procedure in terms of the reagent used and of the chemical and structural features of its target site. The derivative described here, AENS-RNase, with its single probe located in a specific site which makes it sensitive to changes in the environment, can obviously be further utilized to investigate various equilibrium or kinetic aspects of protein folding through measurements of its different fluorescence properties.

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Hydroxylamine Oxidoreductase: A 20-Heme, 200 000 Molecular Weight Cytochrome *c* with Unusual Denaturation Properties Which Forms a 63 000 Molecular Weight Monomer after Heme Removal†

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ABSTRACT: Hydroxylamine oxidoreductase catalyzes the successive dehydrogenation and oxygenation of NH_2OH to HNO_2. Molecular weight (M_r) values of 180 000–200 000 have been reported for the enzyme. Per M_r 200 000, the enzyme contains 18 *c*-type and approximately 2 P-460 hemes [Hooper, A. B., Maxwell, P. C., & Terry, K. R. (1978) *Biochemistry 17*, 2984]. Boiling the enzyme in the presence of 2% sodium dodecyl sulfate ($NaDodSO_4$) and 5% 2-mercaptoethanol followed by polyacrylamide gel electrophoresis in 0.1% $NaDodSO_4$ resulted in five bands with the following designations and molecular weights: I, 225 000; II, 195 000; III, 125 000; IV, 56 500; V, 11 000. Bands I–III and V contained heme. Partial proteolysis of bands I–III produced identical fragments. The ratio of bands I:II:III depended on the presence or absence of 2-mercaptoethanol and the length of heating in $NaDodSO_4$. Production of band I required prolonged treatment and occurred in two discrete steps; bands II and III were apparently intermediates in a sequential denaturation or polymerization. Band IV, which was always present in less than one molecule per molecule of large subunit, was either a contaminant or a hemeless fragment of the large polypeptide. Band V, which was released from the enzyme in $NaDodSO_4$ without 2-mercaptoethanol, was present in a ratio of three or four molecules per molecule of large polypeptide. Some samples of purified enzyme contained heme P-460, 20 molecules of *c*-type heme, and full enzymatic activity, though lacking band IV or V. Further, bands I–III exhibited some enzyme activity on $NaDodSO_4$ gels. Thus the large polypeptide contains all factors necessary for catalysis. Treatment of the enzyme or band I with 2-nitrophenylsulfenyl chloride, which removes thioether-linked heme, resulted in the formation of a M_r 63 000 polypeptide in approximately a 1:1 molar ratio with the band V cytochrome. We conclude that, as isolated, hydroxylamine oxidoreductase probably consists of three molecules of a monoheme *c*-type cytochrome, M_r 11 000, and three tightly complexed molecules of a catalytically active M_r 63 000 protein containing six *c*-type hemes and one P-460 heme.

In nature, essentially all ammonia in aerobic soils or waters is rapidly oxidized to nitrite by the nitrifying bacteria. The nitrifying bacterium *Nitrosomonas europaea* grows autotrophically, utilizing the oxidation of ammonia to nitrite as the sole energy source. Hydroxylamine, or a closely related chemical species which is enzyme bound, is an intermediate in the process. The enzyme hydroxylamine oxidoreductase from *Nitrosomonas* catalyzes the rapid aerobic oxidation of hydroxylamine to nitrite in the presence of phenazine methosulfate (Hooper, 1978). Molecular weight (M_r)[1] values in the range 180 000–200 000 have been reported for the enzyme (Rees, 1968; Maxwell, 1976; Hooper et al., 1978; Yamanaka et al., 1979). On the basis of a particle weight of 200 000, Hooper et al. (1978) estimated that the enzyme contains 18 *c*-type hemes and 2 hemes of a unique CO-binding P-460. The subunit composition of the enzyme is unknown. The present paper describes the dissociation and denaturation pattern and subunit composition of hydroxylamine oxidoreductase based on analysis by polyacrylamide gel electrophoresis in NaDod-

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[1] Abbreviations used: M_r, molecular weight; $NaDodSO_4$, sodium dodecyl sulfate (SDS in figures); sulfenyl chloride, 2-nitrophenylsulfenyl chloride; Tris, tris(hydroxymethyl)aminomethane.

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SO_4. The largest portion of the enzyme is shown to be very resistant to denaturing conditions and to proteolysis. Denaturation occurred in a stepwise fashion giving bands of progressively decreasing mobility on $NaDodSO_4$, indicating stepwise denaturation or polymerization. Chemical removal of the *c*-type heme led to the formation of a single band, M_r 63 000, allowing for the first time a description of the basic repeating structural units of the enzyme. A preliminary report of this work has appeared (Terry et al., 1979).

Experimental Procedures

Growth of Bacteria and Purification of Enzyme. Cells were grown in 90-L batch cultures (Hooper et al., 1972). The cells used for one of the enzyme preparations (sample 5, Table III) were grown for several weeks in semicontinuous fashion in 20-L vessels; on alternate days three-fourths of the volume was harvested and the vessel refilled with sterile media. Enzyme was routinely purified by repeated precipitation in ammonium sulfate, followed by isoelectric focusing on a granulated gel bed (Hooper et al., 1978).

