Apodinitrogenase: Purification, Association with a 20-Kilodalton Protein, and Activation by the Iron–Molybdenum Cofactor in the Absence of Dinitrogenase Reductase†

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ABSTRACT: The *Azotobacter vinelandii* mutant strain UW45 contains a mutation in the *nifB* gene and produces an inactive dinitrogenase protein that can be activated by the addition of purified iron–molybdenum cofactor (FeMoco). This FeMoco-deficient dinitrogenase (Apo I) has now been purified 96-fold to >95% purity and is FeMoco-activatable to 2200 nmol of C_2H_2 reduced/(min·mg of protein). The Apo I complex was found to contain two molecules of a 20-kDa protein, in addition to the $\alpha_2\beta_2$ tetramer found for isolated holodinitrogenase (Holo I). The Apo I complex contained 15 ± 2 mol of Fe per mole, but no Mo. While the presence of dinitrogenase reductase caused a 2-fold stimulation in the activation of the purified Apo I complex by FeMoco, this enhancement resulted from the stabilization of Apo I by dinitrogenase reductase to the denaturing effects of *N*-methylformamide. When the activation was performed in the absence of *N*-methylformamide, there was no enhancement by dinitrogenase reductase alone or by dinitrogenase reductase–Mg–ATP complex. The Apo I complex is more sensitive to O_2 than Holo I, with a half-life in air of 6 min; however, the addition of dithiothreitol to Apo I during the exposure to air (or after exposure) resulted in a half-life very similar to that seen for Holo I. This suggests that sulfhydryl(s) is (are) important for the FeMoco-activation reaction.

Biological N_2 fixation is carried out by many diverse bacteria in the environment. The enzyme responsible for this fixation, nitrogenase, is composed of two separate protein components, dinitrogenase reductase and dinitrogenase (Bulen & LeComte, 1966; Hageman & Burris, 1978). Dinitrogenase reductase (component II), the product of the *nifH* gene, is a dimer of 65–70 kDa (Shah et al., 1984). It contains one 4Fe-4S center and is responsible for the reduction of dinitrogenase one electron at a time, with the concomitant hydrolysis of ATP (Hagemen & Burris, 1978; Shah et al., 1984). Dinitrogenase (Holo I) is an $\alpha_2\beta_2$ tetramer of the *nifK* and *nifD* gene products with a molecular weight of 220 000–240 000 (Shah et al., 1984). This protein contains four 4Fe-4S centers (P clusters) along with an unusual center called the iron–molybdenum cofactor (FeMoco) (Shah & Brill, 1977; Orme-Johnson, 1985). Dinitrogenase is responsible for the actual reduction of N_2 and is also capable of reducing several alternate substrates, including acetylene, cyanide, and cyclopropene (Hardy, 1979).

FeMoco is thought to be the site of N_2 reduction on the dinitrogenase enzyme (Shah et al., 1977; Hawkes et al., 1984; Smith, B. E., et al., 1985). Purification and study of this cofactor have shown it to be a complex organometallic compound containing Mo, Fe, and S in a ratio of 1:(6–8):(8–10), and also to contain an organic component in the form of homocitrate (Shah et al., 1977; Yang et al., 1982; Nelson et al., 1983; Hoover et al., 1989). The products of six *nif* genes have been implicated in the synthesis of FeMoco: *nifQ*, *-B*, *-N*, *-E*, *-V*, and *-H* (Roberts et al., 1978; Imperial et al., 1984, 1988; Hoover et al., 1986; Filler et al., 1986; Robinson et al., 1987). Mutations in any of these genes cause a Nif⁻ phenotype, though NifQ⁻ mutants only do so under low-Mo conditions. When crude extracts of *nifB*, *-N*, and *-E* mutants are made and assayed for nitrogenase activity, active component II is found, but component I is inactive. However, if purified FeMoco is added to the mutant extracts, component I activity is restored (Shah et al., 1977). Therefore, these mutants are unable to synthesize FeMoco but do contain FeMoco-less component I (Apo I) in the extract.

An in vitro assay for FeMoco synthesis has been developed to study the enzymes involved in FeMoco formation (Shah et al., 1986). As first described, this coupled assay involved the mixing of crude extracts of two mutant strains: one with a mutation in the *nifB* gene and the other containing a mutation in *nifN* or *-E*. When mixed in vitro, the mutant extracts complemented each other, forming FeMoco and activating Apo I in the extract, which was then measured by an C_2H_2 reduction assay.

This in vitro synthesis assay has been used in the purification and characterization of several factors involved in FeMoco synthesis. Homocitrate has been found to be essential for FeMoco synthesis and is present in the finished molecule (Hoover et al., 1989). A requirement for component II in the synthesis of FeMoco has also been described (Robinson et al., 1987; Imperial et al., 1988). Recently, the assay has been used to follow purification of the *nifN* and *-E* gene products and physically characterize the complex formed by these proteins (Paustian et al., 1989).

Apo I is not essential for the synthesis of FeMoco in vivo (Ugalde et al., 1984), but it is necessary as a means of assaying the FeMoco that is synthesized during the in vitro reaction. There are two reasons to develop a purification scheme for Apo I: it will be necessary for the eventual complete definition of the in vitro synthesis pathway, and an examination of its

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properties may lead to a better understanding the site of FeMoco incorporation in Holo I.

Two previous attempts to purify Apo I have been published. The isolation of homogeneous Apo I from a *Klebsiella pneumoniae* NifB$^-$ strain has been reported, but the purified protein had a specific activity of 500 nmol of C_2H_2 reduced/(min·mg) when activated with FeMoco, well below the specific activity of purified Holo I [>2000 nmol of C_2H_2 reduced/(min·mg)] (Hawkes & Smith, 1983). The authors attributed the low specific activity and low recovery (about 9%) of Apo I to the instability of the enzyme. A purification of Apo I from *Azotobacter vinelandii* mutant UW45, which contains a mutation in the *nifB* gene, has also been reported (Burgess et al., 1981). This partially purified Apo I had a specific activity of 1900 nmol of C_2H_2 reduced/(min·mg), at 70% purity. Seventy milligrams of Apo I was isolated from 300 g of cells, which is about 25% of the value usually obtained during the isolation of component I.

