*Nature (London) 298*, 534-538.
Ware, C. F., & Kolb, W. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6426-6430.
World Health Organization (1968) *Bull. W.H.O. 39*, 935-936.
World Health Organization (1981) *Eur. J. Immunol. 11*, 668-669.
Yamamoto, K., & Migita, S. (1983) *J. Biol. Chem. 258*, 7887-7889.
Yamamoto, K., Kawashima, T., & Migita, S. (1982) *J. Biol. Chem. 257*, 8573-8576.

# Purification and Properties of the Heat-Released Nucleotide-Modifying Group from the Inactive Iron Protein of Nitrogenase from *Rhodospirillum rubrum*†

Mark R. Pope, Scott A. Murrell, and Paul W. Ludden*
*Department of Biochemistry, College of Agricultural and Life Sciences, and Center for the Study of Nitrogen Fixation, University of Wisconsin—Madison, Madison, Wisconsin 53706*
*Received October 3, 1984*

ABSTRACT: Nitrogenase in *Rhodospirillum rubrum* is regulated in vivo by the covalent modification of the Fe protein. This paper reports the isolation, purification, and properties of the modifying group that has been heat released from the Fe protein. The molecule is isolated from the heated mixture by binding to a boronate affinity column. Purification is achieved on an ion-exchange high-performance liquid chromatography column. Structural properties of the molecule have been investigated by using proton and phosphorus NMR, mass spectrometry, enzyme susceptibility, and chromatographic methods. The heat-released modifying group exhibits an unusual signal in the proton NMR spectrum at 1.26 ppm. The molecule also contains a functional group which can be reduced by borohydride. This group is lost on breakdown of the molecule or upon treatment of the molecule with 5′-nucleotidase. The identity of the base and the pentose of modifying group as adenine and ribose, respectively, is confirmed. Ratios of the known components of the modifying group are established.

Nitrogenase activity in the photosynthetic bacterium *Rhodospirillum rubrum* was shown to be inhibited by the addition of ammonia to cell cultures (Gest & Kamen, 1949; Gest et al., 1950; Schick, 1971). This loss of activity has been termed "switch off" by Zumft & Castillo (1978) and has now been demonstrated in several organisms (Bognar et al., 1982; Carithers et al., 1979; Hallenbeck et al., 1982; Jones & Monty, 1979; Ludden et al., 1978; Yoch, 1980; Zumft & Castillo, 1978). In addition to ammonia, glutamine (Neilson & Nordlund, 1975), darkness, oxygen, the oxidizing dye phenazine methosulfate, and the uncoupler CCCP (Kanemoto & Ludden, 1984) have been shown to cause switch off. The biochemical basis for switch off is the modification of the Fe protein by covalently attached modifying group that consists of pentose, phosphate, and adenine (Ludden & Burris, 1978; Gotto & Yoch, 1982) and some previously unknown material (Ludden et al., 1984); when the modifying group is attached, the Fe protein is inactive. At least part of the modifying group is removed by the activating enzyme (AE) which is isolated from the chromatophore membrane fraction of cell extracts (Ludden & Burris, 1976, 1979; Nordlund et al., 1977, Guth & Burris, 1983; Gotto & Yoch, 1982). In addition to removal of the modifyng group (MG)[1] by AE, MG can be removed, and the Fe protein can be activated by heating the protein under relatively mild conditions (Dowling et al., 1982).

The structure of the MG has been elusive. Although the components of the group are similar to AMP, there is no direct evidence that the component is AMP. Glutamine synthetases from some enteric bacteria are known to be inactivated by the attachment of AMP via a phosphodiester bond to a specific tyrosyl residue (Kingdon et al., 1979; Shapiro & Stadtman, 1968). However, the AMP attached to GS is not thermolabile, and it is susceptible to snake venom diesterase; the modifying group on *R. rubrum* Fe protein is thermolabile and is not a substrate for snake venom diesterase. Similarly, ADP-ribose is a known protein modifier (Hayaishi, 1976; Van Ness et al., 1980). ADP-ribose is also susceptible to snake venom diesterase, and the ratio of adenine to ribose to phosphate is 1:2:2 (Hayaishi, 1976). The stoichiometry of modifying group components found on *R. rubrum* Fe protein was reported to be 1:1:1 per protein dimer by us (Ludden et al., 1982), but others are less definitive (Hallenbeck et al., 1982; Gotto & Yoch, 1982). Michalski et al. (1983) have shown that in toluenized *Rhodopseudomonas capsulata* cells, label from [$^{14}$C]adenine or $\gamma$-$^{32}$P-labeled ATP is incorporated into the MG. Surprisingly, the $\alpha$-P of ATP is not incorporated into MG as would be expected if ATP or NAD were the donor molecules for AMP or ADP-ribose, respectively. $^{3}$H label from both [2-$^{3}$H]adenine or [8-$^{3}$H]adenine are incorporated into modifying group in vivo (Nordlund & Ludden, 1983).

In this paper the purification of the heat-released modifying group ($\Delta$MG) is described. The properties of the molecule were investigated, and information about the structure of the

† This work was supported by funds from the College of Agricultural and Life Sciences, University of Wisconsin—Madison, and by Grant 81-CRCR-1-0703 from the USDA competitive grants program. NMR analysis at the Purdue Biochemical Magnetic Resonance Laboratory was supported by NIH Grant RR01077. P.W.L. is a Steenbock Career Development Award Recipient, and M.R.P. was supported by NIH Cellular and Molecular Biology Training Grant 2 T32 GM07215.
* Address correspondence to this author at the College of Agricultural and Life Sciences.

[1] Abbreviations: MG, modifying group; $\Delta$MG, heat-released modifying group; $H_2\Delta$MG, reduced, heat-released modifying group; DNase, deoxyribonuclease; RNase, ribonuclease; HPLC, high-performance liquid chromatography; EI, electron ionization; CI, chemical ionization.

0006-2960/85/0424-2374$01.50/0 

molecule was obtained by $^{31}P$ and $^{1}H$ NMR and mass spectrometry.