Polyacrylamide Gel Electrophoresis in $NaDodSO_4$. Electrophoresis was carried out in 5 × 100 mm tubes containing 5–12% polyacrylamide [acrylamide:bis(acrylamide) = 30:0.8] (Weber & Osborn, 1969) or in 130 × 95 × 1 mm slabs containing 5–15% polyacrylamide according to the method of Laemmli (1970) except that the gels were overlaid with 5 mm of 3% polyacrylamide. For visualization of both large and small polypeptides in one electrophoresis run, the gel was formed in two steps: 30 or 40 mm of 12% or 15% polyacrylamide, respectively, overlaid with 65 or 55 mm of 5% or 6% polyacrylamide, respectively. Tubes were run at 4 mA/tube (approximately 100 V) until the tracking dye reached the bottom of the tube, approximately 3 h. Slab gels were run at 17.5 mA (50 V) for approximately 7 h. Both polyacrylamide and electrophoresis buffer contained 0.1% $NaDodSO_4$. Unless otherwise noted, gels were stained with Coomassie Brilliant Blue G (Sigma) according to the technique of Fairbanks et al. (1971). This method gave intense bands and did not result in the overstaining found with other methods. The amount of stain in a particular band (I–IV) was proportional to the amount of enzyme (2–15 μg of protein) applied to the gel. Alternatively, gels were stained with Naphthol Blue Black (Amido Black; Sigma) or Procion Brilliant Blue (Colab Laboratories). The cylindrical gels were scanned at 600 nm in a Gilford Model 2400-S spectrophotometer with recorder and Model 2410-S linear transport cuvette holder at a rate of 2 cm min^{-1}. For estimation of heme content, unstained gels were scanned at 410 nm. Peaks of 410- and 600-nm absorbancy were reproduced by xerography, cut out, and weighed on a Sartorius electric balance. Alternatively, gels were stained with benzidine peroxide (Canalco Bulletin, 1963) to detect the presence of heme.

A two-step slab gel (12–6% polyacrylamide) was prerun with 1 mM hydroquinone in the upper buffer chamber in order to locate enzymatic activity. When the hydroquinone had migrated about 2 cm into the gel, the buffer was replaced with fresh buffer lacking hydroquinone. The enzyme was suspended in 2% $NaDodSO_4$ for 2 h at 37 °C. Protein (10 μg) was applied to the gel, and electrophoresis was carried out at 35 mA until the tracking dye reached the bottom of the gel. The gel was soaked in 50 mM Tris buffer, pH 8.0, containing 0.1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma), 10^{-4} M hydroxylamine hydrochloride, and 5×10^{-6} M phenazine methosulfate.

The standard procedure for protein denaturation involved heating at 100 °C for 3 min in the presence of 2% $NaDodSO_4$ and 5% 2-mercaptoethanol. Alternatively, samples were treated by (a) carboxymethylation with iodoacetic acid, (b) succinylation with succinic anhydride, or (c) suspension in 2–8 M urea or (d) in 6 M guanidine hydrochloride plus 1% mercaptoethanol, followed by dialysis against progressively decreasing concentrations of urea from 8 to 2 M. The guanidine-treated enzyme was electrophoresed in buffer containing 2 M urea.

Molecular weights of subunits were determined under fully denaturing conditions with 7.2 μg of enzyme and 5 μg of the following reference proteins: catalase (Sigma); horse heart cytochrome *c*; ovalbumin; myoglobin; RNA polymerase (gift of Dr. Jerry Jendrisak); myosin (gift of Lawrence Taaffe). Gels of 7% or 15% polyacrylamide were used in the M_r range of (5–25) × 10^4 or (1–6) × 10^4, respectively.

Limited Proteolysis. Limited proteolysis was carried out according to the technique of Cleveland et al. (1977). A 10–50-μg sample of hydroxylamine oxidoreductase that had been dissociated in 2% $NaDodSO_4$ and 5% 2-mercaptoethanol by heating for 30 s was applied in a 5-mm slot to a 6% polyacrylamide gel slab. Subunits were separated following electrophoresis for 6 h with a 17.5-mA current. A slice containing the protein bands was laid across the top of a gel slab formed from 60 mm of 15% polyacrylamide overlaid with 30 mm of 3% polyacrylamide. After the addition of 1% agarose around the gel slice, 4 μg of chymotrypsin (Sigma), 4 μg of *Staphylococcus aureus* V_8 protease, or 1.5 μg of papain (Sigma), each containing 0.01% bromphenol blue as a tracking dye, was layered on top, and a 20-mA current was applied until the dye reached the interface between the 3% and 15% gels. After incubation for 30 min with the current off to allow for proteolysis, the system was run at 5 mA for 16 h and the gel stained with Coomassie Blue.

Heme Removal. Chemical removal of thioether-linked (*c*-type) heme was accomplished by incubation for 10 min with 100 equiv (per heme) of sulfenyl chloride (2-nitrophenylsulfenyl chloride; Sigma Chemical Co.) in 66% acetic acid as described by Fontana et al. (1973). After a 3-fold dilution of the incubation mixture with water, the heme derivative was extracted 4 times with an equal volume of ethyl acetate. The aqueous and ethyl acetate phases were concentrated by drying. Recovery of protein in the aqueous phase was determined by the Lowry et al. (1951) procedure.

