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Covalent modification of the iron protein of nitrogenase from *Rhodospirillum rubrum* by adenosine diphosphoribosylation of a specific arginine residue

(photosynthetic bacterium/modified peptide/nitrogen fixation)

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ABSTRACT **Nitrogenase in *Rhodospirillum rubrum* is inactivated *in vivo* by the covalent modification of the Fe protein with a nucleotide. The preparation of two modified peptides derived from proteolytic digestion of the inactive Fe protein is described. The modifying group is shown to be adenosine diphosphoribose, linked through the terminal ribose to a guanidino nitrogen of arginine. The structural features were established by using proton and phosphorus NMR, positive- and negative-ion fast atom bombardment mass spectrometry, and fast atom bombardment/collisionally activated decomposition mass spectrometry. Spectral methods along with chromatographic analysis and sequential degradation established the sequence of the modification site of Fe protein as Gly-Arg(ADR-ribose)-Gly-Val-Ile-Thr. This corresponds to the sequence in the Fe protein from *Azotobacter vinelandii* for amino acid residues 99 to 104.**

Rhodospirillum rubrum is one of a group of microorganisms capable of biological nitrogen fixation. The enzyme complex, nitrogenase, was shown to lose its nitrogen-fixing activity with the addition of ammonia to cell cultures (1–4). Glutamine (3), darkness, oxygen, phenazine methosulfate, and the uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) (4) have also been shown to cause inactivation of nitrogenase activity. This loss of activity can be attributed to a nongenetic regulatory system that covalently modifies the Fe protein, rendering it inactive (5). The modifying group has been shown to consist of ribose, phosphate, and adenine (5–7). The inactive enzyme can be activated *in vitro* by the addition of an activating enzyme isolated from the chromatophore membrane fraction (8, 9). This activation results from the removal of at least part of the modifying group (6, 8–12). The modifying group may also be removed *in vitro* by mild heat treatment, resulting in activation of the enzyme (13).

Previous structural studies of the modifying group have involved the inactive Fe protein (5, 6) or the heat-released form of the modifying group (ΔMG) (7, 13, 14). This paper describes the isolation and characterization of two proteolytically derived modified peptides from Fe protein. Evidence is presented concerning the peptides and the reduced heat-released form of the modifying group (H_2ΔMG) that establishes the modifying group as adenosine diphosphoribose (ADP-Rib). The sequence of the modified peptide and the nature of the linkage to a specific arginine residue is also described. ADP-ribosylation of a number of proteins has been reported (15). Both the cholera toxin-dependent ADP-ribosylation of adenylate cyclase (16, 17) and the ADP-ribosylation of elongation factor in phage T4-infected *Escherichia coli* (18) occur on arginine residues.

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MATERIALS AND METHODS

Growth of *R. rubrum* and Purification of Fe Protein. The conditions for cell growth and collection have been previously described, along with methods for the purification of inactive Fe protein (7).

Preparation and Purification of H_2ΔMG. These procedures have also been described previously (8).

Preparation and Purification of a Modified Hexapeptide from Inactive Fe Protein. Purified Fe protein was treated with DNase and RNase (0.1% each) for 15 min and was chromatographed on a 4.5 × 19 cm Sephadex G-25 column in 100 mM ammonium formate buffer, pH 8.5. This and all subsequent steps were performed aerobically. Fractions containing the Fe protein were collected. Subtilisin (Sigma) was added to the Fe protein solution (1% subtilisin) and proteolysis was allowed to continue for 16 hr.

The modified hexapeptide was separated from the protein digest on an Affi-Gel phenylboronate column (Bio-Rad). The protein digest was applied to a 3-ml (bed volume) column of Affi-Gel 601 equilibrated with 100 mM ammonium formate, pH 8.5. The column was washed with 20 ml of 100 mM ammonium formate, pH 8.5, and the modified peptide was eluted with 100 mM formic acid, pH 2.1. The modified peptide was detected by its absorbance at 260 nm. The peptide was purified by applying it to a Synchropak RP-C8 reversed-phase column (Applied Science Laboratories). Using a Waters HPLC system, with a flow rate of 2.0 ml/min, we chromatographed the modified hexapeptide by using a 2-min isocrat of 0.2% formic acid followed by a 3-min gradient of 0–40% (vol/vol) isopropyl alcohol. The hexapeptide eluted as a sharp peak 3 min after final conditions had been obtained. The samples were then lyophilized to near dryness in a Speed-Vac concentrator (Savant). Peptide was recovered with a yield of 50%.

