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Removal of an adenine-like molecule during activation of dinitrogenase reductase from *Rhodospirillum rubrum*

(ATP binding/phosphatase and diesterase action)

PAUL W. LUDDEN* AND R. H. BURRIS

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706

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ABSTRACT **During the activation of the inactive dinitrogenase reductase from *Rhodospirillum rubrum*, an adenine-like molecule is lost and phosphate is found on both active and inactive forms of the protein. ATP and divalent metals are required for activation of the reduced protein, but ATP is not required for activation of phenazine methosulfate-oxidized dinitrogenase reductase. Snake venom diesterase and spleen diesterase have no effect on the inactive protein; alkaline phosphatase removes phosphate from the activated protein but not from the inactive protein. ATP binds to both active and inactive forms of the protein.**

Dinitrogenase reductase (Rr2) from *Rhodospirillum rubrum* is inactive as isolated (1) and has been shown to have two pentose, two phosphate, and two adenine-like molecules (Ad) attached per 60,000 molecular weight dimer (2). The role of these groups in activation is discussed in this paper.

Other enzymes have been shown to have ribose, phosphate, and adenine covalently attached. The first of these was glutamine synthetase (GS) from *Escherichia coli;* it has up to 12 AMP molecules per dodecamer (3). The adenylylated form of the enzyme is much less active in the biosynthesis of glutamine than is the unadenylylated form. Cleavage of the bond between a tyrosine residue on the protein and the phosphate of AMP by a specific enzyme (ATase) isolated from *E. coli* or by snake venom diesterase (SVD) activates the enzyme. The *E. coli* enzyme can also adenylylate GS with ATP as an AMP donor.

The RNA polymerase of *E. coli* has its template specificity altered by ADP-ribosylation (4). The ADP-ribose donor is NAD^+, and linkage to the protein is through arginine-ribose. ADP-ribose is susceptible to hydrolysis either by SVD or by a glycohydrolase from chromatin material (5), but the two enzymes cleave the ADP-ribose in different places. The adenine/ribose/phosphate ratio is 1:2:2 for RNA polymerase and 1:1:1 for the adenylylated GS.

The dinitrogenase reductases from other organisms do not appear to have the pentose-, phosphate-, and Ad-containing group (2), and they do not require activation. In this paper, evidence is presented to show that the Ad, but not the phosphate group, is removed upon activation. The role of ATP and metals in activation also is established.

MATERIALS AND METHODS

Enzymes. The Rr1 and Rr2 of *R. rubrum* and the activating factor (AF) were purified as described (2, 6). Alkaline phosphatase from *E. coli* was obtained from Sigma. This preparation (type III-R) was essentially free from DNase and RNase activities and had a specific activity of 31 μmol of *p*-nitrophenyl phosphate hydrolyzed per min per mg of protein. The stock solution of phosphatase was diluted 1:60 before use, and 50 μl of the diluted solution was used per assay (0.07 unit per assay). SVD type II also was obtained from Sigma; 1.6 units of the enzyme was dissolved in 2 ml of buffer and 50 μl was used per assay (0.04 units per assay). Because of the lability of this preparation, its activity was checked before it was used on *R. rubrum* Rr2 by measuring its rate of hydrolysis of bis-*p*-nitrophenyl phosphate. The SVD used was low in 5′-nucleotidase activity, inorganic pyrophosphatase activity, and phosphatase activity but had considerable nucleotide pyrophosphatase activity. Spleen diesterase (SpD) from Sigma was dissolved in buffer so that 50 μl contained 0.3 unit; 50 μl was used in each assay. It had considerable phosphatase activity.

Enzymatic Treatment of Rr2. Ten to 20 nmol of Rr2 was treated with enzymes (SVD, SpD, alkaline phosphatase) and AF in the presence of ATP, metal ions, and other compounds. The treatments were carried out anaerobically (unless otherwise noted) in the presence of 1 mM dithionite in 5-ml stoppered serum vials. After incubation for 3–4 hr, the sample, or part of the sample, was transferred to a test tube containing 5 ml of 5% trichloroacetic acid for precipitation of protein and removal of unbound parts of the modifying group. When the entire sample was used, the vial was washed with 1 ml of 0.5% NaOH, and this was added to the trichloroacetic acid. Precipitates to be assayed for Ad were washed in 5 ml of 100 mM acetic acid; this removed interfering trichloroacetic acid. Samples to be assayed for phosphate were digested: 2 ml of 2 M HCl was added to the precipitated samples which then were boiled to dryness; 1 ml of 2 M HCl and 20 μl of 30% H_2O_2 were added, and again the solution was boiled to dryness. Five sequential additions of 100 μl of H_2O_2 with heating to dryness between the additions completed the hydrolysis. Products from each treatment were assayed in duplicate.