Results and Discussion

Progressively Stronger Denaturing Conditions Result in Bands of Progressively Lower Mobility on Polyacrylamide Gel Electrophoresis. The preparation of hydroxylamine oxidoreductase had an apparent molecular weight of 200 000 and was 99% pure by polyacrylamide gel electrophoresis under nondenaturing conditions at several acrylamide concentrations (Hooper et al., 1978). The results of subunit analysis by polyacrylamide gel electrophoresis after varying treatments in the presence of 2% $NaDodSO_4$ are shown in Figure 1 and summarized in Table I. Five bands were observed with designations and molecular weights as follows: I, 225 000; II, 195 000; III, 125 000; IV, 56 500; V, 11 000. The bands did not exhibit subclasses and the molecular weight values remained the same at varying percentages of acrylamide (5–10%, 12%, or 15%).

The amount of protein in each of the higher molecular weight bands was dependent on the treatment of the enzyme prior to polyacrylamide gel electrophoresis. In the presence of $NaDodSO_4$ alone (Figure 1A), the enzyme showed bands of apparent M_r 125 000 (band III), 56 500 (band IV), and 11 000 (band V). Peptides IV and V were not tightly attached

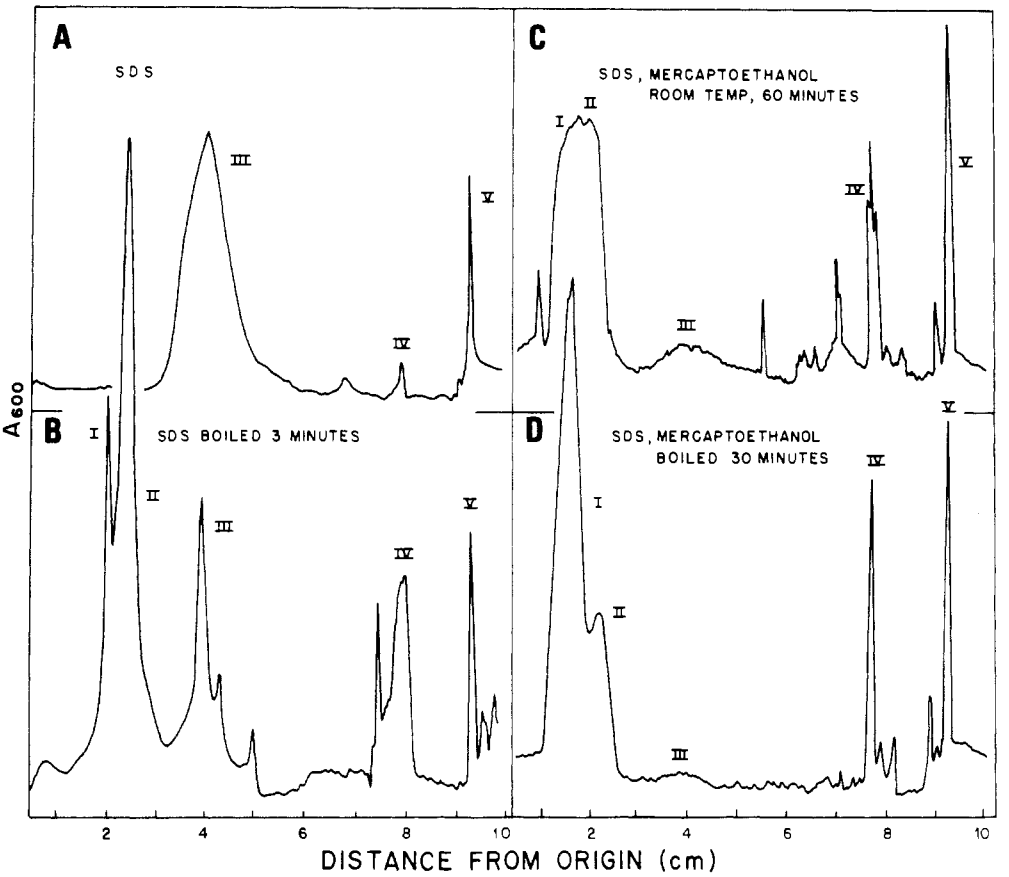

FIGURE 1: Dissociation pattern of hydroxylamine oxidoreductase of *Nitrosomonas*. 7.2 μg of enzyme was treated by (A) suspension in 2% $NaDodSO_4$, (B) suspension in 2% $NaDodSO_4$ at 100 °C for 3 min, (C) suspension in 2% $NaDodSO_4$ plus 5% 2-mercaptoethanol at room temperature for 60 min, and (D) suspension in 2% $NaDodSO_4$ plus 5% 2-mercaptoethanol at 100 °C for 30 min. All samples contained 10% glycerol and 0.01% bromphenol blue. Samples were applied to the top of a two-step cylindrical gel. Electrophoresis was carried out as described under Experimental Procedures. Gels were scanned at a recorder setting of 3.0 OD full scale and a chart speed of 40 s/in.