Preparation and Purification of a Modified Tripeptide. The modified tripeptide was prepared as described for the hexapeptide, with the following differences. Proteolytic digestion was performed with a mixture of subtilisin and Pronase (1% each, proteases from Sigma). Digestion was allowed to proceed for 16 hr. The tripeptide was isolated as described for the hexapeptide, and the crude modified tripeptide was purified by applying it to a Synchropak SAX 300 ion-exchange HPLC column equilibrated with 3 mM ammonium formate buffer, pH 6.5. The column was operated isocratically at 1 ml/min with 3 mM ammonium formate. The tripeptide eluted at about 3.5 min. The collected tripeptide fractions were pooled and lyophilized to dryness. Excess ammonium formate was removed by extraction with HPLC-

Abbreviations: ΔMG, heat-released modifying group; H_2ΔMG, reduced ΔMG; ADP-Rib, adenosine 5′-diphosphoribose; FAB, fast atom bombardment; CAD, collisionally activated decomposition.
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grade methanol. The sample was suspended with a Vortex mixer or sonicated with methanol and centrifuged. The modified tripeptide and hexapeptide are only slightly soluble in methanol, in which the ammonium formate is readily soluble.

Synthesis of Arg(ADP-Rib). Arg(ADP-Rib) was synthesized by the cholera toxin method as described by Moss and Vaughn (17). The product was purified by reversed-phase HPLC on an Alltech C_{18} column. After sample injection the column was washed with 25 mM ammonium acetate, pH 5.0, for 6 min at 1 ml/min. The Arg(ADP-Rib) was then eluted with 20% (vol/vol) methanol in 25 mM ammonium acetate, pH 5.0.

Acid Hydrolysis and Amino Acid Analysis by Two-Dimensional TLC of Dansyl Derivatives. The methods used have been described elsewhere (19). Three to 5 μg of peptide was hydrolyzed in a melting point capillary. After hydrolysis, the samples were treated with dansyl chloride reagent solution. The dansyl derivatives were separated on polyamide chromatography sheets, 5 × 5 cm (Schleicher & Schuell F 1700). The sheets were developed with water/formic acid (200:3, vol/vol) in the first dimension and benzene/acetic acid (9:1, vol/vol) in the second. Dansyl derivatives were visualized by fluorescence under UV light.

Amino Acid Analysis of Modified Peptides. Amino acid analyses were performed by Dan Omilianowski on a Durrum D-550 amino acid analyzer. Samples were hydrolyzed at 105°C for 24 hr in 6 M HCl under reduced pressure.

Sequence Determination by Sequential Degradation of Modified Peptides. The sequence of the hexapeptide was determined by Ron Niece on an Applied Biosystems model 470A gas phase sequencer. Phenylthiohydantoin derivatives of amino acids were determined after Edman degradation by reversed-phase HPLC on an IBM LC 9533 instrument. Ten nanomoles of peptide was analyzed in the sequence determination.

NMR Analysis of Modified Peptides. Samples for NMR analysis were prepared by repeated lyophilization from ^2H_2O (99.99%, Aldrich). Contaminating paramagnetic metals were removed by extraction from the water solution with 8-hydroxyquinoline at 1 mg/ml in chloroform followed by three extractions with chloroform to remove excess hydroxyquinoline.

Proton NMR spectra were obtained on the 470-MHz instrument at the Purdue NMR Center or on a Nicolet 200-MHz instrument. Proton chemical shift assignments were obtained by two-dimensional ^1H–^1H shift correlation spectroscopy at 200 MHz. Phosphorus NMR spectra were recorded on the Nicolet 200-MHz instrument (80.1 MHz for ^{31}P), using 10% (vol/vol) phosphoric acid in ^2H_2O as the chemical shift reference. Samples for ^{31}P NMR contained 2 mM EDTA.

Mass Spectral Analysis. Mass spectra were obtained with the Kratos MS-50 triple analyzer mass spectrometer at the Midwest Center for Mass Spectrometry (University of Nebraska-Lincoln). For negative ion spectra, triethanolamine (Aldrich) was used as the matrix. For positive-ion fast atom bombardment (FAB) spectra, a 5:1 mixture of dithiothreitol/dithioerythritol was used as the matrix.