## Materials and Methods

*Growth of Cells and Enzyme Purification.* *Rhodospirillum rubrum* (ATCC 11170) was grown in batch culture on Ormerod's glutamate–malate medium as previously described (Ludden & Burris, 1978) and collected by a Millipore Pellicon cassette with an HVLP filter. One-half-kilogram batches of cells were lysed in a bead beater (Biospec Products) operated in an anaerobic glove box. Extracts were centrifuged overnight at 50000*g* in a Beckman type 19 rotor. Fe protein was purified from the resulting crude extract as prevously described (Ludden & Burris, 1978). Fe protein labeled with [$^{32}P$]- or [8-$^{3}H$]adenine was prepared as previously described (Ludden et al., 1982). Attempts to label the modifying group with $^{35}S$ were done in a similar manner; 1 mCi of $^{35}SO_4$ was added to the growth medium.

*Heat Release and Purification of Modifying Group (ΔMG).* Purified Fe protein to be used for the preparation of ΔMG was treated with DNase and RNase (0.1% each) and then chromatographed on a 2.5 × 30 cm Sephadex G-25 column in 100 mM ammonium formate buffer, pH 8.5. This and all subsequent steps in the preparation of ΔMG were performed aerobically. The fractions containing Fe protein were pooled and, if necessary, concentrated by lyophilization in a Speed vac concentrator (Savant). Heat release of the molecule was accomplished by heating the protein to 60 °C at pH 8.5 for 3 h in a water bath. Modifying group release was optimal when protein concentrations were above 15 mg/mL.

Heat-released modifying group (ΔMG) was separated from protein on an Affi-gel 601 boronate column (Bio-Rad). The heated fraction was applied to a 2-mL bed volume column of Affi-gel 601 that had been equilibrated with 100 mM ammonium formate buffer, pH 8.5. The column was then washed with 10 mL of ammonium formate buffer, and then the modifying group was eluted with 100 mM formic acid, pH 2.1. The modifying group was detected by its absorbance at 260 nm or by detection of $^{32}P$ or $^{3}H$ by scintillation counting. The pH of the fractions containing modifying group was adjusted to 7.0 by addition of 1 M $NH_4OH$. The material was then applied to a Syncropak AX 300 ion-exchange HPLC column equilibrated with 50 mM $KH_2PO_4$, pH 4.5, and 100 mM NaCl. The column was washed with the same buffer at a flow rate of 2.0 mL/min on a Waters HPLC system. The modifying group was detected by its absorbance at 254 nm and by determination of $^{32}P$ and $^{3}H$ scintillation counting. The fractions containing modifying group were pooled and desalted on an Affi-gel 601 column run as described above. The sample was concentrated by lyophilization on a Speed vac (Savant). At this point, care must be taken not to take the sample to dryness immediately. Rather, the sample should be lyophilized to a volume of 1.0 mL and resuspended in 5 mL of water several times. This process allows the removal of formate from the sample; concentration to dryness in formate buffer causes breakdown of modifying group. The concentrated modifying group can be stored at −20 °C or in liquid nitrogen.

*Preparation of Reduced Heat-Released Modifying Group ($H_2$ΔMG).* The modifying group is released from the Fe protein as described above. After the first boronate column, the pH of the ΔMG fraction is adjusted to 8.0, and $NaBH_4$ is added to a concentration of 10 mM. The mixture is allowed to react at 20 °C for 1 h, then brought to 20 mM $NaBH_4$, and allowed to react for another hour. $H_2$ΔMG is then purified by HPLC as described above. Alternatively, $^{3}H_2$ΔMG is prepared by reacting 1 mCi of $NaB^{3}H_4$ with ΔMG for 1 h followed by the addition of $NaBH_4$ to 20 mM.

*Preparation and Isolation of a Nucleoside Fragment of $H_2$ΔMG.* Purified $H_2$ΔMG was adjusted to pH 8.5 and treated with 5′-nucleotidase at room temperature overnight. The sample was then applied to a Syncropac RP-8 (reverse-phase C8) column and eluted with $H_2O$ at 2.0 mL/min. The nucleoside product eluted at 4.6 min. The pooled nucleoside fractions were concentrated by lyophilization in a Speed vac (Savant).

*Silylation and GC Analysis of ΔMG.* Approximately 100 μg of the nucleoside was lyophilized to dryness and silylated by the method of Lawson et al. (1971). Excess water was removed by the addition, and removal under vacuum, of three 40-μL aliquots of $CH_2Cl_2$. Additions of 80 μL of acetonitrile and 120 μL of bis(trimethylsilyl)trifluoroacetamide were then made. The reaction mixture was left at 60 °C for 24 h to allow for complete silylation of the nucleoside. Silylated nucleotides were analyzed on a Packard Model 417 gas chromatograph with a $^{1}/_{4}$ in. diameter by 6 ft glass column (1% OV-101), temperature programmed at 10 °C/min from 200 to 320 °C with nitrogen as the carrier gas at a 30 mL/min flow rate.

*Analysis of Modifying Group by NMR.* Samples were prepared for proton NMR by repeated lyophilization from $D_2O$ (99.99%; Aldrich). Final sample preparation was performed in a glove bag sparged with dry nitrogen. NMR tubes were baked dry and stored over $P_2O_5$. Samples for phosphorus NMR were prepared in a mixture of $H_2O$ and $D_2O$ containing at least 20% $D_2O$. Samples of Fe protein to be analyzed for phosphorus by NMR were prepared in the presence of 1 mM dithionite, and the NMR tube was loaded and sealed with silicon rubber in an anaerobic glove box.

Proton NMR spectra were obtained on a Nicolet 200-MHz or a Bruker 270-MHz instrument. Alternatively, samples were analyzed on the 470-MHz instrument at the Purdue NMR Center. Phosphorus NMR spectra were recorded on the Nicolet 200-MHz instrument (80.1 MHz for $^{31}P$).

*Mass Spectral Analysis.* Mass spectra for the nucleoside and its trimethylsilyl derivative were obtained on a Kratos MS-25 spectrometer equipped with EI and CI as well as direct probe and GC capabilities. CI was performed with isobutane as the reagent gas.

*Assays.* Protein was determined by microbiuret and, in some cases, by dry weight. Pentose, phosphorus, and adenine were determined as previously described (Ludden et al., 1982).

