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Reaction of Hemerythrin with Disulfides†

Patricia C. Harrington and Ralph G. Wilkins*

Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003
Received May 16, 1984

ABSTRACT: The reactions of hemerythrin from *Phascolopsis gouldii* with the specific sulfhydryl reagents 5,5′-dithiobis(2-nitrobenzoate), 2,2′-dithiodipyridine, and 4,4′-dithiodipyridine were studied at 25 °C. Spectrophotometric measurements showed that 1 mol of disulfide reacted per protein subunit consistent with a single cysteine at residue 50. Reaction leads to dissociation of the octameric structure of the native protein to monomers. The first-order rate constants at 25 °C and pH 9.0 for reactions of methemerythrin [$(1.5 \pm 0.3) \times 10^{-3}$ s^{-1}] and metazidohemerythrin [$(4.0 \pm 0.3) \times 10^{-3}$ s^{-1}] are independent of both the concentration and the nature of the disulfide. The reactions of methemerythrin are strongly inhibited by ClO_4^- ion, which however has no effect on the rates of those of metazidohemerythrin. The first-order kinetic behavior is ascribed to a conformational change involving the protein controlling the reaction, and this slow change appears to dominate a number of the reactions of hemerythrin.

Hemerythrin is an iron-containing respiratory protein in certain marine organisms whose structure and properties have been well characterized (Henderickson, 1978; Loehr & Loehr, 1979; Stenkamp & Jensen, 1979; Wilkins & Harrington, 1983; Klotz & Kurtz, 1984). Interest in the protein has been revitalized by the apparent similarity of its active site to those of several iron proteins from disparate sources. These include ribonucleotide reductase (Sjoberg & Gräslund, 1983; Sjoberg et al., 1983), purple acid phosphatases from beef spleen (Davis & Averill, 1982) and from pig allantoic fluid (also called uteroferrin; Antanaitis & Aisen, 1983; Debrunner et al., 1983), and component A of methane monooxygenase from *Methylococcus capsulatus* (Bath) (Woodland & Dalton, 1984; M. P. Woodland and R. Cammack, private communication).

The native hemerythrin from the coelomic fluid of the sipunculid *Phascolopsis gouldii* is octameric (Klotz & Keresztes-Nagy, 1963; Wilkins & Harrington, 1983). It has a single cysteine at residue 50, and although this is some distance [approximately 15 Å (Stenkamp et al., 1978a)] from the binuclear iron site, a small sequence of residues around 50 plays a critical role in some important reactions of the protein. Treatment with a number of sulfhydryl reagents including salyrganic acid, PCMB,[1] PMB, and NEM promotes dissociation of the octamer to the monomer (Keresztes-Nagy & Klotz, 1963; Garbett et al., 1971; Rao & Keresztes-Nagy, 1973; Clarke et al., 1979). Octameric hemerythrin from *Themiste dyscritum* has cysteine residues at 9 and 50. In many other respects, the proteins from the two sources are very similar (Dunn et al., 1977). The interaction of a number of mercurials with *T. dyscritum* hemerythrin has been recently examined (Clarke et al., 1979). Here again, binding of the bulky PHMB or NEM to the sulfur of cysteine disrupts

† This work was supported by Grant GM-28796 from the National Institute of General Medical Sciences.

[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoate); 2-PDS, 2,2′-dithiodipyridine; 4-PDS, 4,4′-dithiodipyridine; PHMB, *p*-(hydroxymercuri)benzoate; PCMB, *p*-(chloromercuri)benzoate; PMB, *p*-mercuribenzoate; CMNP, 2-(chloromercuri)-4-nitrophenol; NEM, *N*-ethylmaleimide; Mes, 4-morpholineethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; SDS, sodium dodecyl sulfate.

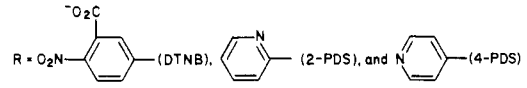
0006-2960/85/0424-0210$01.50/0

subunit interactions (Stenkamp et al., 1978b) and leads to complete dissociation (Clarke et al., 1979).

An interesting and related phenomenon involves the effect of ClO_4^- ion on the properties of the protein. Reactivity of the thiol group and dissociation of the octamer are suppressed even in millimolar concentrations of ClO_4^- (Darnall et al., 1968; Garbett et al., 1971) and in hemerythrin from *T. dyscritum* difference Fourier maps show that ClO_4^- binds near cysteine-9 and -50 and protects them from sulfhydryl reagents (Stenkamp et al., 1978a).

