of the trace of absorbance increase with time.

ATP Hydrolysis Assay. ATP hydrolysis reactions were carried out in 0.5-mL plastic microfuge tubes in an anaerobic glovebox (<2 ppm O_2). Av1 was used as the source of dinitrogenase (Kp2 and Av1 form an active nitrogenase complex) because the recrystallization procedure used for purification of Av1 removes all traces of ATP-hydrolyzing activities. The reactions, which contained the indicated amounts of ATP, [α-^{32}P]ATP, or [γ-^{32}P]ATP, $MgCl_2$, and Av1 in 100 mM MOPS, pH 7.5, were started by the addition of Kp2. Portions of the reactions (1–2 μL) were removed at the indicated times and quenched by spotting onto plastic TLC sheets impregnated with poly(ethylenimine)–cellulose. The TLC sheets were developed in 0.2 M NH_4HCO_3, autoradiograms were pre-

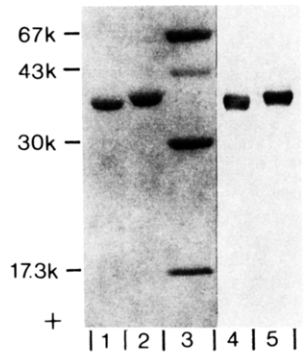

FIGURE 1: Electrophoretic and immunological comparison of Kp2-(UN900) and Kp2(UN1041). Lanes 1–3 show a Coomassie Blue stained SDS gel. Lane 1, 5 μg of purified Kp2(UN900); lane 2, 5 μg of purified Kp2(UN1041); lane 3, molecular weight standards [BSA (67K), ovalbumin (43K), and carbonic anhydrase (30K)]. Lanes 4 and 5 show an immunoblot probed with polyclonal antiserum to dinitrogenase reductase. Lane 4, 1 μg of purified Kp2(UN900); lane 5, 1 μg of purified Kp2(UN1041).

pared, and the spots corresponding to ADP or phosphate were cut out and counted in a liquid scintillation counter.

Preparation of ADP-Ribosylated Kp2. Kp2 was incubated with DRAT, 0.3 mM ADP, 5 mM $MgCl_2$, and 5 mM NAD under anaerobic conditions. The reaction was allowed to continue until there was no detectable acetylene reduction activity remaining when portions of the reaction were assayed in the presence of Kp1. A portion of the Kp2–ADPR was also assayed for acetylene reduction activity in the presence of dinitrogenase reductase activating glycohydrolase (DRAG; Saari et al., 1986) in order to assess what fraction of the Kp2 was still active after removal of the ADP-ribose moiety. In three different experiments, the Kp2–ADPR was maximally activated by DRAG to 44%, 45%, and 78% of its original activity. These values were used to calculate the amount of native Kp2–ADPR to be used in subsequent experiments. Thus, the amounts of Kp2–ADPR shown in Table IV represent only the fraction of Kp2–ADPR in that preparation which was activatable by DRAG.

Gel Electrophoresis and Immunoblotting. SDS–polyacrylamide gel electrophoresis (5% C, 11% T) was performed as previously described (Laemmli, 1970). Gels were stained with Coomassie Blue R-250.

A modification of the method of Towbin et al. (1979) was used for immunoblot detection of Kp2. After electrophoretic transfer to nitrocellulose, proteins were probed with polyclonal antiserum raised against a mixture of Kp2, Rr2, Cp2, and Av2. Horseradish peroxidase conjugated to goat anti-rabbit IgG was used as the color-developing component.

Results

Kp2 was purified to greater than 90% homogeneity from UN1041 and UN900 by using modifications of published procedures (Eady et al., 1972, Shah, 1986). The yield of Kp2 from strain UN1041 was about 30% of that from the wild-type strain.

A comparison of the electrophoretic and immunological properties of Kp2(UN900) and Kp2(UN1041) is shown in Figure 1. Denatured Kp2(UN1041) (lane 2) migrated slightly slower than the wild-type protein (lane 1) in a polyacrylamide gel (37.5 and 36.5 kDa, respectively). The identity of the purified mutant protein as dinitrogenase reductase is demonstrated by cross-reactivity of the 37.5-kDa band with antiserum raised against purified dinitrogenase reductase (lane 5). The native molecular weights of Kp2(UN900) and Kp2(UN1041), as determined by analytical gel filtration on a 1 × 30 cm Superose 12 column (Pharamacia, Piscataway, NJ), were 63K and 67K, respectively. Thus, the mutant protein is a dimer. The published molecular weight for wild-type Kp2, determined by similar methods, is 62K (Eady et al., 1972).