Quantitation of Modifying Group. The modifying group was quantitated by its absorbance at 260 nm. A mM extinction coefficient of 15.1 was used to determine the concentration. Measurements were made on a Cary 14 spectrophotometer (7).

Borohydride Reduction of ADP-Rib. ADP-Rib in 100 mM ammonium formate, pH 8.5, was treated with a 100-fold excess of $NaBH_4$ at room temperature overnight. Reduced ADP-Rib was purified by chromatography on a boronate column. The reaction had gone to completion, as determined by HPLC analysis.

RESULTS

Modified peptides were prepared by proteolytic digestion of purified, inactive Fe protein, followed by isolation by means of a phenylboronate affinity column, which binds the modifying group. Modified peptide was eluted with dilute formic acid. A modified hexapeptide and a tripeptide were prepared by digestion of Fe protein with subtilisin and a mixture of subtilisin and Pronase, respectively. The peptides are substrates for purified activating enzyme from *R. rubrum* (unpublished results).

Acid hydrolysates of the two modified peptides were treated with dansyl chloride, and the resulting products were separated by two-dimensional polyamide TLC. The hexapeptide was observed to contain Gly, Arg, Val, Ile, and Thr, while the tripeptide contained Gly and Arg. In both cases dansylglycine was the most intense spot. Amino acid analysis confirmed these results and provided the ratios of $Gly_2ArgValIleThr$ for the hexapeptide and Gly_2Arg for the tripeptide. We were concerned that glycine produced by acid hydrolysis of adenine might be interfering with the analysis (20). Controls showed that under our conditions less than 0.1 mol of glycine was produced per mol of adenine, indicating that this was not a major source of interference. Sequential Edman degradation performed on the modified hexapeptide established its sequence as Gly-X-Gly-Val-Ile-Thr, in which X is the modified arginyl residue. This implies that the tripeptide is Gly-X-Gly.

Fig. 1*B* shows the ^{31}P NMR spectrum (proton decoupled) of the modified tripeptide. The spectrum is characteristic of pyrophosphate signals. Fig. 1*A* shows the proton-decoupled ^{31}P NMR spectrum of ADP-Rib. The two phosphates are coupled to one another and exhibit an *AB* quartet type signal. This same pattern is displayed in the spectrum of the modified peptide, giving two phosphate pairs at −11.0 and −11.3 ppm and −11.4 and −11.7 ppm. These sets of signals are in an approximate 40:60 ratio and suggest the presence of two stable forms of the molecule.

Fig. 2 shows the proton NMR spectrum of the modified hexapeptide in ^2H_2O acquired at 470 MHz. Table 1 lists the chemical shift assignments obtained from two-dimensional ^1H–^1H chemical shift correlation spectroscopy. The unassigned signals observed between 7.5 and 9.2 ppm are due

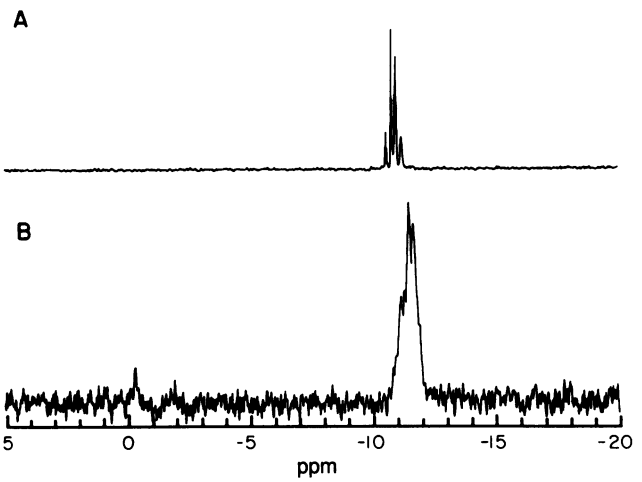

FIG. 1. ^{31}P NMR spectra at 81 MHz. (*A*) Proton-decoupled spectrum of 22 mM ADP-Rib in 50:50 (vol/vol) completely deuterated dimethyl sulfoxide/^2H_2O; 628 scans were acquired at 22°C. (*B*) Proton-decoupled spectrum of 3 mM modified tripeptide from proteolytic digestion of inactive Fe protein in 50:50 deuterated dimethyl sulfoxide/^2H_2O with 2 mM EDTA; 5632 scans were acquired at 22°C. Chemical shifts are relative to an external standard of 10% H_3PO_4 in ^2H_2O.