*Chromatography on Bio-Gel P-2.* $H_2$ΔMG was chromatographed on Bio-Gel P-2 and its elution volume compared to the elution volumes of standards. A 1 cm × 30 cm column of Bio-Gel P-2 (Bio-Rad) equilibrated with buffer containing 50 mM $KH_2PO_4$, pH 7.0, and 8 M urea was used. Elution volumes were determined by monitoring the column effluent for absorbance at 260 nm.

*Reagents.* All reagents for growth of cells and assays were obtained from Sigma and/or U.S. Biochemical Corp. unless otherwise noted. Water used in HPLC analysis was prepared by distillation in glass. Deuterated solvents for NMR were obtained from Aldrich Chemical Co. Tritiated borohydride was from Amersham. 5′-Nucleotidase (EC 3.1.3.5), grade IV, and bacterial alkaline phosphatase (EC 3.1.3.1), type III R, were from Sigma. Syncropac ion-exchange (AX 300) and reverse-phase (RP-P, C-8) columns were purchased from Anspec. Affi-gel 601 boronate resin was purchased from Bio-Rad.

## Results

The modifying group was released by heating purified Fe protein, and the heated Fe protein solution was then applied

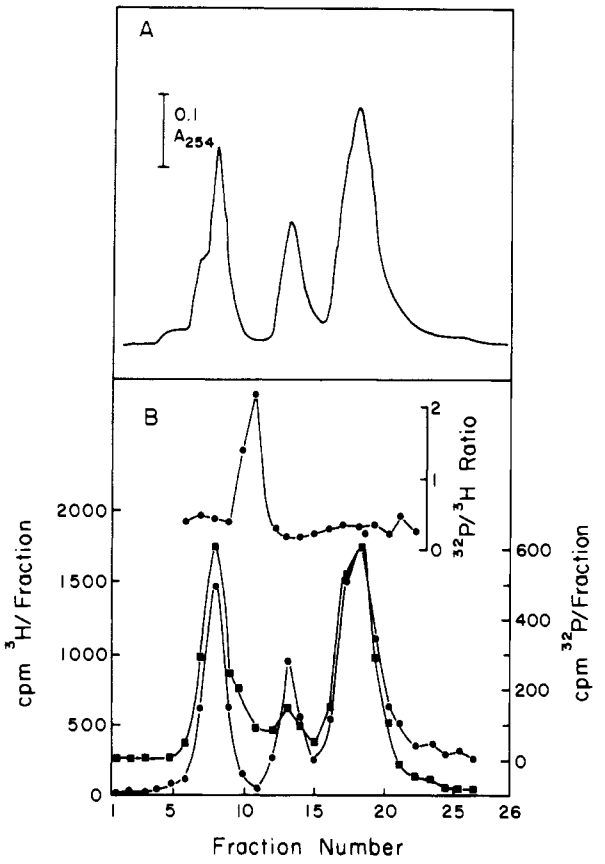


FIGURE 1: Elution profile of heat-released modifying group from ion-exchange HPLC column. Peaks 1–3 referred to in the text are the following: peak 1, left most peak; peak 2, middle peak; peak 3, right peak. (A) Absorbance profile; (B) $^{3}H$ and $^{32}P$ counts in column fractions. (□) $^{32}P$; (○) $^{3}H$.

to a boronate column to separate the released modifying group from protein. Absorbance due to ΔMG, $^{32}P$, and $^{3}H$ coelute from the boronate column. The pooled, pH-adjusted peak fractions were applied to a Syncropac AX 300 ion-exchange HPLC column from which three peaks were eluted; each peak eluted with a peak of [$^{32}P$]- and [$^{3}H$]adenine radioactivity (Figure 1). Peak 3 was always the largest peak; peaks 1 and 2 were variable, and in some cases, peak 2 was not observed. Peak 3 was labile, and the breakdown products included peaks 1 and 2. It was concluded that peak 3 was the material released from the protein because peaks 1 and 2 can be generated from peak 3, while the opposite was not observed. Further evidence for this conclusion will be discussed below. The ΔMG (peak 3) was stable when stored in liquid nitrogen. Peak 2 is the primary breakdown product during lyophilization and is referred to as breakdown product. The $^{32}P/^{3}H$ ratio remains relatively constant except in fractions 7 and 8 (this is the position of orthophosphate in the elution profile) and peak 2 which shows a decreased ratio of $^{32}P/^{3}H$.

The modifying group molecule contains a site that can be reduced by $NaBH_4$. Treatment of ΔMG with $NaB^{3}H_4$ results in a change in the elution position of ΔMG from the ion-exchange HPLC column (Table I). Furthermore, nonexchangable tritium is incorporated into ΔMG by this treatment (Figure 2). The material obtained after $NaBH_4$ treatment is referred to as reduced MG and abbreviated $H_2$ΔMG. The modifying group is much more stable after reduction, although it will still break down to peak 2 during lyophilization with loss of tritium label.

The composition of $H_2$ΔMG was measured by chemical assays for each of the known components of the modifying

Table I: Chromatographic Properties of ΔMG, $H_2$ΔMG, and Standards

| | HPLC[a] (elution time) (min) | GC[b] (elution time) (min) | gel filtration[c] (fraction no.) |
|---|---|---|---|
| ΔMG | 9.4 | 20 | |
| $H_2$ΔMG | 7.6 | | 51 |
| B[d] | 4.5 | 11.0 | |
| 5′-AMP | 4.5 | 12.0 | 58 |
| 3′-AMP | 5.0 | 10.5 | |
| 2′-AMP | 8.8 | 10.5 | |
| ADP | | | 53 |
| ATP | | | 49 |

[a] Chromatography on Syncropak AX-300 column as described under Materials and Methods. [b] Gas chromatography of $Me_4Si$ derivatives as described under Materials and Methods. [c] Gel filtration on Bio-Gel P-2 as described under Materials and Methods. [d] B = breakdown product.