Because of the interesting role that cysteine-50 plays in the chemistry of hemerythrin, we have examined its reactions with three disulfides, RSSR

$$\text{protein-S}^- + \text{RSSR} \rightarrow \text{protein-SSR} + \text{RS}^- \quad (1)$$



These reactions are specific for the thiol group, the RS^- ions are good leaving groups (ensuring complete reaction), and they have high absorbance coefficients at wavelengths shifted from those of the RSSR reagents and protein (Brocklehurst, 1979; Torchinsky, 1981; Riddles et al., 1983). Ellman's reagent (DTNB; Ellman, 1959), in particular, has found steady use for analyzing and probing the environment of thiol groups in a variety of proteins (Riddles et al., 1983).

Materials and Methods

The marine worms *P. gouldii* were obtained from Marine Biological Laboratory, Woods Hole, MA. Oxyhemerythrin was obtained from the coelomic fluid of the worms (Klotz et al., 1957). Methemerythrin was prepared by dialyzing oxyhemerythrin against $Fe(CN)_6^{3-}$ and then several times against the appropriate buffer systems. Other derivatives of the protein were prepared by standard procedures [see Harrington & Wilkins (1981) and Wilkins & Harrington (1983) for references]. The disulfides and other reagents used were commercial products; the potassium salt of DTNB was prepared as described by Wilson et al. (1980). The thiol-modified derivatives were prepared by mixing methemerythrin (0.44–1 mM) and excess reagent (1–3 mM) in pH 8.2 or 9 buffer at 25 °C for 1–2 h. Excess reagent was dialyzed away at 4 °C, and the dialyzate was diluted for use in the kinetic and spectral runs. All studies were carried out in deaerated solutions. Absorbance changes were used to obtain the stoichiometry and kinetics of the disulfide–protein reactions. Absorbance coefficients (M^{-1} cm^{-1}) and wavelengths used (for cleaved anion) were as follows: DTNB, 1.4×10^4, 410 nm (Riddles et al., 1983); 2-PDS, 8.1×10^3, 343 nm (Torchinsky, 1981); 4-PDS, 9.9×10^3, 324 nm (Torchinsky, 1981). Buffer systems used were 0.05 M Tris (pH 8.2) and 0.005 M Tris (pH 9.0). Concentrations of protein were based on the M_r of 13 500 of the monomer and were determined spectrally, $\epsilon_{280} = 35\,400$ (Dunn et al., 1977). Kinetic experiments used a Beckman 24 recording spectrophotometer and a Dionex stopped-flow apparatus interfaced with an OLIS data collecting system. Spectra were recorded on a OLIS updated Cary 14 spectrophotometer. Excess of thiol reagent was used over protein (2–20 μM). First-order kinetics were observed. In experiments using large concentrations of disulfide at high pH, the spontaneous hydrolysis of the disulfide (Wilson et al., 1980) was either allowed for or obviated by use of the initial-rate approach. Good agreement in the rate constants with both methods (where applicable) was obtained. Reproducible data were obtained from different samples of protein taken over a 4-year period.

Table I: Rate Constants for Reaction of Methemerythrin with Excess Thiol Reagents at 25 °C and 0.15 M[a]

thiol reagent	[thiol] (μM)	pH	$k \times 10^3$ (s^{-1})
DTNB	59	9.4	2.0
DTNB	510	9.2	2.0
DTNB	510[b]	9.2	1.8
DTNB	500[c]	9.1	1.8
DTNB	31[d]	9.0	≪1.0[d]
DTNB	30[e]	9.0	3100
DTNB	90[e]	9.0	8900
DTNB	300[e]	9.0	31900
DTNB	33	8.5	0.79
DTNB	328	8.5	0.70
DTNB	33[f]	8.5	≪1.0
DTNB	298[f]	8.5	≪1.0
DTNB	503	8.2	0.47
DTNB	20	8.2	0.42[g]
2-PDS	49	9.0	1.4[g]
2-PDS	496	9.0	1.1
4-PDS	54	8.9	1.5[g]
4-PDS	553	8.9	1.6
CMNP	15	8.2	4.6
CMNP	31	8.2	8.0
CMNP	61	8.2	15
CMNP	30[h]	8.2	13
CMNP	30[h]	8.2	13
CMNP	47[h]	8.2	15
CMNP	60[h]	8.2	18