Table I: Nitrogenase Activity in Extracts of UN900 and UN1041[a]

extract	addition	nmol of ethylene formed
UN1041	none	2
UN1041	46 μg of Kp1	2
UN1041	160 μg of Kp2(UN900)	378
UN900	none	702
UN900	160 μg of Kp2(UN900)	1368

[a] 125 μL of extract was incubated for 20 min in 0.5-mL acetylene reduction assays. Both extract types contained approximately 38 mg mL^{-1} protein. Added Kp2(UN900) was saturating.

Table II: Specific Activities of Kp2(UN900) and Kp2(UN1041) in Substrate Reduction Assays with Kp1[a]

Kp2 (μg)	Kp1 (μg)	nmol of ethylene min^{-1} mg^{-1}	nmol of hydrogen min^{-1} mg^{-1}
220 (UN900)	460	1348	842
220 (UN1041)	460	0	0
460 (UN1041)	46	0	
920 (UN1041)	46	0	

[a] Assay periods were 5 min for Kp2(UN900) and 20 min (C_2H_2 reduction) or 40 min (H_2 evolution) for Kp2(UN1041). Background ethylene (approximately 1 nmol) was the same with no protein present and with Kp1 alone and did not increase with time. There was no measurable background H_2.

Extracts from UN1041 exhibited no acetylene reduction activity when incubated in a nitrogenase assay mixture alone or with Kp1 added (Table I). However, upon addition of a saturating amount of wild-type Kp2, these extracts supported 30% of the level of acetylene reduction activity of extracts from UN900 incubated under similar conditions (Table I). Since the UN1041 extract apparently contains active Kp1, the absence of Kp2 activity is probably not caused by exposure to O_2 during cell breakage or centrifugation.

Purified Kp2(UN1041) shows no detectable (<0.05% of WT) acetylene reduction activity with purified Kp1 when assayed at the same Kp2:Kp1 ratio (2:1) which is optimal for Kp2(UN900) (Table II); increasing the ratio to 80:1 had no effect for Kp2(UN1041). The mutant protein also had no detectable acetylene reduction activity in heterologous systems with Rr1, Cp1, and Av1, all of which were active to some degree with Kp2(UN900) (data not shown). No H_2 evolution was detected when Kp2(UN1041) was incubated in a nitrogenase assay mixture with Kp1 under Ar (Table II); our assay would have detected 1% of the wild-type level.

The apparent lack of electron-transfer activity of Kp2-(UN1041) was not due to the absence of functional 4Fe–4S clusters. Kp2(UN1041) was subjected to one cycle of chemical oxidation and reduction using Indigo Carmine and sodium dithionite. Both the oxidized and the reduced UV–vis spectra (200–600 nm) of Kp2(UN1041) are identical with those of the wild-type Kp2 (data not shown). The $\Delta\epsilon^{430}_{mM}$(ox-red) for the mutant protein was 6.6 mM^{-1} cm^{-1}, compared with 5.8 mM^{-1} cm^{-1} for Kp2(UN900). The EPR spectra of the reduced forms of Kp2(UN900) and Kp2(UN1041) are indistinguishable (Figure 2). The signals at $g = 2.04$, $g = 1.94$ (midpoint), and $g = 1.86$ are similar to published spectra for Kp2 (Eady et al., 1972) and other dinitrogenase reductases (Orme-Johnson et al., 1972); these signals are indicative of 4Fe–4S clusters.