We have developed a better purification protocol for Apo I. This protocol fulfills the following requirements: (i) the protocol is fast and simple, (ii) the purity of the protein is >95% with a specific activity in excess of 2200 nmol of C_2H_2 reduced/(min·mg of protein), (iii) conditions have been found to stabilize the protein so that it can be stored over several months with minimal losses in activity, and (iv) the recovery of active enzyme after the purification is 30% or greater. In this paper we report this purification protocol as well as some of the properties of the isolated enzyme.

Materials and Methods

Materials. ATP, creatine phosphate, creatine phosphokinase, and reactive red 120 agarose type 3000-CL were obtained from Sigma Chemical Co. The HPLC was from LKB. The PL-SAX strong anion-exchange column was from Polymer Labs (Amherst, MA). The DEAE-cellulose used was Whatman DE-52. $Na_2S_2O_4$ was obtained from Virginia Biochemicals. All other chemicals and reagents used were of analytical grade or better.

Bacterial Strains and Growth Conditions. Growth of *A. vinelandii* mutant UW45 and derepression of nitrogenase have been described (Paustian et al., 1989).

Buffer Preparation and Definition. Anaerobic buffers were prepared as described (Shah et al., 1986). The following abbreviations for buffers are used throughout the text. MD buffer is 25 mM Mops–NaOH (pH 7.4) and 1.7 mM $Na_2S_2O_4$. MGD buffer is 25 mM Mops–NaOH (pH 7.4), 20% glycerol, and 1.7 mM $Na_2S_2O_4$.

Activation Assay of Apo I with FeMoco. FeMoco was isolated according to the protocol described previously (Shah & Brill, 1977). Nine-milliliter serum vials to be used for the assays were degassed on a gassing manifold with Ar. The vials were rinsed with 0.3 mL of MD buffer, and then the following was added in this order: 0.1 mL of MD buffer, the fraction to be assayed, 20 μg of component II, and 5 μL of FeMoco preparation. This amount of FeMoco is in excess in the assay and is sufficient to generate >50 nmol of C_2H_2 reduced/min (in separate experiments performed in the presence of excess Apo I). The mixtures were incubated at 30 °C for 5–10 min, and then 0.8 mL of an ATP-generating system (ATP–$Na_2S_2O_4$) prepared as described (Hoover et al., 1986) and an additional 20 μg of component II were added. The vials were brought to atmospheric pressure and assayed for C_2H_2 reduction activity by injecting 0.5 mL of C_2H_2 gas and incubating for 15 min at 30 °C in a water-bath shaker. The reaction was stopped by the addition of 0.1 mL of 4 N NaOH. C_2H_4 formed was measured by using a Shimadzu Model GC 8A gas chromatograph equipped with a Poropak N column. One unit of activity corresponds to 1 nmol of C_2H_2 reduced/min at 30 °C. Protein concentrations were determined by the bicinchoninic acid method (Smith, P. K., et al., 1985).

Purification of Apo I. Frozen UW45 cell paste (200 g) was broken by osmotic shock as described previously (Shah et al., 1972), except that 0.2 mM phenylmethanesulfonyl fluoride and 0.5 mg/L leupeptin were added to the MD buffer used to lyse the cells. The latter two compounds were found to be necessary because Apo I was found to be highly sensitive to protease degradation in the crude extract. The lysed cell suspension was centrifuged at 16000*g* for 50 min at 4 °C. A 5 × 25 cm DEAE-cellulose column was equilibrated with 2 L of 0.1 M NaCl in MD buffer and then washed with 500 mL of MGD, after which the cell extract was applied. The column was washed with 500 mL of MGD followed by 500 mL of 0.1 M NaCl in MGD. Apo I was then eluted with 0.17 M NaCl in MGD, and 50-mL fractions were collected anaerobically.

Active fractions from the DEAE-cellulose column were pooled and diluted with an equal volume of MGD. This was applied to a 5 × 11 cm reactive red 120 column that had been reduced with 1 L of MD buffer and washed with 200 mL of MGD. After application of the diluted fractions, the column was washed with 200 mL of MGD, followed by a wash with 200 mL of 0.15 M NaCl in MGD. Apo I was then eluted from the column with 0.3 M NaCl in MGD buffer. Apo I chromatographed with the front and could be seen as a brown band; this fraction was collected and immediately applied to a 2.5 × 16 cm hydroxylapatite column that had been equilibrated with 75 mL of MGD. After application, the hydroxylapatite column was washed with 70 mL of a buffer consisting of 2 mM KPO_4 (pH 7.4), 10 mM Mops (pH 7.4), 20% glycerol, and 1.7 mM $Na_2S_2O_4$. The column was then developed with a 400 mL (total volume), 2–200 mM KPO_4 gradient (pH 7.4) in the same buffer, except that the 200 mM KPO_4 buffer did not contain 10 mM Mops. Thirty-milliliter fractions were collected anaerobically, with Apo I eluting at 90 mM KPO_4 into three fractions. Active fractions from the hydroxylapatite column (90 mL) were concentrated by adding 42.5 g of $(NH_4)_2SO_4$, and the precipitate collected by centrifugation at 10000*g* for 15 min at 20 °C. The pellet was resuspended in 20 mL of MGD and desalted on a 2.5 × 34 cm Sephadex G-25 column.

At this point Apo I is about 70% pure with only a few major contaminants. The final step of the purification involved the use of a Pharmacia/LKB HPLC. Buffers for use in the HPLC were filtered through a 0.22-μm filter and then sparged with He. After 20 min of sparging, 0.3 mg/mL of $Na_2S_2O_4$ was added and sparging continued during the purification step. All other procedures for the HPLC were according to the manufacturer's suggestions. A 15 × 0.75 cm PL-SAX column was reduced with 18 mL of 0.5 M NaCl in MGD and then equilibrated with 50 mM NaCl in MGD. The desalted Apo I fraction (~20 mL) was then applied to the PL-SAX column. The column was washed with 9 mL of 50 mM NaCl in MGD and then developed with a linear 50–500 mM NaCl gradient (120-mL volume). Four-milliliter fractions were collected, with Apo I eluting at about 175 mM NaCl. After this step Apo I was >95% pure and could be FeMoco-activated to a specific activity of 2200–2400 nmol of C_2H_2 reduced/(min·mg of protein). When stored at −80 °C in this buffer, Apo I was stable for >4 months.