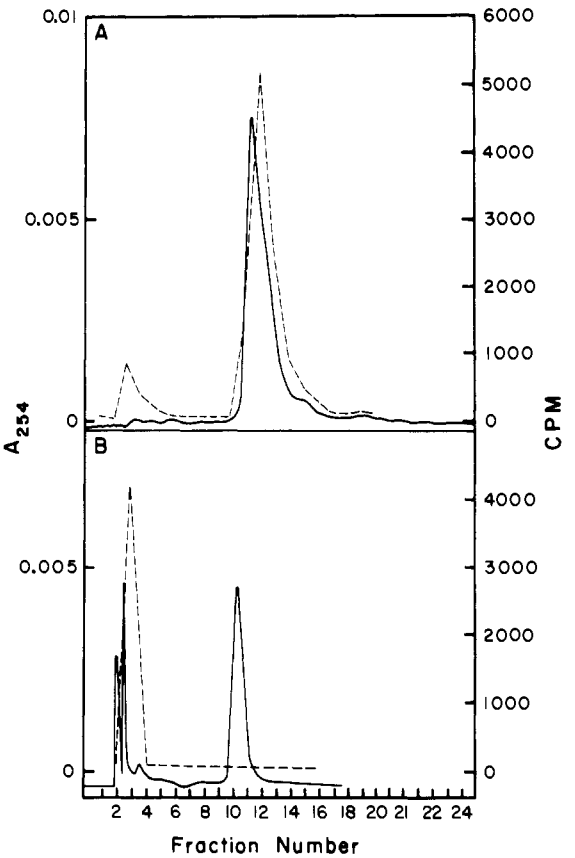


FIGURE 2: Effect of 5′-nucleotidase treatment of $H_2$ΔMG which has been reduced with tritiated borohydride. (A) Comigration of absorbance due to modifying group and $^{3}H$ on ion-exchange HPLC column. (B) Separation of tritiated portion of modifying group from nucleoside after treatment with 5′-nucleotidase on a C8 column. (—) Absorbance at 254 nm; (---) cpm of $^{3}H$ per column fraction.

group on the whole protein. Phosphate, ribose, and adenine were present in a ratio of 1.78:0.87:1 (Table II). It was not possible to detect S from $^{35}SO_4$ in isolated ΔMG; Fe protein was labeled with $^{35}S$ in this experiment.

The UV–visible spectrum of the isolated $H_2$ΔMG was recorded (Figure 3). The spectrum is similar to the spectra for adenine or adenosine, and a millimolar extinction coefficient of 14.1 can be calculated from the concentration of adenine determined by chemical assay and the absorbance at 260 nm seen in Figure 3.

Modifying group can be separated from a variety of standards by gel filtration, ion exchange, chromatography, and gas chromatography (Table I). The data in Table I indicate

Table II: Quantitation of Modifying Group Components in Fe Protein and $H_2\Delta MG$

| | P | ribose | adenine | protein[c] | $^3H/^{32}P$ |
|---|---|---|---|---|---|
| $H_2\Delta MG$[a] | 1.79 ± 0.17 | 0.87 ± 0.09 | 1 | | 6.16 (3.40)[d] |
| Fe protein[b] | 2.10 ± 0.48 | 0.90 ± 0.04 | 1.02 ± 0.06 | 1 | 5.317 (2.99)[d] |
| % yield | 8.19 (9.3)[d] | | 9.3 (11.6)[d] | | |

[a] On the basis of determinations from three separate preparations; data for phosphorus and ribose are based on adenine as determined by $A_{260}$ and an $\epsilon_{mM}$ of 15.1. [b] On the basis of determinations for two separate preparations. Data for phosphorus and adenine are based on specific activity of $^{32}P$ and $^3H$, respectively, as determined for isolated $H_2\Delta MG$. Data for ribose is based on the orcinol assay for ribose. The $^3H$ is from [8-$^3$H]adenine; $H_2\Delta MG$ used in this experiment was prepared from ΔMG and $NaBH_4$ (unlabeled). [c] Protein was determined by biuret and by dry weight with both bovine serum albumin and carbonic anhydrase as standards. [d] First value, preparation 1; second value, preparation 2.

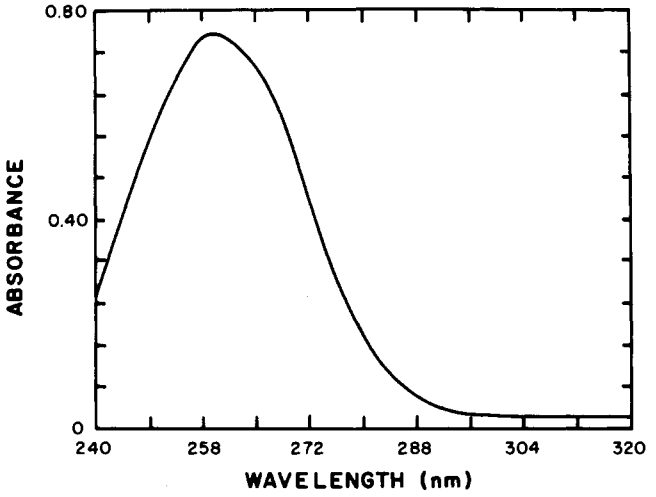


FIGURE 3: UV–visible spectrum of isolated $H_2\Delta MG$.

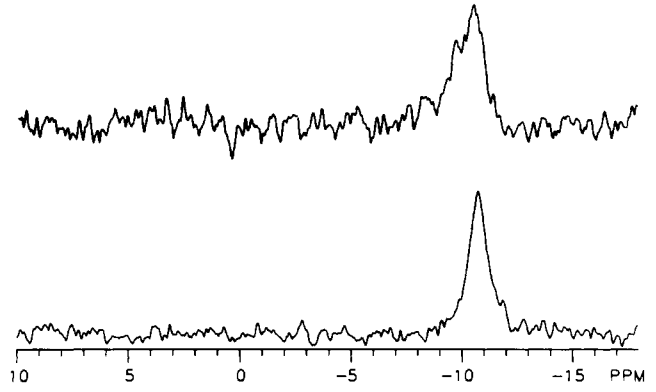


FIGURE 4: $^{31}P$ NMR spectrum of native, reduced Fe protein and isolated $H_2\Delta MG$. Top spectrum, $H_2\Delta MG$; bottom spectrum, native iron protein.

that modifying group is not a common monophosphate, diphosphate, or triphosphate.