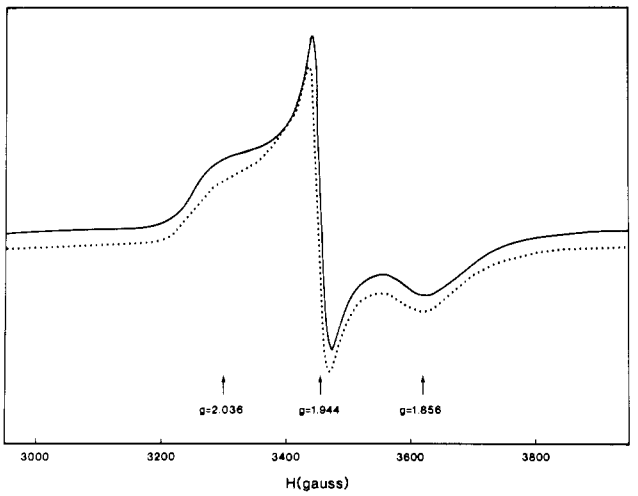

FIGURE 2: EPR spectra of reduced Kp2(UN900) and Kp2(UN1041). Three milligrams of purified Kp2(UN900) (solid trace) or Kp2-(UN1041) (dotted trace) in 0.25 mL of 100 mM MOPS, pH 7.5, was scanned for 100 s (time constant = 100 ms) using a Bruker 200 D instrument at 9.5 K. Other parameters were as follows: microwave frequency = 9.404–9.405 GHz; power = 0.8 mW; gain = 10× (1.25 × 10^4).

Since the protein concentrations of the EPR samples were the same, the fact that the amplitude of the $g = 1.94$ signal ($s = {}^1/_2$) was the same for Kp2(UN900) and Kp2(UN1041) suggests that the fractions of Fe in the $s = {}^1/_2$ and $s = {}^3/_2$ spin states were not altered by the substitution of histidine for arginine at position 101.

Dinitrogenase reductase undergoes a conformational change upon binding of MgATP which results in a decrease in the redox potential of the protein (Zumft et al., 1973), a change in the EPR spectrum under some conditions (Orme-Johnson et al., 1972), and a greatly increased accessibility of the iron–sulfur cluster to metal chelators (Walker & Mortenson, 1973). Addition of Kp2(UN1041) to a cuvette containing the Fe^{2+}-specific chelator bathophenanthrolinedisulfonate (BPS) in anaerobic buffer resulted in the very rapid formation of a small amount of the Fe^{2+}–BPS complex, as indicated by the slight increase in absorbance at 535 nm (Figure 3). This is due to contaminating iron and the release of Fe^{2+} from denatured dinitrogenase reductase. Subsequent addition of 1 mM MgATP to the cuvette caused a slower, but much greater, increase in the A_{535} which eventually stabilized at a value which represents 2.6 Fe^{2+} per mole of Kp2(UN1041) dimer. Similar results were obtained with Kp2(UN900) (2.5 Fe^{2+} per mole of dimer), however, the rate of MgATP-dependent Fe release was significantly faster than with Kp2(UN1041) (Figure 3).

In order to determine whether the slower rate of nucleotide-dependent Fe^{2+} release by the mutant Kp2 was due to a weaker association with MgATP, we compared the effect of MgATP concentration on the initial velocity of Fe^{2+} release from Kp2(UN900) and Kp2(UN1041) (Figure 4). At saturating MgATP (2.5 mM), the rate of Fe^{2+}–BPS complex formation using wild-type Kp2 was 7 times the rate of Fe^{2+} release from Kp2(UN1041) (21.1 and 2.9 nmol of Fe^{2+} min^{-1} mg^{-1}, respectively). Although the MgATP concentration dependence of the BPS chelation reaction is not identical for the two proteins, it is clear that there is not a large difference in the strength of MgATP binding. Fe^{2+} release from both Kp2(UN900) and Kp2(UN1041) approached the maximal level at approximately 2 mM MgATP; the half-maximal rates were at 0.4 and 0.5 mM MgATP for Kp2(UN900) and Kp2(UN1041), respectively.