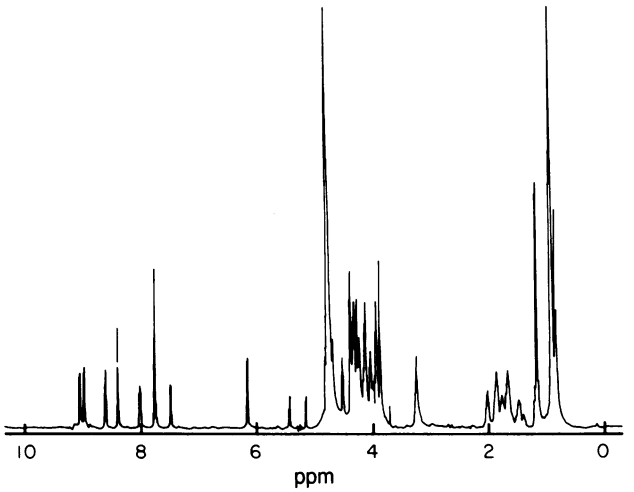

FIG. 2. ^1H NMR spectrum at 470 MHz of 2 mM modified hexapeptide, from proteolytic digestion of inactive Fe protein, in ^2H_2O; 2000 scans were acquired at 22°C. Chemical shifts are relative to an external standard of 0.1% 3-trimethylsilylpropionate in ^2H_2O.

to 8-hydroxyquinoline, which was added to reduce the line-broadening effects of paramagnetic ions in the sample. The signals due to protons of the six amino acid residues were identified by their characteristic chemical shifts (21). The observed coupling in the two-dimensional spectrum confirm these assignments. Signal amplitudes were of the expected relative intensities as determined by integration. The remaining proton signals were assigned to the modifying group and were observed to be similar to the NMR spectrum obtained for Arg (ADP-Rib) (22). The literature evidence suggests that ADP-Rib is covalently linked to the guanidino group of arginine with a specific configuration initially (α), but upon manipulation such as purification by HPLC and preparation for NMR, the anomeric linkage may undergo anomerization to give a mixture of α and β configurations. This anomerization is reflected by a chemical shift difference for the 1′ proton of the terminal ribose linked to the guanidino group and by a chemical shift change for the H-8 proton of the adenine (22). These characteristics are observed in the proton NMR spectrum of the modified hexapeptide (Fig. 2 and Table 1), again suggesting that the modifying group is ADP-Rib. This would also explain the two apparent forms observed in the ^{31}P NMR spectrum. Chemical shift differences for other carbohydrate protons, due to mutarotation, could not be determined due to spectral overlap. Proton NMR of the modified tripeptide (not shown) gave a similar spectrum, without the signals of valine, isoleucine, and threonine.

The positive and negative FAB mass spectra and FAB/collisionally activated decomposition (FAB/CAD) spectra of the modified tripeptide, hexapeptide, and $H_2\Delta MG$ were obtained. The results of high-resolution peak matching for the molecular ions (M + 1 in positive-ion mode) and the CAD spectra have led to the conclusion that the modifying group is ADP-Rib and is bound to the guanidino group of arginine. This confirms the speculations made from the NMR results.

Peak matching for $H_2\Delta MG$ in the positive-ion mode gave a high-resolution mass within 3 ppm of the calculated mass of 562.09516 daltons for the ion $[C_{15}H_{25}N_5O_{14}P_2 + H^+]$, which corresponds to the molecular formula of reduced ADP-Rib, where the site of reduction by borohydride is the aldehyde of the open chain form of the terminal ribose.

Fig. 3 shows a comparison of the negative-ion FAB/CAD spectra of ADP-Rib (M − 1 at m/z 558) (*A*), $H_2\Delta MG$ (M − 1 at m/z 560) (*B*), and borohydride-reduced ADP-Rib (M − 1 at m/z 560) (*C*). The spectra are virtually identical. The primary sites of fragmentation are also shown in Fig. 3. The dominant fragment ions are m/z 346 and 426, which correspond to AMP − 1 and ADP − 1, respectively. Differences in the spectra of ADP-Rib occur in some of the minor fragments, differing by 2 daltons from reduced ADP-Rib and $H_2\Delta MG$, which is consistent with the reduction of the ribose. Ribose phosphate from ADP-Rib gives ions at m/z 292 and 309, whereas the corresponding ions from $H_2\Delta MG$ are m/z 294 and 311 (fragments G and F, respectively).