Gel Electrophoresis. Anaerobic native gel electrophoresis was performed as described (Shah & Brill, 1973). SDS–PAGE was performed as described (O'Farrell, 1975) with

Table I: Purification of Apo I from *A. vinelandii*

sample	total protein (mg)	volume (mL)	total activity (units)[a]	specific activity	x-fold purification	% recovery
UW45 crude extract	11 100	800	272 000	25	1.0	100
DEAE-cellulose	1 540	200	274 000	178	7.3	101
reactive red 120/hydroxylapatite	68	90	108 000	1590	64.5	40
PL-SAX	43	42	100 000	2330	95.5	37

[a] One unit of activity is defined as 1 nmol of C_2H_2 reduced/min at 30 °C (calculated rate from a 15-min assay). The acetylene reduction assay was linear from 5 to 30 min.

modifications (Roberts & Brill, 1980).

Metal Analysis. Metal analysis of Apo I was performed by using a plasma emission spectrophotometer as described previously (Paustian et al., 1989).

Tests of the Requirement of Component II for the Activation of Apo I. Test 1. Two portions of Apo I, each containing ~650 units, were incubated with 5 μL of FeMoco in the presence or absence of 20 μg of component II in an anaerobic 9-mL serum vial. After a 5-min incubation each portion of Apo I was applied to a 1-mL DEAE-cellulose column that had been reduced with 10 mL of MD and equilibrated with 2 mL of MGD. The columns were washed with 2 bed volumes of MGD buffer, and FeMoco-activated Apo I was then eluted with 0.2 M NaCl in MGD into an anaerobic vial (2.5 mL total). Under these elution conditions free FeMoco and component II remain bound to the column (Shah et al., 1985). A 0.1-mL portion was placed in a 9-mL serum vial, to this was added 0.8 mL of ATP–$Na_2S_2O_4$ and 20 μg of component II, and the assay for C_2H_2 reduction was carried out as described earlier.

Test 2. A 20-μL portion of Apo I (~20 μg) in 0.1 mL of MD buffer was incubated with FeMoco in the presence and absence of various chemicals and reagents (see Table II). After a 5-min incubation, 5 μL of 0.2 mM $(NH_4)_2MoS_4$ in *N*-methylformamide was added to a final concentration of 10 nM. The assays were again incubated for 5 min and then assayed for Holo I activity by a C_2H_2 reduction assay. Control assays were also done where the MoS_4^{2-} was added first.

Test 3. Acid-treated Holo I was obtained as described by Nagatani et al. (1974), except that 0.1 M citric acid was used instead of HCl to acidify Holo I. A 20-μL sample of Apo I in 0.1 mL of MD buffer was incubated with 0.1 mL of acid-treated Holo I in the presence and absence of component II. After a 5-min incubation at 30 °C, 0.2 mM $(NH_4)_2MoS_4$ in H_2O was added to a final concentration of 10 nM. The assays were again incubated for 5 min at 30 °C and then assayed for C_2H_2 reduction activity. The acid-treated Holo I had no activity in the presence of excess component II.

Heat Stability of Apo I. Nine-milliliter anaerobic vials were prepared and rinsed with 0.3 mL of anaerobic buffer, and 0.1 mL of MD buffer and 20 μL of Apo I in 20% glycerol were added to each. The vials were then incubated at the temperatures indicated for 5 min. All vials were removed from their water baths and brought to room temperature, after which FeMoco activation assays were performed. Tests with Holo I were performed in a similar manner.

Oxygen Stability of Apo I. A 0.8-mL sample of Apo I was stripped of $Na_2S_2O_4$ in a glovebox, containing less than 1 ppm O_2, by using a PD10 column equilibrated with degassed 25 mM Mops (pH 7.4) and 0.1 mM $Na_2S_2O_4$. The vial containing the stripped Apo I fraction was fitted with a serum stopper, removed from the glovebox, and evacuated on a gassing manifold. Air was injected into the vial to bring it to atmospheric pressure, and the Apo I fraction was incubated in a water bath at 30 °C with vigorous shaking. Twenty-microliter portions were removed from the vial and injected into anaerobic vials containing 0.1 mL of 25 mM Mops (pH 7.4) and 7 mM $Na_2S_2O_4$. After the experiment was complete, all vials were tested for FeMoco activation activity. Another set of aerobic incubations was done in the presence of 1 mM dithiothreitol (DTT). Tests with Holo I were performed in a similar manner.

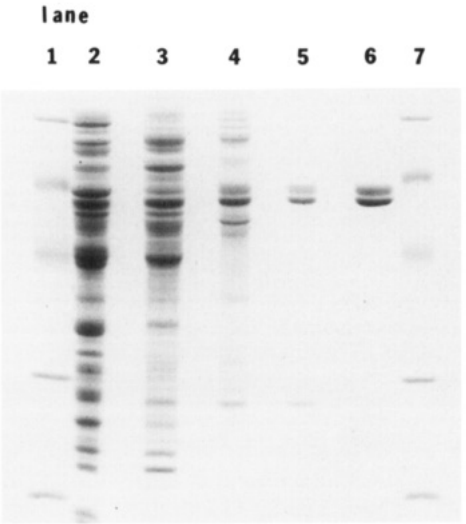

FIGURE 1: One-dimensional SDS gel of Apo I fractions from each purification step. The running gel was 12% polyacrylamide. After electrophoresis the gel was stained with Coomassie blue 250. Lanes 1 and 7, molecular weight standards (20 μg); lane 2, UW45 crude extract (60 μg); lane 3, DEAE-cellulose (22 μg); lane 4, reactive red 120/hydroxylapatite (7.5 μg); lane 5, PL-SAX (5 μg); lane 6, purified Holo I (7.5 μg). The standards used were phosphorylase B (93 kDa), albumin (67 kDa), ovalbumin (45 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.5 kDa).