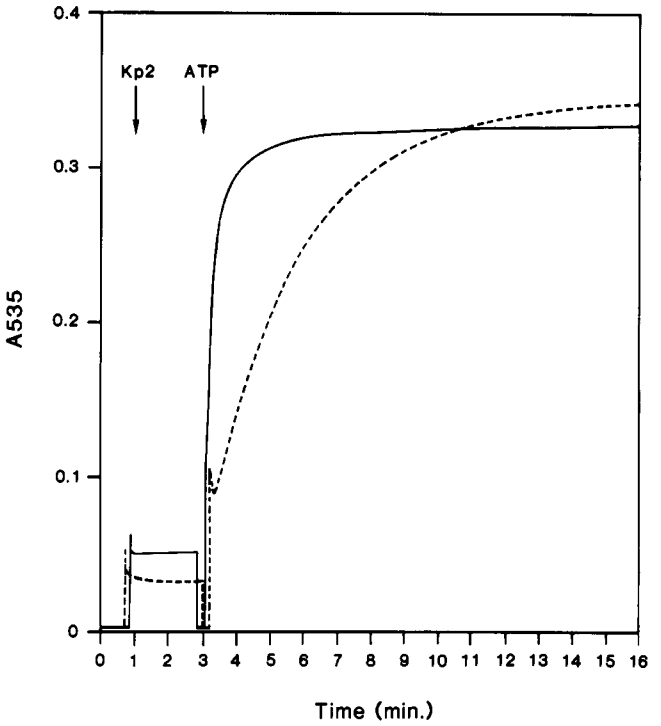

FIGURE 3: MgATP-dependent release of Fe^{2+} from Kp2(UN900) and Kp2(UN1041). 0.4 mg of Kp2(UN900) (solid line) or Kp2(UN1041) (dashed line) was added to an anaerobic cuvette containing 1 mM BPS and 2 mM sodium dithionite in 100 mM MOPS, pH 7.5. After 2 min, MgATP was added to a final concentration of 1 mM.

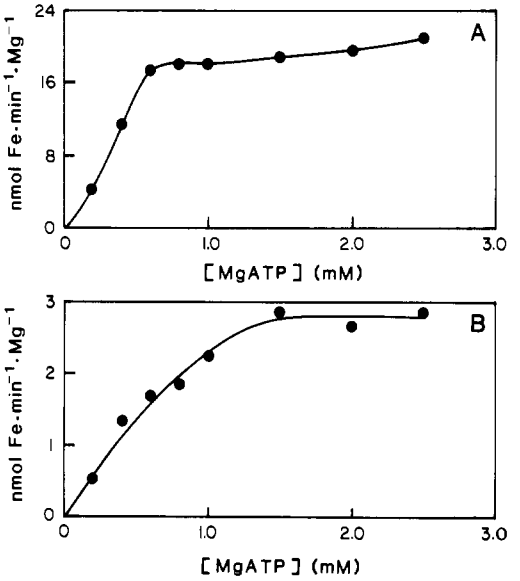

FIGURE 4: Effect of MgATP concentration on the rate of Fe^{2+} release from Kp2(UN900) and Kp2(UN1041). Panel A, Kp2(UN900); panel B, Kp2(UN1041). Anaerobic cuvettes contained 0.25 mM BPS, 10 mM phosphocreatine, 0.05 mg of creatine kinase, 2 mM sodium dithionite, and 0.2 mg of Kp2 in 100 mM MOPS, pH 7.5. Absorbance at 535 nm was allowed to stabilize (approximately 2 min) prior to addition of MgATP.

Since dinitrogenase reductase from UN1041 apparently bound MgATP and underwent an associated conformational change, we next assessed the ability of the mutant protein to hydrolyze ATP. Purified Kp2(UN1041) was incubated with [α-^{32}P]ATP and Mg^{2+} in the presence and in the absence of dinitrogenase from *Azotobacter vinelandii*. Hydrolysis of ATP occurred at a significant rate only when both native Kp2-(UN1041) and Av1 were present (Table III). The rate of ATP hydrolysis approached a maximum as a fixed amount of Av1 was titrated with Kp2(UN1041); this is consistent with

Table III: ATP Hydrolysis by Kp2(UN1041) in the Presence of Dinitrogenase[a]

addition	nmol of ADP formed	addition	nmol of ADP formed
native Kp2(UN1041)	0.7	40 μg of native Kp2(UN1041), Av1	22.6
Av1	0.5	60 μg of native Kp2(UN1041), Av1	25.2
2 μg of native Kp2(UN1041), Av1	3.8	80 μg of native Kp2(UN1041), Av1	26.7
5 μg of native Kp2(UN1041), Av1	7.2	20 μg of O_2-treated Kp2(UN1041)	0.6
10 μg of native Kp2(UN1041), Av1	10.3	20 μg of O_2-treated Kp2(UN1041), Av1	0.6
20 μg of native Kp2(UN1041), Av1	17.6		

[a] Reactions contained 4 mM [α-^{32}P]ATP, 5.0 mM $MgCl_2$, and 40 μg of Av1, when present, in a total volume of 15 μL. The incubation period was 10 min at room temperature. O_2-treated Kp2(UN1041) was exposed to air for 3 h, on ice, and then made anaerobic again.