Positive ion-peak matching for the M + 1 ions of the modified peptides give masses within 0.9 and 0.6 ppm, respectively, of the calculated masses of 830.223549 daltons for $[C_{25}H_{41}N_{11}O_{17}P_2 + H^+]$ and 1143.42371 daltons for $[C_{40}H_{68}N_{14}O_{21}P_2 + H^+]$. These molecular formulas correspond to the peptides Gly-Arg-Gly and Gly-Arg-Gly-Val-Ile-Thr plus ADP-Rib (with the loss of an H_2O molecule). Within the sample of the modified tripeptide a small amount of modified dipeptide was observed [Gly-Arg(ADP-Rib) or Arg(ADP-Rib)-Gly] with M − 1 of m/z 771. Fig. 4 shows the negative-ion FAB/CAD spectra of the modified tripeptide. The essential features of the negative-ion FAB/CAD spectra of the dipeptide and hexapeptide are the same as those shown in Fig. 4 for the tripeptide. In all three peptides the major fragment ion (discounting loss of H_2O) occurs at m/z 426 (L), which corresponds to an ADP fragment. The other major fragments are observed at m/z 346 (M) due to AMP and at m/z 499 (K), which results from cleavage of the ribose ring as is shown in Fig. 4. A key ion in these spectra occurs at m/z 582 (J). This ion is produced by cleavage through the guanidino group, resulting in an imidocarbonylamine group linked to the modifying group. The presence of this ion indicates that ADP-Rib is covalently linked to the arginine guanidino group. Neither NMR nor mass spectrometry indicates conclusively that the modification linkage involves the ribose anomeric site. Indirect evidence, however, does suggest this is the case. While it is possible to reduce ΔMG (7) (and ADP-Rib) with sodium borohydride, reduction does not occur with the modifying group bound to a peptide, suggesting that the anomeric site is involved in the linkage. To test the hypothesis that the modifying group is linked to the Fe protein via a glycosidic bond to a guanidinium nitrogen of arginine, Arg(ADP-Rib) was synthesized and subjected to negative-ion FAB/CAD mass spectral analysis. The result is

Table 1. Assignments of proton NMR signals of the modified hexapeptide

Amino acid residue	Chemical shift, ppm				Adenosyl proton	Chemical shift, ppm	Arginylribose protons	Chemical shift, ppm
	α	β	γ	δ				
Gly-	3.85				A8	8.59, 8.58	R1′α	5.42
-Arg-	4.23	1.84 (CH)	1.62 (CH_2)	3.22 (CH_2)	A2	8.38	R1′β	5.14
-Gly-	3.95, 3.89				A1′	6.14	R2′	4.26
-Val-	4.10	2.00 (CH_2)	0.88 $(CH_3)_2$		A2′	4.72	R3′	4.30
-Ile-	4.33	1.75 (CH)	1.16, 1.46 (CH_2), 0.87 (CH_3)	0.81 (CH_3)	A3′	4.51	R4′	4.14
					A4′	4.36	R5′, R5″	4.02
-Thr	4.13	4.32 (CH)	1.14 (CH_3)		A5′, A5″	4.21		