Table I: Dissociation Pattern of Hydroxylamine Oxidoreductase

band	apparent M_r	heme[a]	% absorption[b] A[c]	B	C	D
I	225 000	0.09	0	16	31	60
II	195 000	0.10	0	42	30	16
III	125 000	0.09	84	17	16	0
IV	56 500	0	3	16	10	10
V	11 000	0.08	13	9	13	14

[a] Ratio of 410-nm absorbancy:600-nm absorbancy measured as under Experimental Procedures. Bands I–III were always red. Benzidine staining and measurement of absorbancy at 410 and 600 nm were carried out on gels run according to protocol D, boiled 30 s. [b] Proportional weight of peak compared to total weight of all peaks. The same amount of protein was applied to each gel. The relative value of total recovered weight was (A) 1.0, (B) 1.4, (C) 1.2, and (D) 1.5. [c] Letters correspond to the pretreatments utilized in panels A–D of Figure 1.

to the enzyme complex since they were dissociated by $NaDodSO_4$ alone. The band V polypeptide was more easily dissociated by $NaDodSO_4$ from the complex than the band IV polypeptide (Table I). In contrast, the larger polypeptide was apparently not fully denatured in $NaDodSO_4$. If the enzyme was boiled for 3 min prior to $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1B), band III was smaller and most of the protein was found in a band corresponding to a M_r of 195 000 (band II). An additional shoulder at M_r 225 000 (band I) appeared, and the band of M_r 56 500 (band IV) increased. Boiling for 20 min or incubation at 37 °C for 18 h caused further diminution in band III (data not shown). Incubation of the enzyme in 2% $NaDodSO_4$ and 5% 2-mercaptoethanol for 1 h at room temperature resulted in a decrease in band III and a corresponding increase in bands I and II (Figure 1C). Boiling the enzyme 30 min in 2% $NaDodSO_4$ and 5% 2-mercaptoethanol (Figure 1D) resulted in a decrease in band II and an increase in band I so that it accounted for 80% of the high molecular weight polypeptide. Significantly, the total recovery of staining material was essentially the same under conditions B–D (Table I).

With 3 min of boiling (2% $NaDodSO_4$ and 5% 2-mercaptoethanol; as in Figure 1D), band I represented approximately 70% of the large polypeptide; after 1 h of boiling, 90% of the stain was in band I, but the total yield of stain on the gel had diminished. Myosin exhibited the same loss of material or reactivity to stain following prolonged boiling. For routine full denaturation, the enzyme was boiled for 3 min. More vigorous treatment, including boiling with 5% dithiothreitol, succinylation, carboxymethylation, or denaturation with guanidine or urea in the presence of 2-mercaptoethanol, resulted in similar dissociation patterns showing only bands I, II, IV, and V.

Bands I–III Consist of the Same Polypeptide. The composition of bands I–III was studied by limited proteolysis with proteases of different specificities. Digestion under the conditions noted under Experimental Procedures yielded at least five peptide fragments for each band. The number and sizes of the peptide fragments from bands I–III were identical, strongly suggesting that band III does not contain two different polypeptides. The yield of proteolytic fragments per unit time was greater, in the order I > II > III, consistent with a pattern of sequential denaturation. The results of limited proteolysis, together with the observation that the large polypeptide can

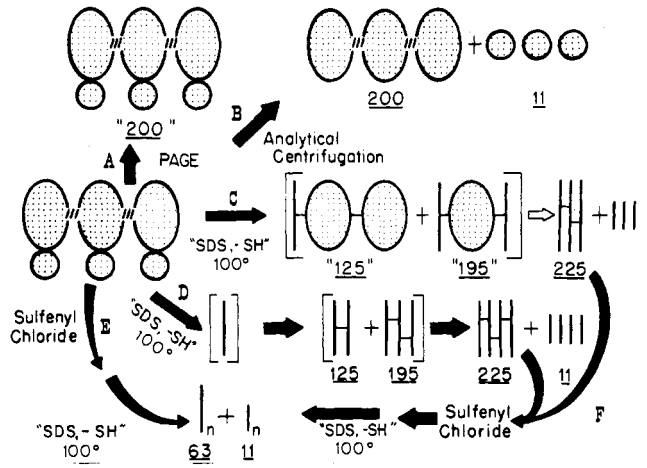

FIGURE 2: Possible pathways for the denaturation and dissociation of hydroxylamine oxidoreductase of *Nitrosomonas*. The filled figures or vertical line figures represent undenatured or denatured forms of putative polypeptides, respectively. Short horizontal lines represent intersubunit bonds; dashed, unusually strong noncovalent bonds; solid, bonds broken after treatment with sulfenyl chloride. "SDS, -SH, 100°" denotes heat treatment in the presence of $NaDodSO_4$ and mercaptoethanol, followed by analysis on polyacrylamide gel electrophoresis (Weber & Osborn, 1969).

Table II: Comparison of Molar Ratios of Subunits Based on Different Protein Stains

stain[a]	staining intensity[b]	molar ratio[c] I and II[d]	IV	V
CB	1	1	0.5	4.4
AB	0.34	1	0.4	4.2
PB	0.07	1	0.6	4.3

[a] CB = Coomassie Brilliant Blue G; AB = Amido Black; PB = Procion Blue. [b] Weight of peaks per microgram of protein, normalized to CB = 1. [c] The weight of each peak was converted to moles by dividing by bands I and II, 225 000 (see text for explanation); band IV, 56 500; and band V, 11 000. [d] A sample of enzyme was dissociated by suspension in 2% $NaDodSO_4$ and 5% 2-mercaptoethanol at 100 °C for 3 min. Under these conditions, band III is not present.

be converted quantitatively to a single form (band I), strongly support the conclusion that bands I–III consist of the same polypeptide. The three forms are apparently interconvertible by a process of denaturation or polymerization (Figure 2C or 2D).