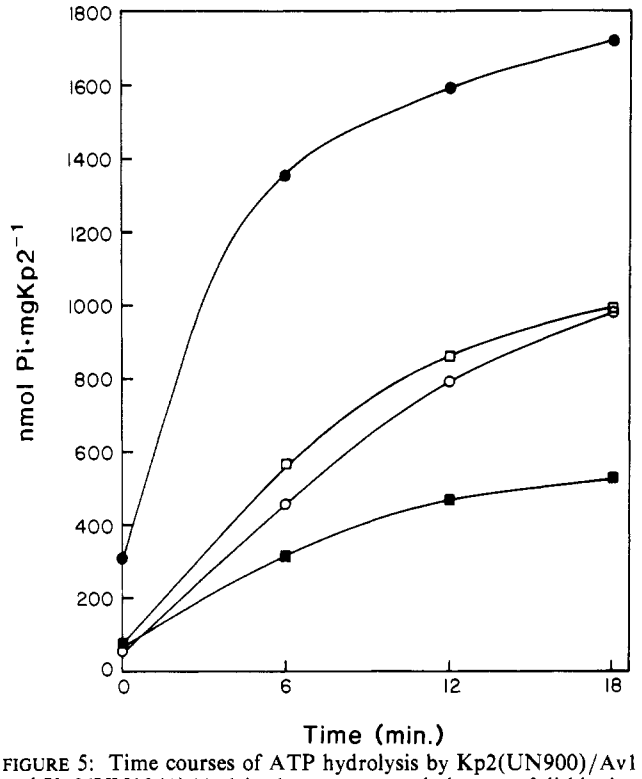

FIGURE 5: Time courses of ATP hydrolysis by Kp2(UN900)/Av1 and Kp2(UN1041)/Av1 in the presence and absence of dithionite. Reactions contained 4 mM [γ-^{32}P]ATP, 5 mM $MgCl_2$, 25 μg of oxidized Kp2, and 33 μg of Av1 in a total volume 20 μL. Reactions lacking Av1 were used to determine background radioactivity. (○) Kp2(UN900); (●) Kp2(UN900) with 10 mM sodium dithionite; (□) Kp2(UN1041); (■) Kp2(UN1041) with 10 mM sodium dithionite.

the interaction of Kp2(UN1041) with a saturable site on Av1. The control incubations with Kp2(UN1041) that had been exposed to air (and then made anaerobic again) demonstrate that the ATP hydrolysis seen in the presence of native Kp2(UN1041) and Av1 is not catalyzed by contaminating activities.

In the experiment described above, the amount of dithionite present was far below stoichiometric with the amount of ATP hydrolyzed. Reductant-independent ATP hydrolysis has been reported for several nitrogenases, including Kp2/Kp1 (Bui & Mortenson, 1969; Iman & Eady, 1980; Cordewener et al., 1987). In order to rigorously assess the effect of reductant on ATP hydrolysis by Kp2(UN1041), we examined the rates of ATP hydrolysis by Kp2/Av1 using Kp2 proteins which had previously been oxidized with Indigo Carmine and Av1 which had been desalted to remove dithionite. In Figure 5, it can

Table IV: Effect of Excess Kp2(UN1041) and Excess ADP-Ribosylated Kp2 on Kp2/Kp1 Activity[a]

addition	nmol of ethylene min^{-1} mg^{-1} [b]	addition	nmol of ethylene min^{-1} mg^{-1} [b]
none	645	110 μg of Kp2–ADPR	670[c]
100 μg of Kp2(UN1041)	708	220 μg of Kp2–ADPR	559[c]
250 μg of Kp2(UN1041)	708	332 μg of Kp2–ADPR	779[c]
500 μg of Kp2(UN1041)	670		

[a] Acetylene reduction assays contained 5 μg of Kp2(UN900) and 20 μg of Kp1 in a total volume of 0.5 mL. The assay period was 20 min. [b] Specific activities are calculated for 5 μg of Kp2(UN900). [c] Ethylene formed by residual non-ADP-ribosylated Kp2 added with Kp2–ADPR was subtracted from the total ethylene formed in an assay; this background level ranged from 28 to 42% of the total ethylene formed.