Results

A summary of the purification of Apo I is shown in Table I. The protocol involved four steps: a DEAE-cellulose weak ion-exchange column, a reactive red 120 agarose column, a hydroxylapatite column, and an HPLC strong anion-exchange column. While the addition of 20% glycerol and the use of low temperature helped to reduce the instability of Apo I, substantial activity was still being lost during our earlier attempts to purify the enzyme. This problem was solved by the addition of the protease inhibitors phenylmethanesulfonyl fluoride and leupeptin, which protected the protein. The purification is routinely accomplished in 3 days with a 30–40% recovery of active Apo I, having a specific activity of 2200–2400 nmol of C_2H_2 reduced/(min·mg of protein).

Composition of the Apo I Protein. Figure 1 shows an SDS–polyacrylamide gel of active fractions from each step in the purification protocol. After the hydroxylapatite step Apo I is >70% of the sample with only a few major contaminants that are removed by the PL-SAX column (Figure 1). A densitometry scan of the purified protein shows equimolar concentrations of the NifK and NifD polypeptides (the products of the *nifK* and *-D* genes), and these coelectrophorese

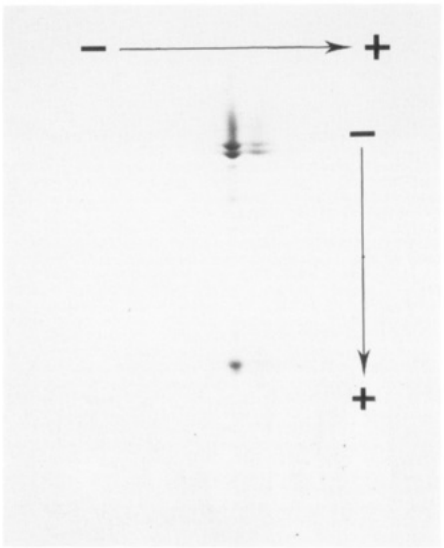

FIGURE 2: Native/SDS two-dimensional polyacrylamide gel of pure Apo I. Twenty micrograms of Apo I was electrophoresed on a native anaerobic slab gel. The lane was then sliced lengthwise and run on an SDS gel. Native gel electrophoresis is represented horizontally; SDS–PAGE is represented vertically.

with NifK and -D from purified Holo I (Figure 1, lanes 5 and 6). Surprisingly, another band of approximately 20 kDa is also present in pure Apo I. This protein, hereafter referred to as the 20K peptide, was present throughout the purification and also cochromatographed with Apo I on a Sephacryl S-300 gel filtration column (data not shown). To further test whether this protein was complexed with Apo I, a native/SDS two-dimensional gel was used as follows: a portion of the purified protein was subjected to anaerobic native PAGE on a slab gel, and the lane containing Apo I was then cut out lengthwise, placed on top of a discontinuous slab SDS–PAGE, and run in a second dimension. The native/SDS two-dimensional gel revealed that Apo I resolves into two bands during native gel electrophoresis (Figure 2). In the second dimension these bands each separate into three protein spots. Two of the spots correspond to the NifK and -D proteins, while the third spot is the 20K protein. A densitometry scan of a one-dimensional SDS gel of pure Apo I revealed that the NifK and -D and the 20K peptide proteins are present in approximately equal molar amounts. All the above evidence taken together strongly suggests that the 20K peptide is tightly bound to the Apo I complex in $\alpha_2\beta_2\delta_2$ hexamer, and this complex will be operationally defined as Apo I in the rest of the paper.

We employed the native/SDS two-dimensional gel described above as a way of assaying the dissociation of the 20K peptide from Apo I. We hoped to dissociate the 20K peptide from the NifKD complex by various treatments and then test the separated proteins for their roles in FeMoco activation. The following treatments neither dissociated the 20K peptide from Apo I nor destroyed FeMoco activatability: temperatures of 45 and 55 °C; treatment with 0.01–0.05% *N*-tetradecylsulfobetaine 14; incubation with 0.3 M NaBr (a chaotropic salt); incubation with DTT or β-mercaptoethanol; or incubation with 2 or 4 M urea. However, 6 M urea did dissociate the NifKD complex from the 20K peptide. Apo I was then subjected to treatment with 6 M urea and chromatographed on a Sephacryl S-300 gel filtration column developed with MGD, which separated the Apo I complex from the 20K peptide and also removed the urea. When assayed for FeMoco activation, neither the NifKD complex nor the 20K peptide had activity, nor could activity be detected when the two

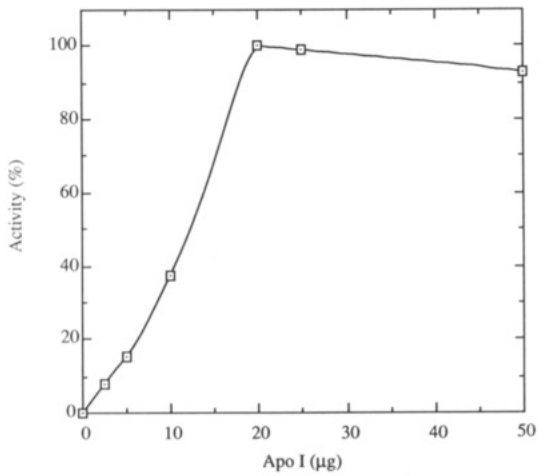

FIGURE 3: Titration of FeMoco with Apo I. A constant amount of FeMoco was titrated with increasing amounts of Apo I by using the standard activation assay. One hundred percent activity corresponds to 690 nmol of C_2H_2 reduced in a 15-min assay.

fractions were mixed together with FeMoco. Apparently, the 6 M urea destroyed the activatability of the proteins during dissociation, and it could not be restored.