The properties of the modifying group can be studied by NMR techniques. The phosphorus NMR spectrum of the native, reduced Fe protein and that of the isolated $H_2\Delta MG$ are shown in Figure 4. The spectra exhibit a single peak at −10.3 ppm relative to an external standard of 10% phosphoric acid. The spectra shown are not proton decoupled. A known quantity of inorganic phosphate was added to the Fe protein anaerobically, and integration of the resulting spectrum indicated that there are two phosphates per protein dimer (data not shown).

The phosphorus spectrum of the breakdown product (peak 2, Figure 1) of ΔMG has also been compared to the spectrum of standard adenine nucleotide monophosphates (Ludden et al., 1984). The spectrum of breakdown product differs from the standards in the position of its chemical shift and by its noticeable lack of splitting.

Samples of $H_2\Delta MG$ and the breakdown product were pH titrated, and their $^{31}P$ spectra were recorded. A plot of

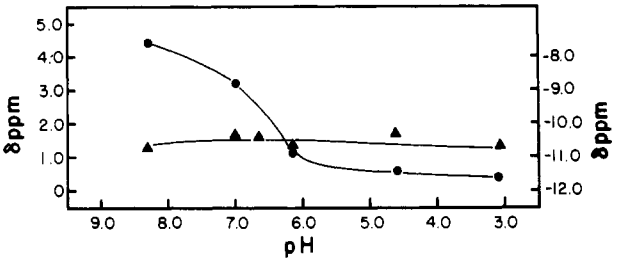


FIGURE 5: Effect of pH on the chemical shift of $^{31}P$ NMR spectra of $H_2\Delta MG$ and the breakdown product of ΔMG. (●) Breakdown product; (▲) $H_2\Delta MG$.

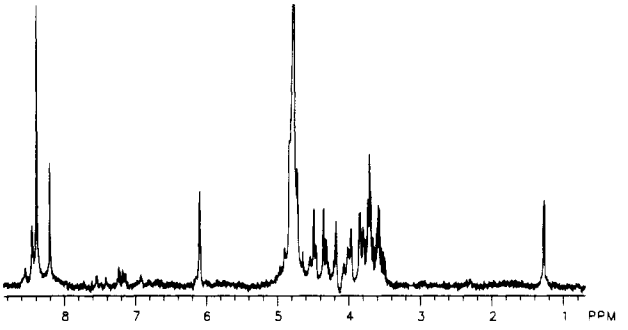


FIGURE 6: Proton NMR spectrum of $H_2\Delta MG$. Spectrum recorded on 470-MHz NMR.

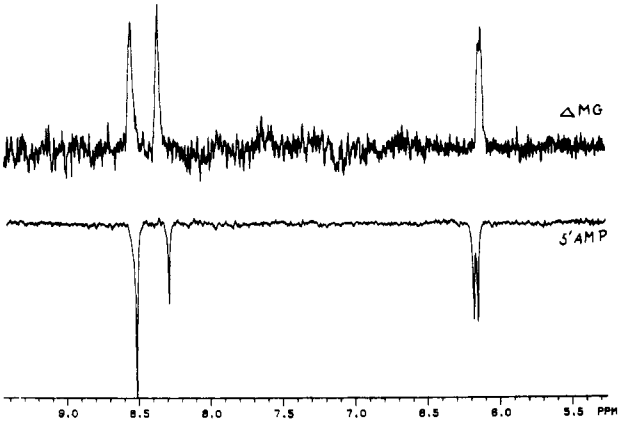


FIGURE 7: Proton NMR spectrum of ΔMG in the 6–9 ppm range. Top spectrum, ΔMG; bottom spectrum, 5′-AMP standard. Spectrum recorded on 200-MHz NMR.

chemical shifts ($\delta$) vs. pH (Figure 5) shows that the chemical shift of $H_2\Delta MG$ changes very little over the pH range, whereas the $^{31}P$ signal of the breakdown product has a significant change in chemical shift with a midpoint at pH 6.9. This indicates that the phosphate of $H_2\Delta MG$ is a disubstituted pyrophosphate, while that of the breakdown product is a phosphomonoester.

The proton spectrum of $H_2\Delta MG$ is shown in Figure 6. In addition to signals due to $H_2\Delta MG$, a large peak due to formate is seen at approximately 8.4 ppm, and a large peak due to HDO is seen around 4.7 ppm. The spectrum exhibits peaks

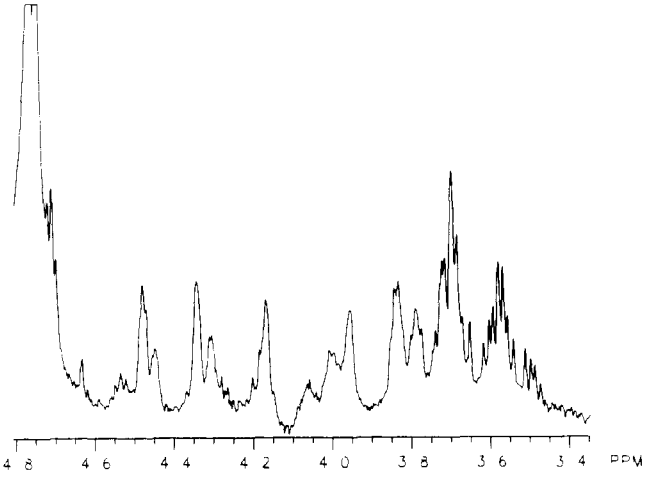


FIGURE 8: Proton NMR spectrum of $H_2\Delta MG$ in the 3.4–5.0 ppm region of the spectrum. Spectrum recorded on 470-MHz NMR.

characteristic of adenine, ribose, and some unusual components. An expanded spectrum of ΔMG for the region between 6 and 9 ppm is compared to the spectrum of AMP in this region (Figure 7). The sample used in this experiment was not reduced by $NaBH_4$ and was repeatedly lyophilized to remove any formate. In this region, peaks characteristic of the 8- and 2-protons of the adenine ring are seen at 8.363 and 8.113 ppm, respectively. Similarly, a peak characteristic of the 1′- (anomeric) proton of ribose in AMP is seen at 6.131 ppm.