The effect of different enzymes on the activity of the Rr2 was tested. Samples taken from the mixtures described above were assayed for their acetylene reduction activity.

Assays. The orcinol assay (7) was used for pentose; phosphate was measured with Chen's modification of the molybdate assay (8). Adenine was measured by its fluorescence after treatment with glyoxal hydrate (9). Assays for these compounds have been described in detail (2). Protein was precipitated with 5% trichloroacetic acid and then estimated by the microbiuret method. Nitrogenase activity was measured by the acetylene

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Abbreviations: GS, glutamine synthetase; SVD, snake venom diesterase; SpD, spleen diesterase; EPR, electron paramagnetic resonance; Ado-*PP*[NH]*P*, adenosine 5′-β,γ-imidotriphosphate; BPS, bathophenanthroline disulfonate. The MoFe proteins from *Rhodospirillum rubrum* and *Clostridium pasteurianum* (molybdoferredoxin, component I) are called dinitrogenase, Rrl or Cpl, and the Fe proteins (azoferredoxin, component II) are called dinitrogenase reductase, Rr2 or Cp2 (modified from ref. 21). AF refers to activating factor and Ad refers to the adenine-like moiety found on the inactive form of Rr2.

* Present address: Department of Biochemistry, University of California, Riverside, CA 92521.

reduction technique (10). Walker and Mortenson's assay (11) for release of Fe-S centers from Rr2 was performed as modified by Ljones and Burris (12).

Preparation of Activated Rr2. Purified Rr2 was activated in a mixture containing 5 mM ATP, 25 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.1 mg of creatine phosphokinase, and 20 mM creatine phosphate. Ten to 20 mg of Rr2 and 3–4 ml (protein not determined) of purified AF were added and the mixture was incubated anaerobically at room temperature for 1 hr. It then was put on a 0.6 × 5 cm anaerobic DEAE-cellulose column. AF and small molecules were washed away with 150 mM NaCl/50 mM *N*-tris(hydroxymethyl)methylglycine (Tricine)/1 mM $MgCl_2$ [Tricine was used instead of Tris because it binds Mn^{+2} but not Mg^{2+}; Mn^{2+} interferes with evaluation of the protein by electron paramagnetic resonance (EPR)]. The Rr2 was eluted from the column with 400 mM NaCl in 50 mM Tris acetate, pH 7.6. Rr2 prepared in this manner had a specific activity of 600–1000 nmol of acetylene reduced per min per mg of protein, and it did not require AF or Mn^{2+} to exhibit high activity. These preparations, however, were not completely active because addition of AF and Mn^{2+} increased activity 10–20%. It has not been possible to separate active from inactive protein.

UV Spectra. UV spectra of proteins and other compounds were recorded with a Cary 14 spectrophotometer. Proteins were precipitated with 1% perchloric acid to remove dithionite and Fe-S centers before recording the spectra.

Thin-Layer Plates. Polyethyleneimine plates from Brinkman were washed and run as described by Randerath and Randerath (13). Solvents were 1.6 M LiCl in water and 1.6 M LiCl plus 0.17 M acetic acid in water. Phosphate-containing compounds were localized on the plates with the molybdate reagents. A thin coat of 1 M HNO_3 containing 0.4% $(NH_4)_2MoO_4$ was sprayed onto the plate with an atomizer; the plates were heated to dryness under a 250-W heat lamp and then were sprayed with a solution of 10% ascorbic acid and heated again. Five nanomoles of phosphate or phosphate-containing compounds gave easily detected blue spots.