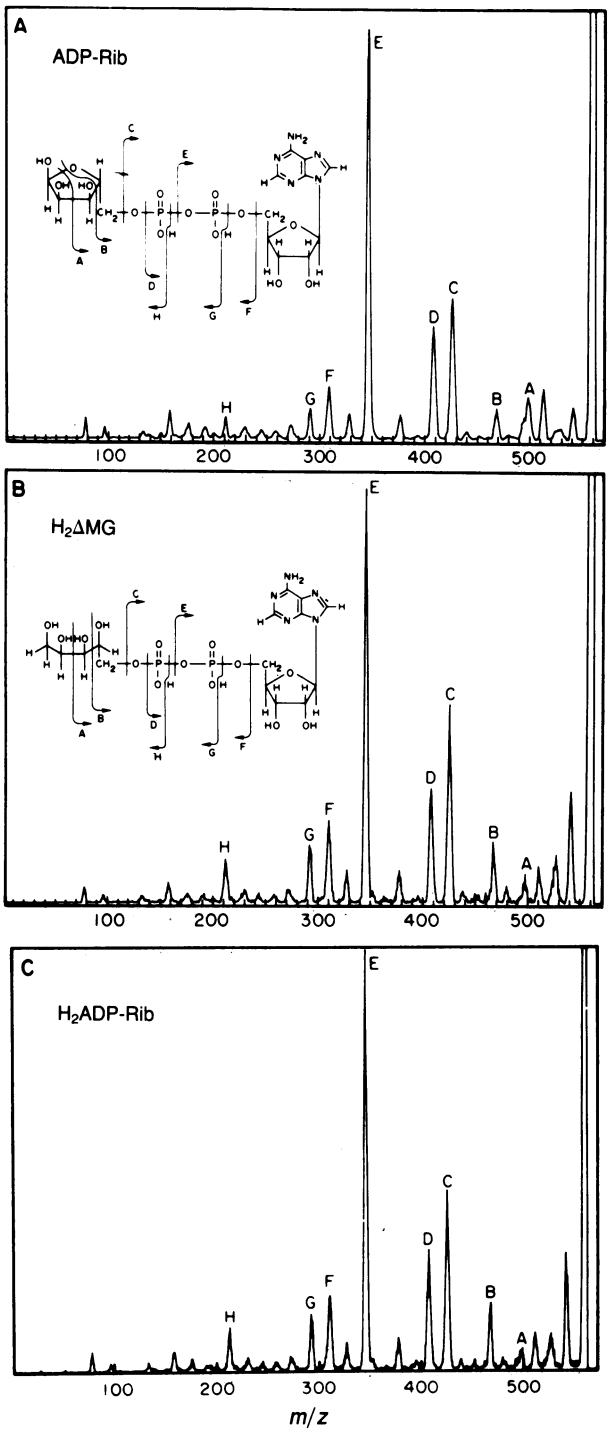

FIG. 3. Comparison of negative-ion FAB/CAD spectra and fragmentation of ADP-Rib (*A*), $H_2\Delta MG$ (*B*), and borohydride-reduced ADP-Rib (*C*).

shown in Fig. 5. The fragment ion observed at m/z 582 supports the hypothesis. Fragment ions are also observed at m/z 346, 426, and 499.

DISCUSSION

Previous structural studies of the covalent modification of the Fe protein of *R. rubrum* have concentrated on ΔMG (7). In this study, both $H_2\Delta MG$ and peptides with the bound nucleotide have been investigated.

Two modified peptides have been obtained by using various degrees of proteolytic digestion. Amino acid analysis

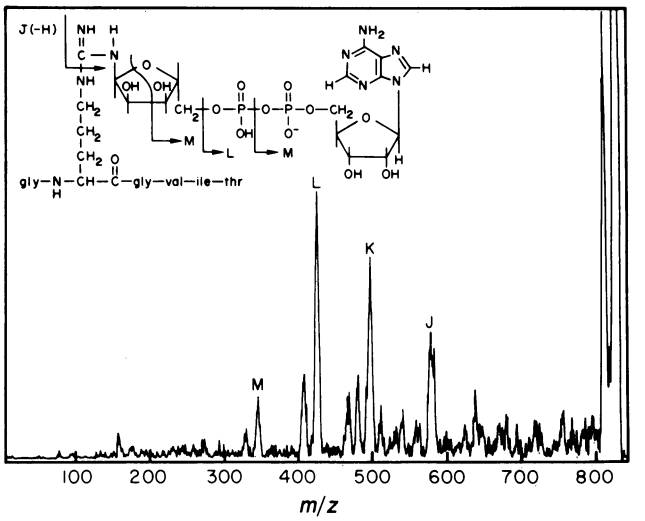

FIG. 4. Negative-ion FAB/CAD spectrum of the modified tripeptide (M − 1 = 828) from inactive Fe protein.

and sequential degradation have established the peptides as Gly-X-Gly and Gly-X-Gly-Val-Ile-Thr, in which X is arginine with ADP-Rib linked through the guanidino group. Comparison of this sequence with the known sequence of the Fe protein from *Azotobacter vinelandii* (23) provides an exact sequence match from residue 99 through residue 104. This region is found to be highly conserved in the Fe proteins of other nitrogen-fixing bacteria. The sequence is completely conserved in the Fe protein of *Klebsiella pneumoniae*, and isoleucine is substituted for valine in the proteins from *Clostridium pasteurianum* and *Anabaena*. It should be noted that the site of modification falls within a region of the protein predicted to possess β-sheet or α-helical structure (23), and the modified arginine is only three amino acids removed from one of the proposed cysteine ligands of the iron–sulfur center (24). The close proximity of the modification site to the ligand of the active center leads to the speculation that the inhibition results either from a physical blocking of the binding site for the MoFe protein or from a conformational change that inhibits the protein–protein interaction. Previously, we have established by ^{31}P NMR and chemical analysis that the modifying group contains two phosphates in a pyrophosphate linkage (7). The present results confirm this and show the pyrophosphate coupling pattern, which could not be ob-