The region from 3 to 5 ppm is expanded in Figure 8. This spectrum is from the same data set as shown in Figure 6. In this region, the peaks characteristic of pentose and five or six additional protons that cannot be attributed to ribose are seen. Peaks characteristic of ribose are seen at 4.710 (2′), 4.478 (3′), 4.342 (4′), 3.840 (5′), and 3.821 (5″). These assignments were made by decoupling experiments. The coupling constants for the ribose protons of $H_2\Delta MG$ and its nucleoside were determined. For $H_2\Delta MG$ they are the following: $1'-2' = 5.7$ Hz; $2'-3' = 5.3$ Hz; $3'-4' = 3.5$ Hz. For the nucleoside they are the following: $1'-2' = 6.3$ Hz; $2'-3' = 5.1$ Hz; $3'-4' = 3.2$ Hz; $4'-5' = 2.9$ Hz; $4'-5'' = 3.5$ Hz; $5'-5'' = -12.9$ Hz.

In addition to peaks in the 3.5 ppm region, there is a peak at 1.26 ppm. This peak is observed in every preparation and integrates for a single proton. We are unaware of any report of a nucleotide exhibiting an NMR signal for a single proton in this region. The signal is a doublet, and the splitting is lost when the decoupler is placed at 4.05 pm (Figure 9). Breakdown of the HPLC peak 3 to HPLC peak 2 does not result in loss of the NMR signal of 1.26 ppm. The reducible site is lost on breakdown. The remaining protons in the spectrum can be characterized as two sets of two protons which are coupled to each other. An additional proton is coupled to the 1.26 ppm peak.

The isolated $H_2\Delta MG$ is a substrate for 5′-nucleotidase. The nucleotidase used in these experiments was checked for activity against 2′- and 3′-AMP and observed to have no activity with these compounds. The products of 5′-nucleotidase treatment of $H_2\Delta MG$ can be separated on an HPLC reverse-phase column (Figure 2). A UV-absorbing peak can be separated from a non-UV-absorbing peak which contains $^3H$ from $NaB^3H_4$ reduction of ΔMG. The peak containing $^3H$ also can be shown to contain $^{32}P$ (data not shown).

The adenine-containing fragment from 5′-nucleotidase treatment was purified and analyzed by using mass spectrometry and NMR. Mass spectral analysis was performed

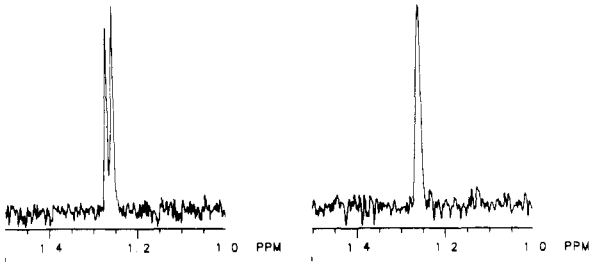


FIGURE 9: Proton NMR spectrum of $H_2\Delta MG$ of the 1.26 ppm signal. Left signal, not decoupled; right signal, decoupler placed at 4.05 ppm. Spectrum recorded on 470-MHz NMR.

by using EI and CI ionization modes. Ion peaks at $m/z$ 267 and 135 characteristic of adenosine and adenine, respectively, were observed in the EI mode, and the corresponding ion peaks at $m/z$ 268 and 136 were observed in the CI mode.

Proton NMR analysis of the nucleoside fragment obtained by 5′-nucleotidase treatment resulted in the chemical shift and coupling constant values listed above. On the basis of comparisons to the value of known adenine nucleosides (Klinke et al., 1980a,b), we conclude that the pentose of the modifying group is ribose rather than arabinose, xylose, or lyxose.

## Discussion

The modifying group has been purified and characterized. A heat release of the molecule followed by purification on a boronate column and an ion-exchange HPLC column results in a 10% yield of the modifying group. Three peaks of heat-released material were observed in the elution profile of the HPLC column. Because the material was heat released, we were concerned that the material might have rearranged during heat release. The conclusion that peak 3 represents the modifying group as it is released from the protein rests on the following facts: (1) the chemical shifts of the $^{31}P$ NMR signals of peak 3 and of the modifying group on the whole protein are in the same position; (2) peak 3 breaks down to yield peaks 1 and 2, indicating that peaks 1 and 2 are derived from peak 3; (3) peaks 1 and 2 do not yield peak 3. Evidence that a peptide containing the modifying group is a substrate for the activating enzyme for Fe protein will be presented later.[2] This peptide also has a $^{31}P$ NMR signal identical with the $^{31}P$ NMR signals of Fe protein and $H_2\Delta MG$. It is interesting to note that the phosphorus of the MG can be observed on the reduced, native protein. The fact that the MG phosphorus is observed indicates it is not near (<10 Å) the paramagnetic iron–sulfur center of the reduced Fe protein.

The isolated modifying group contains adenine, ribose, and phosphate in a single molecule. It has always been assumed that this would be the case; however, data presented here confirmed this for the first time. The molecule can be labeled with [$^{32}P$]orthophosphate and [8-$^3H$]adenine in vivo; thus, both of these components are present. Chemical assays (Table I) and NMR analysis (Figure 6 and 8) indicate that all three compounds are present in the isolated modifying group. The stoichiometry of the components of $H_2\Delta MG$ is 1:1:2 (adenine:ribose:phosphate). This is in contrast to our previous report of 1:1:1 based on chemical assays of the components on the whole protein (Ludden et al., 1982). The data presented in this paper for the $H_2\Delta MG$ are based on chemical assays of phosphate and ribose normalized to adenine. Adenine was determined by its $A_{260}$ using an $\epsilon_{mM}$ of 15.1. Use of the experimentally determined $\epsilon_{mM}$ for $H_2\Delta MG$ (14.1) does not

[2] M. R. Pope and P. W. Ludden, unpublished results.

change the conclusions. Furthermore, calculations for the amount of adenine and phosphorus on the whole protein also show a 1:2 ratio. These calculations are based on the specific radioactivity of adenine and phosphorus in the purified compound. It is not clear why the phosphomolybdate chemical assays for phosphorus produce low values for P/Fe protein. However, chemical assays for phosphorus carried out on the batches of protein used in these experiments also show a ratio of 1:1 phosphorus to Fe protein. It can be stated that the values for phosphorus based on specific activity in the purified molecule are not overestimates of the phosphorus present due to contamination. The ratio of $^{3}H/^{32}P$ is essentially the same for the $H_2\Delta MG$ as it is for Fe protein used in these experiments. Treatment of the protein for phosphorus analysis involves treatment of the protein with DNase and RNase followed by precipitation of the protein with trichloroacetic acid. This treatment results in less than 10% loss of either $^{3}H$ or $^{32}P$. The ratio of 2:1 phosphorus to Fe protein is confirmed by quantitation of the phosphorus by NMR using inorganic phosphate as an internal standard (data not shown).