Reagents. AMP, ATP, adenosine 5′-β,γ-imidotriphosphate (Ado*PP*[NH]*P*), creatine kinase, bathophenanthroline disulfonate (BPS), and phenazine methosulfate were obtained from Sigma. Phosphoribosyl pyrophosphate was obtained from P-L Biochemicals. Dithionite was from Baker, and creatine phosphate was from Pierce.

RESULTS

SVD has been useful in the study of GS because it removes AMP and activates the enzyme; it also removes ADP-ribose from other proteins. However, treatment with SVD caused minimal changes in the groups associated with Rr2 and did not activate the protein in the presence or absence of ATP. Although reducing agents may inhibit the activity of SVD (14), we found it to be capable of hydrolyzing bis-*p*-nitrophenyl phosphate under the conditions of our assay (i.e., anaerobically in the presence of dithionite). SpD, which unlike SVD requires a free 5′-hydroxyl group on ribose for activity, did not activate Rr2. SpD has not been reported to activate any modified protein, but the ineffectiveness of SVD prompted us to try SpD. Although alkaline phosphatase does not affect inactive Rr2, it removes up to 50% of the phosphate from activated Rr2. This 50% may represent the percentage of the Rr2 that has been activated. The maximal specific activity we have observed for Rr2 is 1200 compared to 980–3100 for other dinitrogenase reductases.

There are several possible explanations for the fact that phosphate on the inactive protein is not accessible to alkaline phosphatase. The phosphate may be attached to the protein as a phosphomonoester in such a way that alkaline phosphatase cannot attack it. The phosphate on the protein may occur as a phosphodiester, or the two phosphates per nitrogenase reductase may be joined in a pyrophosphate linkage.

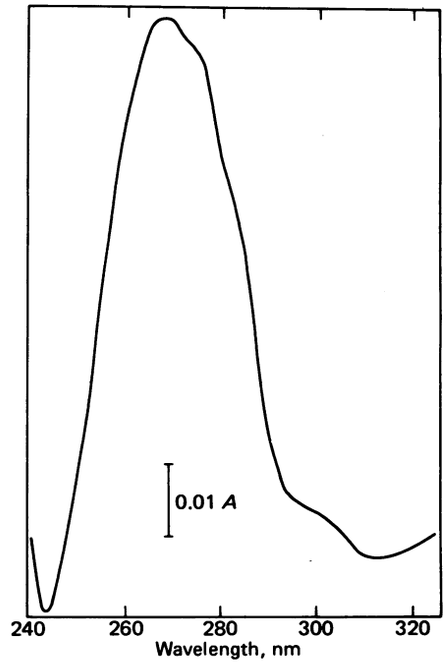

FIG. 1. Difference spectrum, inactive Rr2 minus active Rr2.

Fig. 1 shows the difference spectrum for inactive minus active Rr2. Each protein was precipitated in the same way to remove dithionite and Fe-S centers, and the inactive protein was not exposed to ATP at any time, so the difference cannot be attributed to incomplete removal of ATP. The peak at 268 nm corresponds to the extra peak exhibited in the UV spectrum of Rr2 (2, 6). Quantitation of the peak yields ΔA_{268} of 3.5 at a protein concentration of 1 mM. This probably represents a lower limit for the ϵmM of any molecule removed from the Rr2 because activation is not complete.

Table 1 shows the effect of AF on the phosphate and Ad content of Rr2. Ad leaves the protein upon activation, whereas phosphate remains attached.

Fig. 2 shows the time course for activation of the Rr2 and for loss of Ad. As Ad is lost, activity increases; the relationship appears to be causal.

When inactive Rr2 was hydrolyzed in 1 M HCl for 1 hr at 100°C, a UV-absorbing phosphate-containing soluble material was released. Although the solution was contaminated with tyrosine, it was greatly enriched in the compound that absorbs at 268 nm (Fig. 3). When this solution was run on polyethyleneimine thin-layer plates with 1.6 M aqueous LiCl, all detectable fluorescent material remained at the origin. The fluorescence spectrum of the original hydrolysate resembled the

Table 1. Effect of AF on the phosphate and Ad content of Rr2

	nmol/nmol Rr2			
	Inactive	Active	Δ	%
Phosphate	2.1	1.93	0.17	8.1
Ad	1.94	1.06	0.88	45.3

Activation was carried out for 3 hr before measurement of the changes in phosphate and Ad content. All measurements were done with a single batch of protein, and the values shown represent the average of duplicate samples.