Table V: ATP Hydrolysis by ADP-Ribosylated Kp2 in the Presence of Dinitrogenase[a]

addition to preincubation	addition to ATP hydrolysis assay	nmol of P_i formed
DRAT	40 μg of Av1	6.6
NAD	40 μg of Av1	6.0
NAD	40 μg of Av1, 20 mM dithionite	23.3
DRAT, NAD	40 μg of Av1	2.7
DRAT, NAD	40 μg of Av1, 20 mM dithionite	3.1

[a] Kp2(UN900) was preincubated for 90 min as described for preparation of ADP-ribosylated Kp2. Ten micrograms of ADP-ribosylated Kp2 was added to ATP hydrolysis reactions containing 5 mM [γ-^{32}P]ATP and 6.25 mM $MgCl_2$ in a total volume of 10 μL. The reactions were incubated for 10 min at room temperature. ATP hydrolysis was dependent on the presence of Av1; reactions lacking Av1 were used to calculate background radioactivity.

be seen that the rates of Av1-dependent ATP hydrolysis by oxidized Kp2(UN900) and Kp2(UN1041) are similar. Addition of excess dithionite to the Kp2(UN900) incubation results in a severalfold increase in the ATP hydrolysis rate; this is expected since electron transfer to dinitrogenase is known to require ATP hydrolysis (Kennedy et al., 1969). In contrast, addition of reductant to the Kp2(UN1041) reaction caused a 2-fold decrease in the rate of ATP hydrolysis. This experiment strongly suggests that ATP hydrolysis by Kp2(UN1041)/Av1 occurs without electron transfer, even when reductant is present.

The lack of substrate reduction activity in Kp2(UN1041)/Kp1 incubations indicates that electrons are not being transferred to dinitrogenase by the mutant protein, yet the fact that it hydrolyzes ATP in the presence of Av1 suggests that it interacts with dinitrogenase in some way. If Kp2(UN1041) forms a nitrogenase complex with Kp1, but then is unable to transfer electrons, it should be possible to inhibit the wild-type nitrogenase reaction with the mutant protein. We incubated a dilute mixture of Kp2(UN900) and Kp1, at a molar ratio of 1, in acetylene reduction reactions containing various amounts of Kp2(UN1041) (Table IV). Surprisingly, there was no inhibition of acetylene reduction activity even with a 100-fold molar excess of Kp2(UN1041). This result prompted us to examine the ability of ADP-ribosylated Kp2 to hydrolyze ATP, since our previous experiments suggested that ADP-ribosylated dinitrogenase reductase does not form a complex with dinitrogenase (Murrell et al., 1988). Kp2 which was previously treated with DRAT and NAD was still able to hydrolyze ATP in the presence of Av1, albeit at a reduced rate (Table V). Addition of dithionite to the ATP hydrolysis incubations did not cause a significant increase in the amount of ATP hydrolyzed by the ADP-ribosylated Kp2;

thus, the hydrolysis is not catalyzed by residual non-ADP-ribosylated Kp2. Samples of Kp2 from the DRAT preincubations were also analyzed by SDS–PAGE in order to confirm that the protein was ADP-ribosylated (data now shown).

Since the evidence that ADP-ribosylated Kp2 does not form a nitrogenase complex with Kp1 is indirect (i.e., by analogy with the Cp2/Av1 system), we tested the ability of ADP-ribosylated Kp2 to inhibit binding of unmodified Kp2 to Kp1. As was the case for Kp2(UN1041), there was no inhibition of acetylene reduction activity in dilute, equimolar Kp1/Kp2 mixtures even at a 66-fold excess of ADP-ribosylated Kp2 (Table IV).

Discussion

Dinitrogenase reductase from *K. pneumoniae* strain UN1041 has a histidine residue substituted for arginine-101. Since arginine-101 is possibly at the interface of the nitrogenase complex, the mutant protein was purified and characterized in order to gain some insight into the role of the DRAT-target arginine in the interaction of dinitrogenase reductase with dinitrogenase.

The purified Kp2(UN1041) appears to be native by several criteria: (1) it migrates as a dimer on a gel filtration column; (2) it contains a functional 4Fe–4S cluster; (3) it binds MgATP and undergoes an associated conformational change and; (4) when oxidized, it supports dinitrogenase-dependent ATP hydrolysis uncoupled from electron transfer at a rate comparable to the wild-type protein. Thus, the differences we have reported between Kp2(UN900) and Kp2(UN1041) are probably not due to an inherent, overall instability of the mutant protein.

According to the current model for nitrogenase function, dinitrogenase reductase acts simply as a reductant for dinitrogenase; it has no known role in the reduction of nitrogenase substrates (Hageman & Burris, 1978). Since Kp2(UN1041) has a redox-active 4Fe–4S cluster, its inability to support any proton or acetylene reduction in the presence of Kp1 can be interpreted as an inability of the reduced protein to transfer electrons to dinitrogenase. The replacement of arginine-101 with histidine could prevent formation of a normal nitrogenase complex with Kp1, or it could prevent electron transfer once the complex has formed. For the purposes of this discussion, "nitrogenase complex" will refer only to the complex of dinitrogenase reductase and dinitrogenase which forms immediately prior to electron transfer.