Ribose was determined by orcinol assay both in preparations of $H_2\Delta MG$ and Fe protein. The ratio of 1:1 ribose to adenine in the $H_2\Delta MG$ is also confirmed by integration of peaks in the $^{1}H$ NMR spectrum. The anomeric proton integrates to a single proton relative to the 2- and 8-protons of adenine. It is possible that the unknown moiety was a sugar and that it is not detected as a pentose after reduction.

Evidence for a pentose in the modifying group was originally obtained by use of a nonspecific assay for pentose, the orcinol assay (Ludden & Burris, 1978). It was also established that the pentose was not a deoxy sugar (Ludden & Burris, 1978). Evidence presented in this paper indicates that the pentose is ribose. This conclusion is based on the chemical shifts of the carbon-bound protons of the carbohydrate and coupling constants for the interaction for the carbohydrate protons as compared to standards (Klinke et al., 1980a,b). The coupling data presented here rule out the possibility that arabinose, xylose, and lyxose are the carbohydrates of the modifying group (Klinke et al., 1980b; Davies, 1978). The loss of the component containing the reducible site slightly affects the chemical shifts of the ribose protons (data not presented).

The phosphorus of the modifying group appears to be a disubstituted pyrophosphate. This conclusion is based on the similarity of phosphorus signal observed for $H_2\Delta MG$ and Fe protein with the $^{31}P$ NMR signals observed for the pyrophosphates of NAD and ADPR. Although not shown here, splitting of the phosphorus signal of the isolated peptide has been observed. This conclusion is also based on the lack of a shift in the phosphorus signal when the pH of the sample is titrated from pH 5 to 8. A pH-dependent shift of the phosphorus signal is seen for the breakdown product (peak 2, Figure 5) with a midpoint at pH 6.9. Thus, it would appear that the breakdown involves conversion of the phosphorus linkage from a pyrophosphate to a phosphomonoester. This might be accomplished by a ring opening or loss of a piece of the molecule. Because the reducible site (or its reduced product) is lost on breakdown, we favor the latter conclusion. Note that the $^{3}H/^{32}P$ ratio observed for the breakdown product of $\Delta MG$ (peak 2, Figure 1) is increased relative to that of peak 3. We interpret this as meaning that the breakdown process involves cleavage between the two phosphorus' of the $\Delta MG$, with a loss of half of the phosphorus.

The linkage of the components to each other and to the protein have not been established. Because the phosphorus of the isolated molecule appears to be in a disubstituted pyrophosphate linkage, and because the NMR signal of the phosphorus does not change when the $\Delta MG$ is isolated, it is unlikely that phosphorus is directly involved in the linkage to the protein. The isolated $H_2\Delta MG$ and the breakdown product are substrates for 5′-nucleotidase.

## ACKNOWLEDGMENTS

We acknowledge the helpful advice and discussions with Dr. H. K. Schnoes, Dr. T. Farrar, Dr. P. Hart, Dr. E. Mooberry, Dr. E. Urlich, and M. Micke during the course of this investigation.

## REFERENCES

Bognar, A., Desrasiers, L., Libman, M., & Newman, E. B. (1982) *J. Bacteriol. 152*, 706–713.

Carithers, R. P., Yoch, D. C., & Arnon, D. I. (1979) *J. Bacteriol. 137*, 779–789.

Davies, D. B. (1978) *Prog. Nucl. Magn. Reson. Spectrosc. 12*, 135–225.

Dowling, T. E., Preston, G. G., & Ludden, P. W. (1982) *J. Biol. Chem. 257*, 13098–13992.

Gest, H., & Kamen, M. D. (1949) *Science (Washington, D.C.) 109*, 558–560.

Gest, H., Kamen, M. D., & Bregoff, H. M. (1950) *J. Biol. Chem. 182*, 153–170.

Gotto, J. W., & Yoch, D. C. (1982) *J. Biol. Chem. 257*, 2868–2873.

Guth, J. H., & Burris, R. H. (1983) *Biochim. Biophys. Acta 749*, 91–100.

Hallenbeck, P. C., Meyer, C. M., & Vignais, P. M. (1982) *J. Bacteriol. 149*, 708–717.

Hayaishi, O. (1976) *Trends Biochem. Sci. (Pers. Ed.) 1*, 9–10.

Jones, B. L., & Monty, K. J. (1979) *J. Bacteriol. 139*, 1007–1013.

Kanemoto, R. H., & Ludden, P. W. (1984) *J. Bacteriol. 158*, 713–720.

Kingdon, H. S., Shapiro, B. M., & Stadtman, E. R. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1703–1710.

Klinke, G., Cano, I., Ludemann, H. D., Mengel, R., & Robins, M. J. (1980a) *Z. Naturforsch., C.: Biosci. 35C*, 853–864.

Klinke, G., Cano, I., Ludemann, H. D., Mengel, R., & Robins, M. J. (1980b) *Z. Naturforsch. C: Biosci. 35C*, 865–877.

Lawson, A. M., Stillwell, R. N., Tacker, M. M., Tsaboyama, K., & McCloskey, J. A. (1971) *J. Am. Chem. Soc. 93*, 1014–1023.

Ludden, P. W., & Burris, R. H. (1976) *Science (Washington, D.C.) 194*, 424–426.