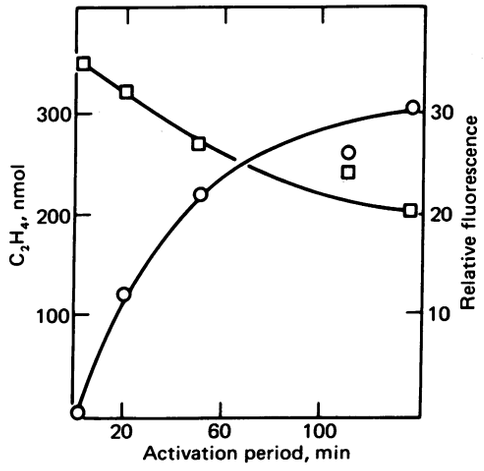

FIG. 2. Loss of Ad during activation. Samples were taken from an activation mixture containing Rr2, AF, ATP, Mg^{2+}, and Mn^{2+}. Activity of Rr2 (O) is shown as nmol of C_2H_4 formed per 5 min, the amount of Ad remaining on the protein (□) is shown by relative fluorescence.

spectrum of tyrosine, so apparently the tyrosine in the sample remained at the origin. The Ad showed no fluorescence before treatment with glyoxal hydrate/acetic acid. The samples used on the thin-layer plates had not been treated with glyoxal hydrate. Staining for phosphate revealed a spot just above the origin; AMP (R_F, 0.5) ran far ahead of this spot. When the Rr2 hydrolysate was treated with alkaline phosphatase, the spot just above the origin disappeared and a new spot corresponding to free phosphate was detected near the front. When 100 mM acetic acid was added to the LiCl solvent, the phosphate-containing compound ran with an R_F of $\approx$0.5.

Role of ATP and Metals in Activation. ATP was required for activation of reduced Rr2. AMP, ADP, Ado*PP*[NH]*P*, GTP, or UTP would not substitute for ATP; active dinitrogenase reductases have a specificity for ATP as a substrate. The concentration of metal ions required for optimal rates of activation of the Rr2 (1) are in excess of the molar ATP concentration required, and this indicates that metals have two roles in activation: to complex with ATP and to bind at some other site on the AF or Rr2.

ATP binds to both active and inactive Rr2. This was demonstrated with Walker and Mortenson's (11) assay for ATP-dependent exposure of Fe-S centers to Fe^{2+} chelators. Fe^{2+} chelation is revealed by an increase in absorbance at 535 nm as a Fe^{2+}–BPS complex forms. Active and inactive Rr2 released its Fe-S centers at essentially the same rates in assay mixtures (1 ml) containing 2.7 nmol of protein, 1 mM BPS, and 1 mM MgATP. The rates were substantially slower than for Cp2 under similar conditions (12), and the background rate in the absence of ATP was slower than that for Cp2. The reactions in the presence of ATP were allowed to go to completion, and 3.5–4.8 atoms of Fe^{2+} were released per molecule of Rr2. NAD^+, an inhibitor of activation, did not induce the opening of the Rr2 nor did it inhibit the ATP-dependent release of Fe^{2+} (Fig. 4). These results are in contrast to those recently reported by Carithers *et al.* (15) who found no effect of ATP in the release of Fe^{2+} from partially purified Rr2. It should be noted that α,α-dipyridyl has an ϵ mM of 8.4 at 520 nm. Less than 16 nmol of Rr2 in a 2.5-ml assay (6.4 nmol/ml) was used in these experiments and 6.4 nmol of Rr2 should contain 25 nmol of Fe. Calculations show that the maximum A_{520} observed should be 0.21 (based on an ϵ mM of 8.4 for α,α-dipyridyl) if the protein were pure. Because an A_{520} of >0.5 is reported, we suggest that they are observing Fe other than that in the Rr2.

Very little AF-dependent ATP hydrolysis occurred during activation. In one experiment, 4 nmol of ATP was hydrolyzed during the activation of 37 nmol of Rr2. We were unable to detect pyrophosphate as a product of the activation reaction. ATP hydrolysis does not appear to be linked to activation.