Molar Ratios of Polypeptides. The proportionality of peak weight to protein applied was constant, allowing calculation of approximate molar ratios of the subunits. We have noted previously (Hooper et al., 1978) that this enzyme and cytochrome *c* were overreactive in the Lowry assay as compared with bovine serum albumin. For that reason three different stains were used in order to minimize the possibility of a variation between bands of the staining intensity per milligram of protein. As seen in Table II, although the staining intensity varied over a 14-fold range depending on the stain used, the ratio of subunits in a particular enzyme preparation remained the same. Because bands I and II were shown to contain the same polypeptide with a true M_r of 225 000, the values for those bands were summed. On average of several different enzyme preparations, the ratio of (I + II):IV:V was 1.0:0.5:3.5.

Enzymic Role of Subunits. On a molar basis, the ratio of band IV and V polypeptides to the large polypeptide varied between different enzyme preparations (Table III). Of special interest was sample 5, prepared from cells grown semicontinuously as described under Experimental Procedures. For unknown reasons, the resulting purified enzyme was unusual

Table III: Comparison of Molar Ratios in Various Enzyme Preparations

sample of enzyme by isoelectric focusing[a]	sp act.[b]	molar ratio[c] I and II	IV	V
1[a]	30	1	0.5	4.4
2	23	1	0.4	2.7
3	28	1	0.25	2.2
4	26	1	0.14	3.1
5	18	1	0.12	0.45

[a] Enzyme used for other data in present paper. [b] μmol of nitrite produced min^{-1} (mg of protein)$^{-1}$ in the presence of 10^{-4} M hydroxylamine and 5×10^{-6} M phenazine methosulfate. [c] See Table II.

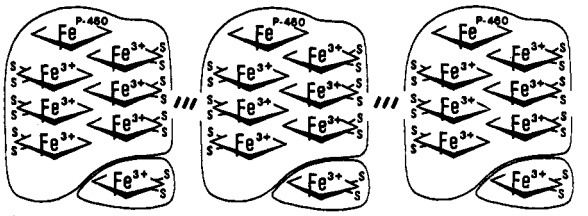

FIGURE 3: Model for subunit composition and distribution of *c*-type and P-460-type hemes of hydroxylamine oxidoreductase of *Nitrosomonas*.

in that it had low molar ratios of subunits IV (0.12) and V (0.45). This was a reproducible phenomenon. Significantly, the initial specific activity and the ratio of heme P-460:*c*-type heme as determined by dithionite-reduced *minus* oxidized absorption spectra were approximately the same as in other samples of enzyme. However, sample 5 was much less stable than all other samples, losing 50% activity every 2 days while stored at 4 °C. These results indicate that, although they have possible stabilizing properties, proteins IV and V are probably not involved directly in the enzyme activity. That conclusion is supported by the fact that band III (Figure 1A) and, to a lesser extent, bands II and I (Figure 1B), but not band IV or V, exhibited hydroxylamine dehydrogenase activity on the gel as indicated by the deposition of reduced formazan of a tetrazolium dye in the presence of hydroxylamine.

Content of c-Type Heme and Heme P-460. Bands I–III and V but not IV were red, reacted positively to benzidine staining, and thus contained heme. In gels which were overloaded with enzyme (28 μg of protein), no trace of blue stain was seen in band IV. The ratio of absorbancy at 410 nm to protein stain was approximately the same in peaks I–III and V and mammalian cytochrome *c* (Table I; Figure 3A,C), indicating a similar ratio of moles of heme:moles of peptide. If the ratio of heme:particle weight is assumed to be the same in each subunit as in the native enzyme (18:200 000; Hooper et al., 1978), proteins I and V have approximately 20 and 1 *c*-type hemes, respectively. The fact that heme P-460 is apparently at or very near the hydroxylamine dehydrogenase site (Hooper & Terry, 1977) and the presence of hydroxylamine dehydrogenase activity in the M_r 225 000 polypeptide allow tentative location of heme P-460 in the large polypeptide.

Nature of Band IV Polypeptide. The amount of band IV polypeptide was variable and less than 1 mol/mol of the large polypeptide. Peptides produced by limited proteolysis of bands I–III included a band equal in mobility to IV. Preliminary results indicated that the peptide products of proteolysis of both the band I polypeptide and band IV polypeptide included smaller peptides of identical molecular weight. Polypeptide IV appears to be either a tightly bound contaminant or a fragment derived from III following breakage of a specific