Ludden, P. W., & Burris, R. H. (1978) *Biochem. J. 175*, 251–259.

Ludden, P. W., & Burris, R. H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6201–6205.

Ludden, P. W., Okon, P., & Burris, R. H. (1978) *Biochem. J. 173*, 1001–1003.

Ludden, P. W., Preston, G. G., & Dowling, T. E. (1982) *Biochem. J. 203*, 663–668.

Ludden, P. W., Murrell, S. A., Pope, M., Kanemoto, R., Dowling, T. E., Saari, L. L., & Triplet, E. (1984) in *Advances in Nitrogen Fixation Research* (Veeger, C., & Newton, W. E., Eds.) pp 181–187, Nijhoff/Junk, Wageningen.

Michalski, W. P., Nicholas, D. J. D., & Vignais, P. M. (1983) *Biochim. Biophys. Acta 743*, 136–148.

Neilson, A. H., & Nordlund, S. (1975) *J. Gen. Microbiol. 91*, 53–62.

Nordlund, S., & Ludden, P. W. (1983) *Biochem. J. 209*, 881–884.

Nordlund, S., Eriksson, U., & Baltscheffsky, H. (1977) *Biochim. Biophys. Acta 462*, 187–195.
Schick, H.-J. (1971) *Arch. Mikrobiol. 75*, 102–109.
Shapiro, B. M., & Stadtman, E. R. (1968) *J. Biol. Chem. 243*, 3769–3771.
Van Ness, B. G., Howard, J. B., & Bodley, J. W. (1980) *J. Biol. Chem. 255*, 10717–10720.
Yoch, D. C. (1980) *Biochem. J. 187*, 273–276.
Zumft, W. G., & Castillo, F. (1978) *Arch. Microbiol. 117*, 53–60.

# Proteolysis of Smooth Muscle Myosin by *Staphylococcus aureus* Protease: Preparation of Heavy Meromyosin and Subfragment 1 with Intact 20 000-Dalton Light Chains†

M. Ikebe and D. J. Hartshorne*

*Muscle Biology Group, Departments of Biochemistry and Nutrition and Food Science, University of Arizona, Tucson, Arizona 85721*

*Received September 11, 1984*

ABSTRACT: The proteolysis of gizzard myosin by *Staphylococcus aureus* protease produces both heavy meromyosin and subfragment 1 in which the 20 000-dalton light chains are intact, and conditions are suggested for the preparation of each. Cleavage of the myosin heavy chain to produce subfragment 1 is dependent on the myosin conformation. Proteolysis of myosin in the 10S conformation yields predominantly heavy meromyosin, and myosin in the 6S conformation yields mostly subfragment 1 and some heavy meromyosin. Two sites are influenced by myosin conformation, and these are located at approximately 68 000 and 94 000 daltons from the N-terminus of the myosin heavy chain. The latter site is thought to be located at the subfragment 1–subfragment 2 junction, and cleavage at this site results in the production of subfragment 1. The time courses of phosphorylation of both heavy meromyosin and subfragment 1 can be fit by a single exponential. The actin-activated $Mg^{2+}$-ATPase activity of heavy meromyosin is markedly activated by phosphorylation of the 20 000-dalton light chains. From the actin dependence of $Mg^{2+}$-ATPase activity the following values are obtained: for phosphorylated heavy meromyosin, $V_{max} \sim 5.6\ s^{-1}$ and $K_a$ (the apparent dissociation constant for actin) $\sim 2$ mg/mL; for dephosphorylated heavy meromyosin, $V_{max} \sim 0.2\ s^{-1}$ and $K_a \sim 7$ mg/mL. The actin-activated ATPase activity of subfragment 1 is not influenced by phosphorylation, and $V_{max}$ and $K_a$ for both the phosphorylated and dephosphorylated forms are $0.4\ s^{-1}$ and 5 mg/mL, respectively. The $Mg^{2+}$- and $Ca^{2+}$-ATPase activities of subfragment 1 are distinct from those of heavy meromyosin, and the $Mg^{2+}$-ATPase activity of subfragment 1 is not affected by phosphorylation. It is clear from these results that the phosphorylation of the 20 000-dalton light chain of subfragment 1 is not required for actin-activated ATPase activity. It is suggested that regulation via phosphorylation involves the interaction of the myosin heads with other parts of the molecule, possibly in the subfragment 2 region.

It is accepted that phosphorylation–dephosphorylation of the two 20 000-dalton light chains of myosin is involved in the regulation of contractile activity in smooth muscle (Adelstein & Eisenberg, 1980; Walsh & Hartshorne, 1982). Initiation of contraction and an increase in the actin-activated ATPase activity of myosin require phosphorylation, and the reverse process, i.e., dephosphorylation, accompanies relaxation and the loss of actin-activated ATPase activity (Güth & Junge, 1982). Although this general pattern for the function of myosin phosphorylation is well documented, its precise in vivo role is still unresolved. There are suggestions that in order to explain the observed biochemical and physiological data, additional $Ca^{2+}$-dependent regulatory mechanisms must be implicated (Marston, 1982; Murphy & Mras, 1983). Clearly the effects of light chain phosphorylation must be understood at the molecular level before one can evaluate its physiological role.

Recently it has been shown that monomeric smooth muscle myosin can exist in two distinct conformations (Suzuki et al., 1982) referred to as 10S and 6S to reflect their differences in sedimentation rates. The large conformational change is due to an apparent folding of the molecule as visualized by electron microscopy (Onishi & Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983). Several factors influence the 10S–6S transition, including the ionic strength of the solvent and the $MgCl_2$ concentration (Ikebe et al., 1983), but of particular interest is the observation that phosphorylation of myosin favors the extended 6S form (Trybus et al., 1982; Craig et al., 1983; Onishi et al., 1983). Associated with the conformational change of myosin there is an alteration of ATPase activities, and it was shown that the 10S and 6S states are characterized by distinct enzymatic properties (Ikebe et al., 1983). This relationship prompted the suggestion that the

† This work was supported by Grants HL 23615 and HL 20984 from the National Institutes of Health.

* Address correspondence to this author at the Department of Nutrition and Food Science.

0006-2960/85/0424-2380$01.50/0 