The ATP hydrolysis experiments presented here suggest that Kp2(UN1041) does interact with dinitrogenase in some way and that, at least for the oxidized form of the protein, the interaction has the same functional consequences that it has with wild-type Kp2. However, it is not known whether the the interaction between dinitrogenase reductase and dinitrogenase during reductant-independent ATP hydrolysis is the same as that during electron transfer between the two proteins; our data suggest that it is not. We have shown that both Kp2(UN1041) and ADP-ribosylated Kp2(UN900), neither of which appears to form a nitrogenase complex with dinitrogenase, are able to hydrolyze ATP in the presence of Av1. The implication of these results is that dinitrogenase reductase interacts with dinitrogenase in more than one way and that the nitrogenase complex which forms during electron transfer is different than the association of the two proteins during reductant-independent ATP hydrolysis. This hypothesis does not require that there be two different binding sites for dinitrogenase reductase on the dinitrogenase protein; it is possible that different forms of dinitrogenase reductase interact differently with the same site on dinitrogenase. Our data suggest that the substitution of histidine for arginine-101, like the ADP-ribosylation at this site, disrupts specifically that interaction between dinitrogenase reductase and dinitrogenase which occurs during electron transfer.

ATP hydrolysis is not the only function of dinitrogenase reductase which can occur independently of electron transfer to dinitrogenase. The synthesis of the dinitrogenase Fe–Mo cofactor requires dinitrogenase reductase (Filler et al., 1986; Robinson et al., 1987); both Kp2(UN1041) and ADP-ribosylated Kp2 support FeMo-co biosynthesis in vitro (J. Imperial, unpublished data) and, in the case of Kp2(UN1041), in vivo (Table I). Thus, our data are consistent with the hypothesis that arginine-101 interacts with dinitrogenase during electron transfer, but it is clear that this amino acid is not as critical for other functions of dinitrogenase reductase.

It is likely that a bulky ADP-ribose moiety bound to arginine-101 of dinitrogenase reductase sterically hinders the formation of a nitrogenase complex; this mechanism of inhibition is less likely to be the case for the more subtle alteration of arginine to histidine in Kp2(UN1041). Our results with the nucleotide-dependent Fe^{2+} release from Kp2(UN1041) suggest an alternative explanation. The single 4Fe–4S cluster of dinitrogenase reductase is believed to reside between the two protein subunits (Gillum et al., 1977; Hausinger & Howard, 1983). The binding of MgATP to dinitrogenase reductase causes a transition to a more open conformation, resulting in increased exposure of the metal center and its cysteine ligands (Walker & Mortenson, 1973; Hausinger & Howard, 1983). The slower rate of MgATP-dependent Fe^{2+} release by Kp2(UN1041) relative to wild-type Kp2 suggests that substitution of histidine for arginine at position 101 causes a decreased accessibility of the 4Fe–4S cluster to chelators, possibly by partial impedance of the MgATP-dependent transition to an open conformation. Thus, it may be the inability of Kp2(UN1041)–MgATP to enter a fully open conformation that prevents the formation of a nitrogenase complex capable of electron transfer.

In summary, we have purified a mutant dinitrogenase reductase which appears by some structural and functional parameters to be similar to the wild-type protein, but which is incompetent for electron transfer to dinitrogenase. Our results clearly demonstrate the importance of arginine-101 to dinitrogenase reductase function and are consistent with the hypothesis that this amino interacts with dinitrogenase during electron transfer. The fact that Kp2(UN1041) and ADP-ribosylated Kp2(UN900) both undergo one type of functional interaction with dinitrogenase, but that neither of the modified proteins appears to form a nitrogenase complex has led us to propose that there are two different types of interactions between dinitrogenase reductase and dinitrogenase. We have suggested that the nucleotide-dependent transition of dinitrogenase reductase to an open conformation might be a prerequisite for electron transfer to dinitrogenase but that such a conformational change is not as critical for the interaction between dinitrogenase reductase and dinitrogenase which occurs during reductant-independent ATP hydrolysis.

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Registry No. Arg, 74-79-3; His, 71-00-1; nitrogenase, 9013-04-1.

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