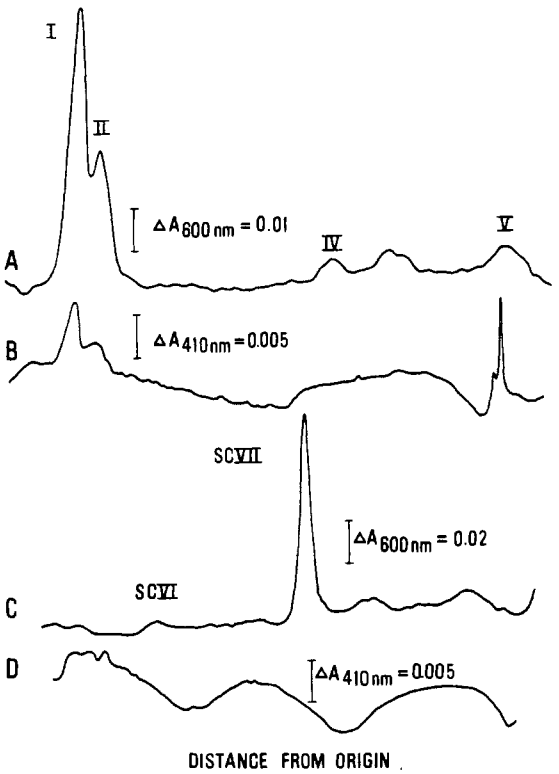

FIGURE 4: Dissociation following treatment with sulfenyl chloride. Electrophoresis was carried out on slabs as described under Experimental Procedures. Enzyme (A, B) or sulfenyl chloride treated enzyme (C, D) was suspended in 2% $NaDodSO_4$ plus 2-mercaptoethanol at 100 °C for 3 min and 5 μm applied to the gel. After electrophoresis, the gels were sliced and each lane was immersed in water and scanned at 410 nm. The gel was then stained with Coomassie Blue as described and scanned again at 600 nm. During immersion in water, the smaller proteins diffused somewhat, resulting in broad peaks. Migration is from left to right.

peptide bond and loss of heme during enzyme purification. Possible precedent is found in the oxidation of a specific histidine residue and breakage of peptide bonds upon treatment of superoxide dismutase with H_2O_2 (Bray et al., 1974). In a process involving oxidative destruction of heme P-460, hydroxylamine oxidoreductase also readily reacts with low concentrations of H_2O_2 (Hooper & Terry, 1977).

Dissociation of Large Polypeptide to M_r 63 000 Fragment by Treatment with Sulfenyl Chloride. If truly a single polypeptide, the M_r 225 000 subunit would be extremely unusual in terms of the large number of *c*-type hemes. On the other hand, if the large subunit is not a single polypeptide, the nature of the linkage holding the components together must be unique. To test the hypothesis that the large subunit is an aggregate held together by forces dependent on the presence of the large number of *c*-type hemes, the subunit composition was determined following the removal of *c*-type hemes with 2-nitrophenylsulfenyl chloride (sulfenyl chloride). This compound reacts quickly and quantitatively with thioether linkages of *c*-type cytochromes to form the nitrophenylcysteine disulfide and release the heme (Fontana et al., 1973). Although sulfenyl chloride also forms a derivative of peptidyl tryptophan, it has the advantage of causing minimal breakage of peptide bonds as compared with other heme removal techniques.

For convenience, preparations of the enzyme with lower contents of bands IV and V were used for characterization of the substructure of the large subunit. Parts A and B of Figure 4 show the distribution of Coomassie Blue staining and heme absorbance, respectively, following polyacrylamide gel electrophoresis of the enzyme under the conditions of Figure 1C with enzyme 5 (Table III). Analysis by polyacrylamide gel electrophoresis of the products of treatment of the enzyme with sulfenyl chloride indicated that heme had been completely removed (less than 0.1 molecule of heme per M_r 11 000, based on the ratio of 410-nm absorbancy:absorbancy of Coomassie Blue with reference to subunit I, II, or III or mammalian cytochrome *c* as standard) from all enzyme fragments (Figure 4B,D). Concomitantly, the high molecular weight bands I, II, and III were almost completely absent. The major product of treatment with sulfenyl chloride was a single band (designated band SC VII) with a particle weight of 62 700 as estimated on the basis of mobility on polyacrylamide gel electrophoresis in comparison with proteins of known molecular weights which had not been treated with sulfenyl chloride. The SC VII protein exhibited a homogeneous peak and the same value of particle weight on gels of 5%, 7%, 12%, or 14% acrylamide.

Table IV: Recovery of Protein Fragments following Treatment with Sulfenyl Chloride

	$M_r \times 10^{-3}$	protein (mg)	recovery % protein	recovery nmol
(1) native enzyme	225	4.0 [b]	100	18
(2) sulfenyl chloride treated	225	2.8 [b,c]	70	12
(3) band SC VI	120	0.64		
(4) band SC VII	63	1.53	54 [d]	34 [d]
(5) new low M_r fragments	35 [a]	0.37 [b]	9	11
(6) total recovered on gel		2.54	63	

[a] Estimated mean value. [b] Corrected for 0.2 mg of low molecular weight fragments present both before and after sulfenyl chloride treatment. [c] Corrected for 0.22 mg of undissociated bands I–III. [d] Assuming band SC VI to be a dimer of band SC VII.