When Rr2 was exposed to air, the Ad became susceptible to removal by AF in the absence of ATP (Table 2), but metal ions still were required. Alkaline phosphatase still could not remove the phosphate from O_2-inactivated Rr2 (Table 3). Note that

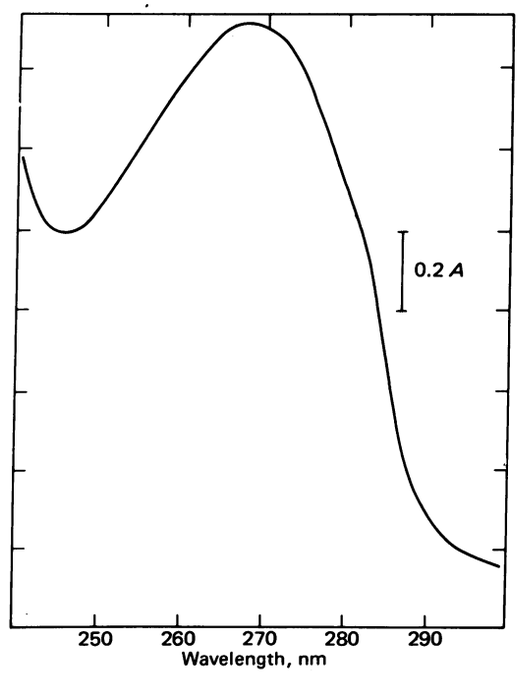

FIG. 3. UV spectrum of 1 M HCl hydrolysate of inactive Rr2.

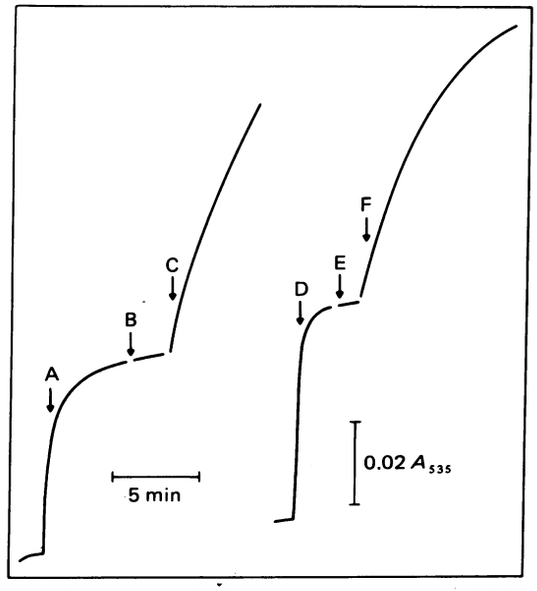

FIG. 4. Exposure of Fe-S centers to the chelator BPS in the presence of ATP. The formation of the Fe^{2+}–BPS complex was measured as an increase in absorbance at 535 nm. The initial ΔA on addition of Rr2 is from chelation of free Fe in the protein solution. Total volume, 1 ml. Additions: A, 2.78 nmol of inactive Rr2; B, 1 μmol of NAD^+; C, 1 μmol of ATP; D, 2.7 nmol of active Rr2; E, 1 μmol of NAD^+; F, 1 μmol of ATP.

Table 2. Removal of Ad from O_2-denatured Rr2 (10 nmol) by AF

AF, ml	Mg, mM	Mn, mM	Ad remaining on protein, nmol
0	0	0	13.9
0	0	0	10.5
0.1	0	0	10.4
0.1	0	0	10.4
0.1	25	0.5	6.5
0.1	25	0.5	6.5

After aerobic treatment for 20 min, samples were made anaerobic and AF was added. Samples were treated for 3 hr and then precipitated for Ad assay.

phosphate was removed from O_2-treated Rr2 in the presence of AF only; the reason for this is not clear.