Since Holo I, as typically purified, lacks the 20K peptide, it was reasonable to expect that the in vitro activation of Apo I with FeMoco might decrease the affinity of the factor for the NifKD complex. When FeMoco-activated Apo I was analyzed by the native/SDS two-dimensional gel system, no dissociation was observed (data not shown). Also, when FeMoco-activated Apo I was subjected to reactive red 120 or PL-SAX chromatography, the 20K peptide remained bound to the NifKD complex (data not shown).

Metal Analysis of Apo I. When Apo I protein and Holo I were analyzed for Fe and Mo, Apo I was found to have 15 ± 2 mol of Fe and no Mo per mole of protein, while Holo I contained 33 ± 4 mol of Fe and 2 ± 0.25 mol of Mo per mole of component I. The values for Holo I are in good agreement with previous reports of the metal content for this protein (Shah et al., 1984). The limit of detectability for Fe was 0.026 ppm and for Mo was 0.021 ppm, which corresponds to 0.4 mol of Fe and 0.2 mol of Mo per mole of protein.

Titration of FeMoco with Apo I. A titration of FeMoco with Apo I is shown in Figure 3. The activation of Apo I is linear up to about 700 nmol of C_2H_2 reduced in a 15-min assay, at which point the FeMoco is completely utilized, and the protein was activated in this titration to a maximum specific activity of 2300 units/mg of protein. The curve shows a somewhat sigmoidal response to Apo I addition. This concentration dependence was observed in all the assays throughout the purification of the protein. Low activity at very low concentrations of Apo I is likely due to the inactivation of Apo I by *N*-methylformamide (NMF), the solvent used for FeMoco isolation (see below).

Requirements for Activation of Apo I in Vitro. The purified Apo I preparation from mutant UW45 is capable of being activated in the assay described without the addition of any other proteins or factors. This indicates that no other component or factor from the crude cell extract is required to activate Apo I in vitro. However, the assay we are using is performed in two parts. In the first part, Apo I is activated by incubation for 5 min with FeMoco. In the second part, the amount of activated Apo I is measured by an C_2H_2 reduction assay that requires the addition of component II and ATP–$Na_2S_2O_4$. It is therefore possible that component II may be necessary for activation and, during the second part of the

Table II: Requirements for Activation of Apo I by FeMoco[a]

initial addition to Apo I	first treatment	second treatment	act. (nmol)[c]
	Test 1		
	FeMoco	DEAE	180
component II	FeMoco	DEAE	252
	Test 2		
	FeMoco	MoS_4^{2-}	120
	MoS_4^{2-}	FeMoco	9
component II	FeMoco	MoS_4^{2-}	268
component II	MoS_4^{2-}	FeMoco	14
component II, oxidized	FeMoco	$MosS_4^{2-}$	124
component II, oxidized	MoS_4^{2-}	FeMoco	3
BSA	FeMoco	MoS_4^{2-}	124
ATP–Mg^{2+}	FeMoco	MoS_4^{2-}	123
component II + ATP–Mg^{2+}	FeMoco	MoS_4^{2-}	277

[a] The factors to be tested were first mixed with Apo I and then incubated in the presence of FeMoco or MoS_4^{2-} for 5 min at 30 °C. After the first treatment, a DEAE column (test 1) or incubation with MoS_4^{2-} or FeMoco for 5 min at 30 °C (test 2) was used to stop the activation reaction. MoS_4^{2-} was added in the first incubation to demonstrate its ability to block FeMoco activation. [b] A DEAE-cellulose column was used to separate FeMoco from Apo I in test 1. [c] Ten micrograms of Apo I was used for each activity assay, in test 2. In test 1, 0.1 mL of DEAE eluate was assayed (see Materials and Methods). The values reported are nmol of C_2H_2 reduced in a 15-min incubation.

assay, could be activating Apo I with FeMoco. The assay we are using would not differentiate between these two possibilities, so the following two experiments were done.

In the first experiment, Apo I was mixed with an excess of FeMoco in the presence and absence of component II. After a 5-min incubation, the mixtures were passed over an anaerobic DEAE-cellulose column and the reconstituted Apo I eluted with 0.2 M NaCl in MGD. Under these conditions free FeMoco and component II stay bound to the column (Shah et al., 1985). The eluted, FeMoco-activated Apo I was then assayed for C_2H_2 reduction activity. Apo I, reconstituted both with and without component II, had activity, but the activity of the reaction including component II was significantly higher (Table II). The protein concentration of the two eluted fractions was the same.

In the second experiment, the compound $(NH_4)_2MoS_4$ was used to inhibit FeMoco insertion, providing a way to quench the activation reaction. From previous work it is known that MoS_4^{2-} binds to Apo I, blocking activation by FeMoco (Shah et al., 1985), and inhibits Apo I activation 93% when present at concentrations of >10 nM. Apo I was mixed with FeMoco with and without component II present. After a 5-min incubation, $(NH_4)_2MoS_4$ was added to a concentration of 10 nM and incubated another 5 min. The MoS_4^{2-}-treated Apo I was then assayed for activity. Again, FeMoco by itself was able to activate pure Apo I, but the addition of component II increased the degree of activation (Table II). Oxygen-inactivated component II and bovine serum albumin were not able to replace active component II. The addition of ATP–Mg in the activation reaction with or without component II had no effect.