Treatment of the enzyme with sulfenyl chloride sometimes produced an additional band (designated band SC VI), M_r 120 000 (data not shown). This band was clearly differentiated from band III by mobility on polyacrylamide gel electrophoresis and the absence of heme. Two observations supported the hypothesis that band SC VI polypeptide was not itself a separate subunit but rather a dimer of the M_r 63 000 polypeptide and possibly an intermediate in the degradation of the M_r 225 000 polypeptide. First, with increasing periods of incubation with sulfenyl chloride, band SC VI diminished in size whereas band SC VII increased. Second, as measured on the gel summarized in Table IV, the value of the molar ratio of the M_r 63 000:M_r 120 000 bands was 5:1, and the M_r 63 000 polypeptide clearly made up *at least* 50% of the enzyme. Thus, the M_r 120 000 band cannot exist with two M_r 63 000 bands since the resulting $\alpha\beta_2$ enzyme would have a M_r of 420 000.

For direct demonstration of the conversion of the M_r 225 000 peptide polypeptide to the M_r 63 000 form (Figure 2F), band I was isolated (in low yields) by 48-h electrophoresis from the gel into 8 M urea. $NaDodSO_4$, which interfered with the sulfenyl chloride reaction, was removed by electrophoresis before collection of the protein band. Sulfenyl chloride treatment following subsequent dialysis in 50% acetic acid resulted in the formation of band SC VII.

The M_r 225 000 Protein Is a Simple Polymer of the M_r 63 000 Protein. The absence of bands other than band SC VII in the sulfenyl chloride dissociated enzyme (Figure 4C) suggests that the large molecular weight subunit may be a trimer or tetramer of band SC VII and that no other peptides are present. Quantitative analysis of the dissociation as shown

in Table IV supported that conclusion. Sulfenyl chloride treatment of the enzyme or mammalian cytochrome *c* resulted in recovery in the aqueous phase of 70% or 92%, respectively, of the protein as determined by the Lowry procedure. Equal 5-μg samples of the aqueous phase of treated enzyme or mammalian cytochrome *c*, when analyzed by polyacrylamide gel electrophoresis, resulted in the recovery of the same total amount of Coomassie Blue staining (91%). On a molar basis, no other single protein band was equivalent to either the M_r 225 000 or 63 000 polypeptides. If random loss of polypeptide fragments during sulfenyl chloride treatment is assumed, 12 nmol of the M_r 225 000 polypeptide was dissociated and 34 nmol of the band SC VII protein recovered. If the large polypeptide is assumed to be a simple trimer or tetramer, the molar recovery of the M_r 63 000 protein was therefore 92% or 71%, respectively. Even if it is assumed that the "new low molecular weight fragments" produced by sulfenyl chloride treatment represented degradation products of a single M_r 35 000 polypeptide, that polypeptide would be present in a ratio of only 1 per band SC VII polypeptide (Table IV, line 5). The data of Table IV show that, even if selective and complete loss of a subunit and selective and complete recovery of the M_r 63 000 polypeptide during the sulfenyl chloride treatment are assumed, the M_r 63 000 protein makes up at least 54% of the M_r 225 000 protein. On the basis of the total amount of protein applied to or recovered from the analytical gels, the M_r 63 000 protein makes up 78% or 85%, respectively, of the M_r 225 000 protein. Although we consider it unlikely, we cannot presently rule out the possibility that the M_r 225 000 polypeptide consists of two molecules of the M_r 63 000 polypeptide and one or more other subunits which were selectively lost during the treatment with sulfenyl chloride.

The protein molecular weight of band VII polypeptide is 59 000, assuming all hemes to have been removed and sulfenyl chloride to have derivatized each of the cysteine and tryptophan residues. Therefore, in the native state of the enzyme, each molecule of band VII protein contains 5.6 ($\simeq$6) molecules of *c*-type heme and 0.7 ($\simeq$1) molecule of heme P-460, and the particle weight is approximately 63 300. These calculations are based on the presence of 73 tryptophans, 18 *c*-type hemes, and 2.2 P-460 irons per M_r 189 000 protein (Hooper et al., 1978). We conclude that band I polypeptide of apparent M_r 225 000 as determined by polyacrylamide gel electrophoresis contains at least a dimer and possibly a trimer (M_r 189 000) or a tetramer (M_r 252 000) of the M_r 63 000 polypeptide (Figure 2).

Stability of Subunits. In addition to being resistant to denaturation in $NaDodSO_4$, the native enzyme was completely resistant to proteolysis by chymotrypsin, *S. aureus* protease, trypsin, or subtilisin. Bands III, II, and I were progressively more easily digested by proteases. Bands SC VI and SC VII resulting from treatment with sulfenyl chloride were so rapidly digested by proteases that peptides in the M_r 10 000–50 000 range were not obtained. Band V cytochrome and mammalian cytochrome *c* acquired a similar increased sensitivity to proteases after sulfenyl chloride treatment. Band SC VII was much more rapidly digested by protease than band IV.