The fact that Ad could be removed from O_2-inactivated Rr2 in the absence of ATP suggested that an attempt be made to activate oxidized Rr2 in the absence of ATP. Rr2 was oxidized with 1 mM phenazine methosulfate under O_2-free conditions and, after treatment as described in Table 4, 20-μl aliquots were assayed for acetylene reduction in the presence and absence of AF. The acetylene assays in the presence of AF showed that the Rr2 was not destroyed during the incubation, whereas the acetylene assays in the absence of AF showed whether or not the Rr2 was activated during the incubation. Although a trace of AF was carried over when the 20-μl aliquot was diluted into the 0.5-ml acetylene reduction assay mixture, there was no Mn^{2+} in the assays lacking AF, so the observed activity is clearly due to Rr2 that has been activated during incubation in the absence of ATP but in the presence of phenazine methosulfate.

DISCUSSION

Fig. 5 presents a working hypothesis for activation. MgATP or oxidation of Rr2 is portrayed as causing a change in position of the Ad. The exposed Ads are removed from the protein by AF in a reaction dependent on divalent metal. The activated Rr2 can be modified further by removal of phosphates with alkaline phosphatase. The removal of the phosphates does not change the activity of the enzyme. The position of pentose is not entirely clear at this time because of the inadequacy of ribose assays in the presence of protein. Our available data indicate that pentose, like phosphate, is not removed during activation of Rr2. Furthermore, we have no data at present to indicate whether the pentose moiety present is ribose or some other 5-carbon sugar (the orcinol reaction does not distinguish among pentoses). It does seem clear from spectral data presented here (Figs. 1 and 3) and previously (2) that the compound bound to Rr2 is not AMP- or ADP-ribose. The migration of the compound on polyethyleneimine thin-layer plates compared to the migration of AMP agrees with this conclusion as does the inability of phosphodiesterase to cleave the compound from the enzyme.

Table 3. Accessibility of phosphate on O_2-denatured Rr2 (6.6 nmol)

AF, ml	Alkaline phosphatase, units	Phosphate remaining, nmol
0	0	10.6
0	0.07	10.6
0.2	0.07	7.2
0.2	0	7.2

After aerobic treatment, the samples were made anaerobic and AF was added. After treatment for 3 hr, the samples were precipitated and digested for phosphate analysis.

Table 4. Activation of oxidized Rr2 in the absence of ATP

Incubation		C_2H_2 assays	
ATP, mM	AF, ml	AF, nmol/25 min	AF, nmol/10 min
0	0.1	20	36
5	0.1	26	22
0	0	0	16

The incubations were for 20 min under H_2 in the presence of 1 mM phenazine methosulfate. The Rr2 used was from the second DEAE column. All incubation mixtures contained 100 μl of Rr2, 25 mM Mg^{2+}, and 0.5 mM Mn^{2+}. C_2H_2 assays with AF contained 25 mM Mg^{2+} and 0.5 mM Mn^{2+}; while assays without AF contained 10 mM Mg^{2+} and 0 Mn^{2+}.

The working model presented in Fig. 5 assumes that all components of the modifying group are linked together. Because Ad is removed during activation, it is regarded as outermost from the protein. It is clear that phosphate is not removed during activation, and available data indicate that pentose also remains bound. Because phosphate becomes labile to alkaline phosphatase after activation, it must be linked to pentose as a phosphomonoester and not as a phosphodiester between the pentose and the protein. Thus, the pentose appears to be a part of the modifying group bound directly to the protein.

Although there is considerable information concerning the activation of Rr2, we have been unable to reverse the activation. This failure may be due to the lack of the proper Ad donor or to the lack of the enzyme required to add Ad to the protein, or both. We have tried to inactivate the active protein with the activation mixture plus various nitrogen compounds (NH_4^+, glutamate, and glutamine). AMP, ADP, ATP, NAD^+, and $NADP^+$ have been tested as adenine donors, and purified AF, crude extract, and crude chromatophores have been tested as inactivators without success.