From the above two experiments it is clear that extra factors are not required for the activation of Apo I with FeMoco, but the addition of component II stimulated the activation. One possible explanation for this phenomenon would be that Apo I is unstable in the presence of NMF, the solvent into which FeMoco is extracted, and that component II protects Apo I from this inactivation. To test this hypothesis, Apo I was incubated in 4% NMF (the concentration used to provide FeMoco in the assays), in the presence and absence of component II. If NMF is denaturing the protein and component

Table III: Role of Component II in Protection of Apo I from NMF

initial addition to Apo I[a]	first incubation[b]	first incubation time (min)	second incubation[c]	act. (nmol)[d]
	FeMoco	5	NMF	178
	NMF	5	FeMoco	130
component II	FeMoco	5	NMF	243
component II	NMF	5	FeMoco	207
	FeMoco	20	NMF	165
	NMF	20	FeMoco	67
component II	FeMoco	20	NMF	224
component II	NMF	20	FeMoco	162
	H^+–Holo I	5	MoS_4^{2-}	292
component II	H^+–Holo I	5	MoS_4^{2-}	301
component II (no Apo I)	H^+–Holo I	5	MoS_4^{2-}	0

[a] Twenty micrograms of component II was added where indicated. [b] Five microliters of FeMoco or NMF was added, and the samples were incubated at 30 °C for the indicated time. H^+–Holo I is acid-treated crystalline Holo I. [c] Five microliters of FeMoco or NMF was added, and the samples were incubated at 30 °C for 5 min and an C_2H_2 reduction assay was performed. [d] Ten micrograms of Apo I was used for each activity assay. The values reported are nmol of C_2H_2 reduced in a 15-min incubation.

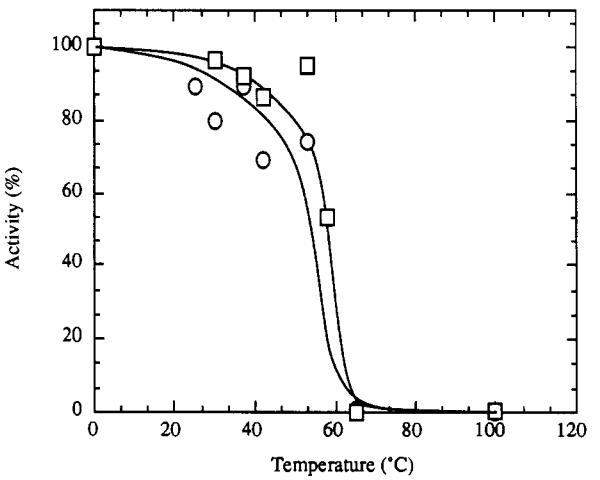

FIGURE 4: Heat stability of Apo I. Apo I, or Holo I, was diluted into MD buffer and incubated at the indicated temperature for 5 min. The vials were cooled and then assayed for activation activity (Apo I) or C_2H_2 reduction activity (Holo I). ○, Apo I; □, Holo I. One hundred percent activity for the amounts of each protein used is 473 nmol of C_2H_2 reduced (Apo I) or 2400 nmol of C_2H_2 reduced (Holo I) in a 15-min assay.

II protects the enzyme, the rate of inactivation of Apo I without component II should be faster than the rate of inactivation in the presence of component II. As shown in Table III, Apo I is rapidly inactivated in the presence of NMF and this inactivation is time dependent. Interestingly, addition of FeMoco before NMF protects Apo I from inactivation, suggesting that Holo I is more stable in NMF than Apo I. The inclusion of component II also protects Apo I, which indicates that component II is physically interacting with Apo I and preventing its inactivation. If component II and FeMoco are both added before NMF, maximum protection is observed.

To test whether component II still had an enhancing effect on activation of Apo I in the absence of NMF, the following experiment was performed. Apo I was activated with acid-treated Holo I, an aqueous source of FeMoco, in the presence and absence of component II. Under these conditions component II did not have an enhancing effect upon the activation of Apo I (Table III).

Stability of Apo I to Heat and O_2. Apo I is stable up to temperatures of 55 °C and then rapidly denatures (Figure 4). Apo I and Holo I were found to have very similar denaturation curves, with Apo I being only slightly less stable than Holo

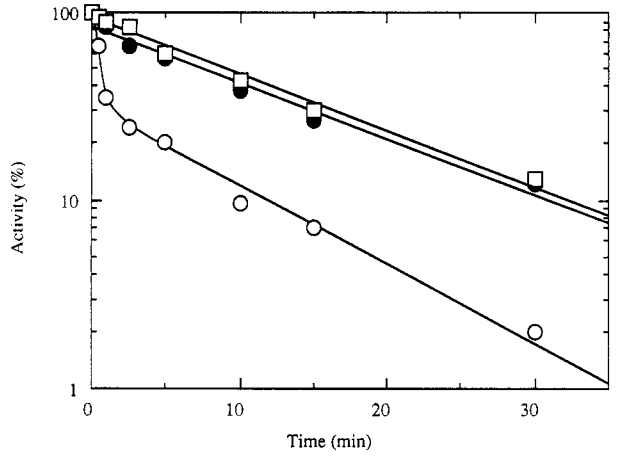

FIGURE 5: O_2 denaturation of Apo I. A sample of Apo I in 25 mM Mops (pH 7.4) and 0.1 mM $Na_2S_2O_4$ was exposed to O_2 in the presence or absence of 1 mM DTT. Samples (20 μL) were taken at the indicated times and injected into 0.1 mL of 25 mM Mops (pH 7.4) and 7 mM $Na_2S_2O_4$. After the last time point, FeMoco was added to each vial and an activation assay was performed. ○, Apo I, no DTT; ●, Apo I + DTT; □, Holo I. One hundred percent activity for the amounts of each protein used corresponds to 323 nmol of C_2H_2 reduced (Apo I) or 1550 nmol of C_2H_2 reduced (Holo I) in a 15-min assay.

I. This suggests that FeMoco may not be involved in interactions that are important for the structural integrity of the $\alpha_2\beta_2\delta_2$ complex.

Apo I rapidly loses activatability when exposed to O_2 with a half-life of 6 min, but the inactivation is biphasic in nature with a rapid drop to 50% activity and then a slower decline (Figure 5). The addition of DTT to the buffer during the oxygen treatment experiment blocked this rapid inactivation, and the protein then had a half-life identical with that of Holo I (11 min for both). Inactivation of Apo I could also be reversed by the addition of DTT after exposure to O_2, and the level of activity was the same as if DTT was present during inactivation. The DTT is likely preventing the oxidation of a free sulfhydryl on the Apo I complex.