Subunit Composition of the Enzyme in Solution. Because this enzyme exhibits a very unusual pattern of denaturation or polymerization, a complete description of the subunit composition of the enzyme is not possible. The present report establishes the M_r 63 300 subunit as a basic repeating unit of hydroxylamine oxidoreductase of *Nitrosomonas* as isolated in solution. There are three to four molecules of the M_r 11 000 cytochrome per molecule of the M_r 225 000 cytochrome (Tables II and III). If the M_r 225 000 polypeptide is assumed to be a trimer or tetramer of the M_r 63 300 polypeptide, it follows that the M_r 63 300 subunit exists in equimolar quantity with the M_r 11 000 subunit in the native enzyme. The particle weight of the enzyme is 200 000 based on measurements of sedimentation velocity, sedimentation equilibrium and electron microscopy (Rees, 1968), sedimentation analysis (Maxwell, 1976), or polyacrylamide gel electrophoresis under nondenaturing conditions (Hooper et al., 1978). Yamanaka et al. (1979) estimated a particle weight of 175 000–180 000, utilizing chromatography with Sephadex G-150 or analysis by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. We suggest, as a model for future work, that the native enzyme in solution is a hexamer consisting of three molecules of the M_r 11 000 monoheme *c*-type cytochrome and three molecules of the M_r 63 300 subunit, each of which contains six *c*-type hemes and one P-460 heme (total M_r 220 000; Figure 3). Quantitative analysis of the electron paramagnetic resonance spectrum indicates the presence of a multiple of approximately nine hemes per molecule (J. D. Lipscomb and A. B. Hooper, unpublished results), lending support to the present report of a multiple of eight hemes per molecule. The enzyme can apparently exist as an enzymatically active but relatively unstable trimer of the M_r 63 300 subunit (Table III, line 5; Figure 2B).

Model: Existence of a M_r 225 000 Polypeptide Which Undergoes Sequential Denaturation. The data are consistent with the hypothesis that bands I–III are different conformations of a M_r 225 000 polypeptide and that sequential denaturation results in bands of successively lower mobility on polyacrylamide gel electrophoresis (Figure 2C). A similar effect of conformation on apparent molecular weight has been seen with lysozyme (Dunker & Kenyon, 1976) and cytochrome oxidase (Hundt & Kadenbach, 1977). We can only speculate on the forces holding the protein in the undenatured form. The protein contains enough cysteine residues in excess of those bound to *c*-type heme to contain intramolecular disulfide bonds (Hooper et al., 1978) which may have been unusually protected from reaction with mercaptoethanol. Resistance to full denaturation has been noted in other *c*-heme proteins (Cusanovich, 1971).

The existence of a M_r 225 000 polypeptide would imply the existence of an enzyme containing also three or four molecules of the M_r 11 000 cytochrome and having a total M_r of 270 000. That value is significantly greater than the values of 180 000–200 000 determined by sedimentation equilibrium (Rees, 1968; Maxwell, 1976), suggesting that a M_r 225 000 form does not exist prior to treatment with denaturants but, according to the denaturation model, is formed rapidly under mild denaturing conditions and undergoes stepwise denaturation (Figure 2C). We cannot completely rule out the possibility that a M_r 260 000–270 000 form of the enzyme does exist in solution, but it readily dissociates into a M_r 180 000–200 000 form (which is seen in the present work as the M_r 225 000 form) under conditions of analysis by sedimentation velocity (Rees, 1968; Maxwell, 1976) or Sephadex chromatography (Yamanaka et al., 1979) (Figure 2B). The small amounts of the M_r 11 000 subunit might easily have been ignored in the previous analysis of the enzyme. The resulting high molecular weight form would be expected to have had enzyme activity but to be relatively unstable as seen here with sample 5, Table IV.

The nature and biological significance of the bonds holding the M_r 63 300 monomers in the M_r 225 000 band I are unknown. Although unlikely, it is possible that the large protein

is a continuous polypeptide which is cleaved at specific loci. Possible interpeptide bridges dependent on the presence of *c*-type heme include (a) heme–thioether bonding, (b) cross-links formed from putative reactive intermediates in the degradation of specific labile hemes such as P-460 (Hooper & Terry, 1977), (c) Fe–O–Fe "oxo" diheme bridges, and (d) stable noncovalent heme polymers (Brown et al., 1976). The protein polymer may also contain resistant interpeptide disulfide bonds which are cleaved either directly by sulfenyl chloride or by mercaptoethanol only after the removal of heme. Alternatively, the interpeptide bonds might involve groups other than heme or cysteine which react with sulfenyl chloride.

Model: Possible Sequential Polymerization. The successive increase in amounts of bands III, II, and I with increasingly drastic denaturing conditions would also have occurred if a true subunit, possibly the M_r 63 300 form detected after treatment with sulfenyl chloride, was able to form unusually stable aggregates in the denaturants (Figure 2D). The observed apparent M_r values of 125 000, 195 000, and 225 000 are, in fact, close to the values expected for dimers, trimers, and tetramers of the M_r 63 000 form. According to this model, formation of the higher polymers would have been facilitated by boiling in 2-mercaptoethanol or dithiothreitol or following treatment with urea, succinic anhydride, iodoacetic acid, urea, or guanidine hydrochloride. The fact that this protein contains an unusually large number of *c*-type heme moieties (six per M_r 63 000) and that the unique heme P-460 moiety (approximately three per enzyme) is unusually sensitive to oxidative degradation by H_2O_2 (Hooper & Terry, 1977) suggests the possible involvement of a specific heme site in cross-linking. Unexplained apparent cross-linking phenomena which may have occurred by this mechanism have been reported. For example, cytochrome *c* peroxidase migrates in native gels with decreased mobility following heme degradation by hydrogen peroxide (Erman & Yonetani, 1975), and two subunits of cytochrome oxidase aggregate irreversibly when heated in the presence of $NaDodSO_4$ and 2-mercaptoethanol (Hundt & Kadenbach, 1977).

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