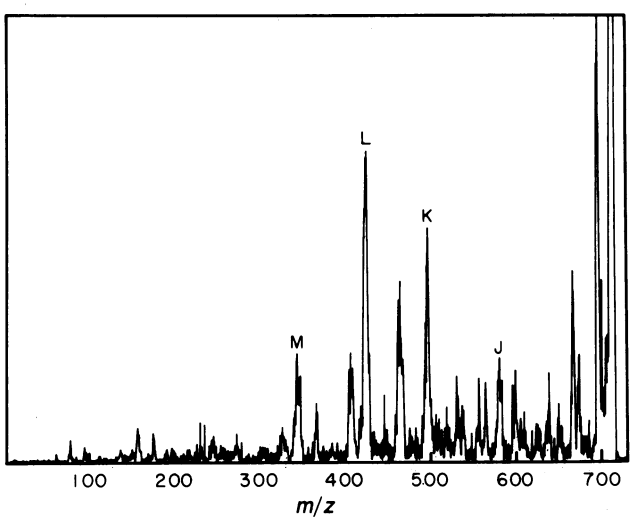

FIG. 5. Negative-ion FAB/CAD spectrum of Arg(ADP-Rib).

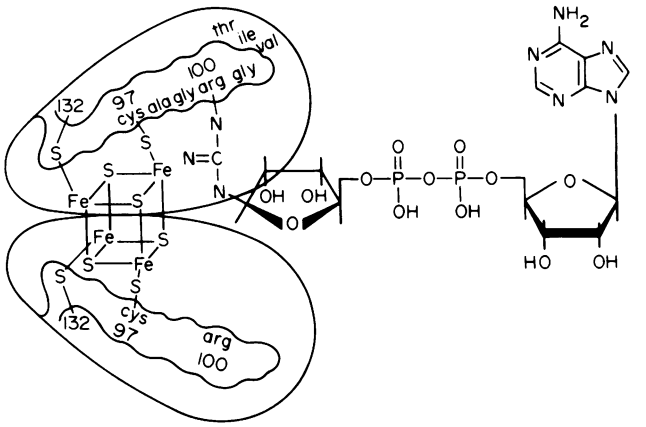

FIG. 6. Model of ADP-ribosylation of Fe protein.

served previously due to line broadening. The proton NMR spectrum of the modified hexapeptide contains the signals expected for the amino acids present and peaks that indicate the presence of an ADP-Rib moiety. The proton and phosphorus NMR spectra suggest the sample to be a mixture of α and β configurations of the ribosyl anomeric carbon. It would be difficult to accept an enzymatic system that would attach ADP-Rib in both configurations; however, a more likely explanation is that anomerization occurs during purification, as has been observed for Arg(ADP-Rib) prepared with cholera toxin (22).

The results of the FAB and FAB/CAD mass spectra provide solid evidence, in conjunction with the NMR results, that the modifying group is ADP-Rib. The major fragments observed for the modified peptides and $H_2\Delta MG$ are all consistent with the ADP-Rib structure. Further, high-resolution analysis using peak matching yields masses that are in good agreement with the calculated masses for $H_2\Delta MG$ and the two modified peptides, in which the modifying group is ADP-Rib. Positive-ion FAB/CAD spectra of $H_2\Delta MG$ and reduced ADP-Rib also confirm this structure (data not shown). The m/z 582 ion observed in the negative-ion CAD spectra of all of the modified peptides and synthetic arg(ADP-Rib) indicates that ADP-Rib is linked to the arginine residue through the guanidino group. Fig. 6 shows our current model of the modification of Fe protein from *R. rubrum*. The β configuration of linkage to arginine is shown; however, we have not established this configuration. Because only one of the two potential sites is modified, it is proposed that the two sites are close enough that modification of one site blocks ADP-ribosylation of the second. This result may have some implications regarding the symmetry of Fe protein subunit association.

NAD is the donor molecule for other ADP-ribosylations (17, 18). The identification of ADP-Rib as the modifying group raises the possibility that NAD may be the donor molecule.

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