Discussion

Apo I was purified 96-fold to a specific activity of 2200–2400 nmol of C_2H_2 reduced/(min·mg of protein), with an overall recovery of 30–40%. The inclusion of glycerol in the buffers and the addition of protease inhibitors stabilized Apo I, allowing its purification. The protein was judged to be >95% pure by one-dimensional SDS–polyacrylamide gel electrophoresis. This analysis also revealed the presence of a 20-kDa peptide associated with the NifK and -D proteins, forming an $\alpha_2\beta_2\delta_2$ complex. The complex had 15 ± 2 mol of Fe per mole of Apo I and no Mo. The complex was very oxygen-sensitive, but the addition of DTT increased the half-life of Apo I to that of Holo I.

Glycerol is known to have a stabilizing influence on many proteins, and it is able to enhance the stability of the Apo I protein during its purification. However, even in the presence of glycerol, the Apo I protein still lost activity. The addition of phenylmethanesulfonyl fluoride and leupeptin to the crude extract prevented this inactivation and stabilized the protein completely. The presence of proteases in the crude extract may explain the previously observed instability of Apo I from *K. pneumoniae* (Hawkes & Smith, 1983). If purified Apo I was added to a crude UW45 extract in the absence of protease inhibitors and electrophoresed on a two-dimensional native/SDS gel, the peptides corresponding to NifK and -D and the 20K protein were degraded and could not be located, while the background pattern of protein spots remained the same. If protease inhibitors were included in the UW45 extract, before the addition of Apo I, and the mixture was electrophoresed in an identical manner, the three Apo I spots were located in similar positions in the gel as those shown in Figure 2 (data not shown). These experiments suggest that Apo I is more susceptible to protease degradation than other proteins in the extract, and this may indicate a site on Apo I that is hypersensitive to protease attack.

The purified Apo I protein has equimolar amounts of the NifK and -D proteins but also includes another peptide of ~20 kDa, tightly bound to the Apo I complex, forming an $\alpha_2\beta_2\delta_2$ hexamer. We were unable to dissociate the 20K peptide from the NifKD complex and still retain FeMoco activation activity, nor were we able to demonstrate a lessened affinity between the 20K peptide and NifKD after FeMoco addition, which leaves the question of why this protein is present in Apo I and not in Holo I. It is possible that an enzyme in *nif*-derepressed cells is responsible for the removal of the 20K peptide after FeMoco activation and we did not have it present in our tests. Interestingly, the purification protocol of Apo I from *K. pneumoniae* also yielded a low molecular weight protein copurifying with Apo I (Hawkes & Smith, 1983). The appearance of a similar peptide in another unrelated organism suggests that the 20K peptide is performing some function for Apo I and is not an artifact of the purification method. It was an obvious possibility that the 20K peptide was encoded by one of the *nif* genes. We therefore separated the 20K peptide on an SDS gel and microsequenced the protein. Comparison of the first five amino acids of the amino terminus to the DNA sequence of known *nif* genes suggests that the protein is not *nif*-encoded. It is possible that the 20K peptide is one of those associated with a nitrogenase complex as isolated by Shethna (Shethna et al., 1968) and others (Haaker & Veeger, 1977), but definitive assignment will require further analysis.

Metal analysis of Apo I revealed the presence of 15 ± 2 mol of Fe per mole of Apo I and no Mo. If the amount of Fe in Apo I is subtracted from the amount in Holo I, an estimate of 18 ± 2 Fe are present in the FeMoco molecule, with the remaining Fe being found in the P-clusters. This analysis is dependent upon the accuracy of the protein concentration measurement and is subject to error. It is important to remember that the Apo I complex also contains two 20K peptides and Fe may also be associated with these proteins. Measurement of the metal content of Apo I from *K. pneumoniae* revealed the presence of 0.4–0.9 mol of Mo per mole of Apo I (Hawkes & Smith, 1983). Our results show that Apo I isolated from an *A. vinelandii nifB* point mutant does not contain Mo. It is possible that the difference in bacterial species could explain this inconsistency, but we consider this unlikely.

It was recently reported that the Mg–ATP–component II complex is involved in FeMoco insertion (Robinson et al., 1989). The purification of Apo I enabled us to quantitatively test whether this or other factors in the crude cell extract are necessary for the insertion of FeMoco. It was possible to activate Apo I with FeMoco alone, suggesting that no other proteins from the crude cell extract are necessary for FeMoco insertion. However, after the activation, component II is needed to measure C_2H_2 reducing activity in the second part of the assay. It is therefore possible that component II is involved in FeMoco activation, and it was necessary to separate these two steps of the assay to test this hypothesis. Using two different techniques to eliminate the possibility of FeMoco activation during the C_2H_2 reduction assay (removal of Fe-

Moco by DE-52 chromatography or blockage of FeMoco activation with MoS_4^{2-}), we have demonstrated that component II is not required for the activation of Apo I.

However, the presence of component II was observed to enhance the activation of Apo I. Examination of this enhancement showed that (i) Apo I is more sensitive to NMF than Holo I, (ii) component II protects Apo I from inactivation by NMF, and (iii) if acid-treated Holo I is used as a source of FeMoco, instead of NMF-extracted Holo I, no component II effect on Apo I activation is observed (Table III). These results indicate that the enhancement of activation by component II can be attributed entirely to protection of Apo I from NMF. The addition of Mg–ATP in the activation reaction, with or without component II, had no effect on activation of Apo I by FeMoco. Robinson et al. (1989) claim the insertion of FeMoco into Apo I involves a component II–Mg–ATP complex binding to Apo I. Our results are inconsistent with their conclusions. However, the Apo I we have characterized was isolated from UW45, an *A. vinelandii* strain, containing a mutation in the *nifB* gene. Robinson et al. (1989) investigated Apo I in a strain containing a mutation in the *nifH* gene. This difference in strain background may explain the difference in results obtained.

The protection of Apo I from NMF by component II, even in the absence of FeMoco activation, strongly suggests some physical interaction between these two proteins. This interaction is occurring in the presence or absence of Mg–ATP. Evidence for a Mg–ATP-independent interaction of component II and Holo I has also been presented recently, and our results are consistent with these findings (Willing et al., 1989).