No link has been established between activation of Rr2 and regulation of the nitrogenase system *in vivo*. *R. rubrum* and *S.*

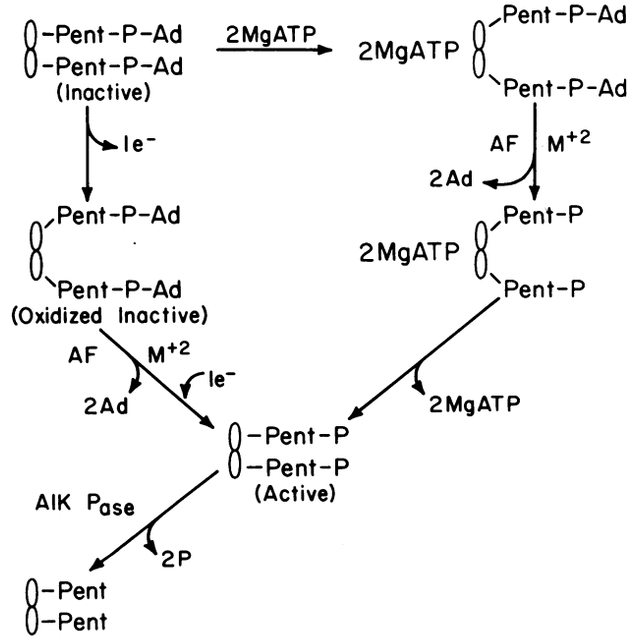

FIG. 5. Working hypothesis for activation of Rr2. Pent, pentose; P, phosphate; Ad, adenine-like moiety; 0, subunit of Rr2.

lipoferum dinitrogenase reductases are different from other dinitrogenase reductases because they are isolated in an inactive state. Only Rr2 has been shown to contain pentose, Ad, and phosphate, and *R. rubrum* is unique among N_2-fixing bacteria in that its nitrogenase system completely loses activity *in vivo* within a few minutes of the addition of ammonia to a culture (16, 17). It is attractive to conclude that the activation system for Rr2 is a part of this ammonia feedback system of regulation. Whole cells of *R. rubrum* exhibit a lag in nitrogenase activity when they are transferred from the dark to light (18). The Rr2 we have isolated may be inactive because the cells were collected in the dark, but we have not discovered a way to collect the cells so that active Rr2 can be isolated directly from them. Carithers *et al.* (15) have recently reported that crude extracts of *R. rubrum* grown on limiting ammonia exhibit nitrogenase activity in the absence of Mn^{2+}. This activity can be presumed to be due to Rr2 in its active state. We have demonstrated that purified Rr2 can be converted to an active form and that AF and Mn^{2+} then are no longer required for activity (1, 2).

The system of activation described here is unlike the systems involving adenylation or ADP-ribosylation of some other proteins because in this system only part of the modifying group is removed upon activation. Also, a preliminary change in the conformation of the modified enzyme then allows activation.

It has recently been suggested by Scherings *et al.* (19) that the AF from *R. rubrum* might function in a system for the protection of Rr2 from O_2, as they observe in *Azotobacter vinelandii*. This is unlikely for several reasons: AF itself is O_2 labile; unlike the *A. vinelandii* protein, purified AF is colorless in solution; AF is recovered in far smaller amounts (on a protein basis) than is Rr2, and a trace amount of AF (again on a protein basis) can activate a large amount of Rr2.

Nordlund *et al.* (20) have concluded that a stable complex of AF and the nitrogenase system is formed which is required for enzyme turnover. This conclusion is based on the saturation that is seen when crude preparations of the nitrogenase system and partially purified AF are titrated against each other. We have attempted similar experiments (unpublished) and have obtained inconclusive results. It is possible to saturate purified Rr2 with AF, but the maximal activity observed depends on the batch of AF used. We attribute this to the presence of O_2-inactivated AF (O_2-inactivated AF inhibits activation) and to the presence of ATPase in less-purified preparations of AF. Thus, saturation experiments should be interpreted with caution, because increasing levels of two substances inhibitory to the nitrogenase system (inactive AF and ATPase) are being added with AF. We have demonstrated (1, 2) that it is possible to separate AF from activated Rr2 on a DEAE-cellulose column and to obtain active Rr2 that shows no lag in activity and that does not require high Mg^{2+} or Mn^{2+} concentrations for activity. Rr2 that has been activated and separated from AF shows only one protein band on polyacrylamide gels, and this is identical to the band for inactive protein. The banding patterns of O_2-denatured active and inactive Rr2 also are identical (2). We have demonstrated one difference between active and inactive Rr2—i.e., the loss of Ad from the inactive protein during activation. We conclude that the role of AF is the removal of the Ad and that the AF is not required for turnover of the enzyme once the Rr2 is activated.

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