Investigation into the stability of Apo I revealed that the protein is nearly as heat-stable as Holo I but is much more O_2-sensitive. The presence of DTT during O_2 exposure blocked the rapid oxidation of Apo I. The complex could also be reactivated by the addition of DTT after inactivation in the presence of O_2. The above results suggest that a sulfhydryl group on the Apo I complex is required for activatability, and it will be interesting to discover the location of this group. The denaturation of Apo I in the presence of air is biphasic, with a rapid decline in activity to approximately 50%, and then a slower loss in activity upon further incubation. This suggests that there are two discrete types of oxidation affecting the molecule. Given the complexity of the coupled assay and the range of possibilities, it is difficult to speculate on this phenomena with specific models. In the presence of DTT, Apo I has a half-life in air similar to that observed for Holo I. This indicates that FeMoco is not the most O_2-sensitive part of the Holo I complex and that something else, possibly the P-clusters, is more rapidly destroyed in the presence of O_2.

It is now possible to rapidly purify milligram quantities of pure Apo I that can be activated to >2200 nmol of C_2H_2 reduced/(min·mg of protein). With the pure protein in hand we will be able to further investigate FeMoco synthesis and also perhaps identify the point of FeMoco attachment.

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Registry No. Nitrogenase iron–molybdenum cofactor, 72994-52-6.

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Biosynthesis of Quinoxaline Antibiotics: Purification and Characterization of the Quinoxaline-2-carboxylic Acid Activating Enzyme from *Streptomyces triostinicus*†

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ABSTRACT: A quinoxaline-2-carboxylic acid activating enzyme was purified to homogeneity from triostin-producing *Streptomyces triostinicus*. It could also be purified from quinomycin-producing *Streptomyces echinatus*. Triostins and quinomycins are peptide lactones that contain quinoxaline-2-carboxylic acid as chromophoric moiety. The enzyme catalyzes the ATP–pyrophosphate exchange reaction dependent on quinoxaline-2-carboxylic acid and the formation of the corresponding adenylate. Besides quinoxaline-2-carboxylic acid, the enzyme also catalyzes the formation of adenylates from quinoline-2-carboxylic acid and thieno[3,2-*b*]pyridine-5-carboxylic acid. No adenylates were seen from quinoline-3-carboxylic acid, quinoline-4-carboxylic acid, pyridine-2-carboxylic acid, and 2-pyrazinecarboxylic acid. Previous work [Gauvreau, D., & Waring, M. J. (1984) *Can. J. Microbiol. 30*, 439–450] revealed that quinoline-2-carboxylic acid and thieno[3,2-*b*]pyridine-5-carboxylic acid became efficiently incorporated into the corresponding quinoxaline antibiotic analogues in vivo. Together with the data described here, this suggests that the enzyme is part of the quinoxaline antibiotics synthesizing enzyme system. The enzyme displays a native molecular weight of 42 000, whereas in its denatured form it is a polypeptide of M_r 52 000–53 000. It resembles in its behavior actinomycin synthetase I, the chromophore activating enzyme involved in actinomycin biosynthesis [Keller, U., Kleinkauf, H., & Zocher, R. (1984) *Biochemistry 23*, 1479–1484].

Quinoxaline antibiotics are chromodepsipeptides produced by several *Streptomyces* strains. They can be divided into two groups, namely, the triostins and the quinomycins (Okumura, 1983). Both groups of compounds possess the same peptide backbone characterized by the antiparallel arrangement of two quinoxaline-2-carboxylic acid tetrapeptides which are connected with each other via ester bonds to form octadepsipeptide rings as shown in Figure 1. A peculiar characteristic of these compounds is that they each contain a cross bridge which arises in the case of triostins by formation of a disulfide bridge between the *N*-methylcysteine residues present in the peptide chains. Correspondingly, in the case of quinomycins the cross bridge is formed by a dithioacetal linkage between a *N*-methylcysteine and a *N*,*S*-dimethylcysteine residue (Dell et al., 1975). Both types of antibiotics are active against Gram-positive bacteria and display inhibitory activity against a variety of tumors (Lee & Waring, 1978; Waring & Wakelin, 1974).

Studies on the biosynthesis of quinoxaline antibiotics revealed that the constituent amino acids are derived from the naturally occurring ones. The quinoxaline-2-carboxylic acid portion of the antibiotics is derived from tryptophan, whereas the methyl groups of the *N*-methyl amino acids are donated by methionine (Yoshida & Katagiri, 1969). The biosynthetic relationship between triostins and quinomycins has been established by showing that protoplasts of *Streptomyces echinatus* are able to convert externally added triostin A into quinomycin A (Cornish et al., 1983). It is suggested that the thioacetal cross bridge in the quinomycins arises from the methylation of the disulfide bond in triostin with *S*-adenosyl-L-methionine. A cell-free system of quinomycin A (echinomycin) biosynthesis has been described (Arif et al., 1970). However, a characterization of the enzymes involved in the various biosynthetic steps has as yet not been presented.

Studies on the controlled biosynthesis in vivo of both triostins and quinomycins have shown that new compounds could be obtained when intact mycelia of *Streptomyces triostinicus* or *S. echinatus* were fed with several structural analogues of the chromophore quinoxaline-2-carboxylic acid (Gauvreau & Waring, 1984a,b; Santikarn et al., 1983). Among others, quinoline-2-carboxylic acid or thieno[3,2-*b*]pyridine-5-carboxylic acid (Figure 2) was efficiently incorporated into the corresponding triostin or quinomycin analogue. The data indicate that quinoxaline-2-carboxylic acid and its analogues act as free intermediates in the biosynthetic process. It appears to be likely that they have to be activated by a specific enzyme belonging to the enzyme system responsible for the biosynthesis of quinoxaline antibiotics.

During the biosynthesis of the bicyclic chromopentapeptide lactone actinomycin in *Streptomyces chrysomallus*, the chromophoric precursor of these antibiotics, 4-methyl-3-hydroxyanthranilic acid (4-MHA) has been shown to be a free intermediate that is activated as an adenylate. The activating enzyme in this case has a M_r between 52 000 and 55 000 and

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