[a] Using 5–12 μM protein, no effect on k. [b] Contains 25 μM cysteamine disulfide. [c] Contains 124 μM cysteamine disulfide. [d] Unfolded with 3.1 mM SDS. [e] Protein treated with 6 M guanidinium chloride for 90 min at 25 °C. [f] Protein incubated with 100 mM ClO_4^- for 2 h at 25 °C. [g] Theoretical absorbance changes were 93 ± 4% calculated on the basis of one SH/monomer unit. [h] Metazidohemerythrin. Free N_3^- ion dialyzed away.

Results and Discussion

Most of the study was conducted with the met form of hemerythrin in which the two irons are in the oxidation state 3+. Since all the experiments were at pH ≥7.8, the base form (probably methydroxohemerythrin) was involved (Kereszstes-Nagy & Klotz, 1965; Bradić & Wilkins, 1983). For the three disulfides examined in (1), absorbance changes indicate 0.90–0.95 SH group has reacted in each hemerythrin monomeric unit, in agreement with the one Cys-50 residue present (see footnotes *g* and *c* of Tables I and II). The methemerythrin–thiol reaction products (protein–SSR) bind azide and thiocyanate ion. The spectra of the 2-PDS and 4-PDS derivatized products (protein–SSR) are similar to those of the original methemerythrin. The spectral characteristics of the DTNB derivatives (protein–SSR) are altered markedly. For DTNB–methemerythrin, $\epsilon_{420nm} = 4.8 \times 10^3$ and $\epsilon_{324nm} = 1.87 \times 10^4$; for the azide adduct, $\epsilon_{429nm} = 6.6 \times 10^3$ and $\epsilon_{327nm} = 2.1 \times 10^4$; for the thiocyanate complex, $\epsilon_{420nm} = 1.0 \times 10^4$ and $\epsilon_{328nm} = 1.75 \times 10^4$ M^{-1} cm^{-1} at pH 9.0 see Figure 1. Solutions of the DTNB derivatives are stable for at least several days when stored at 4 °C. Gel filtration chromatography on a Sephadex G-100 column (Clarke et al., 1979) indicated that the products of the reactions with the three thiol reagents are monomeric. The octameric structure of native methemerythrin had been disrupted, a not unexpected result in view of the binding of the bulky thiols at Cys-50.

The stoichiometric reactions are obviously suitable for kinetic studies. Excess disulfide was used, and the reactions were nicely first order in protein concentration. The results are collected in Tables I and II. The rates with methemerythrin are quite slow, requiring about 30 min or so for completion, and the first-order rate constants are *independent of both the concentration* (over a wide range) *and the nature of the disulfide*. Even addition of the powerful disulfide of cysteamine,

Table II: Rate Constants for Reaction of Hemerythrin Derivatives with DTNB at 25 °C and 0.15 M

DTNB (μM)	pH	$k \times 10^3$ (s^{-1})
Methemerythrin–Azide Adduct[a]		
25	8.9	4.0
30[b]	9.0	4.0
250	8.9	3.6
88	8.2	2.5
99	8.2	2.7[c]
281	8.2	3.0
Methemerythrin–Thiocyanate Adduct[d]		
103	9.1	0.3[e]
103[b]	9.1	0.07[e]
Semimethemerythrin–Azide Adduct[f]		
103	8.2	2.4[c]
Oxyhemerythrin[g]		
98	8.2	1.3
Deoxyhemerythrin[h]		
100	8.2	0.06[e]

[a] Contains 0.1 mM free N_3^-. No effect on rate of N_3^- < 1 mM. [b] Protein incubated with 100 mM ClO_4^-. [c] Theoretical absorbance changes were 93 ± 4% calculated on the basis of one SH/monomer unit. [d] Contains 3.5 mM free SCN^-. [e] Initial rate method. [f] Prepared from one-electron reduction of methemerythrin and 3 mM N_3^- then added. [g] Contains air, saturated. [h] Prepared from deazaflavin–EDTA–$h\nu$ reduction of met (Bradić et al., 1980).

R′SSR′ (Wilson et al., 1980), to DTNB and methemerythrin does not accelerate the production of the nitrothiobenzoate anion. It could, in principle, accomplish this by reacting directly with protein–S^- and liberating $R'S^-$, which reacts rapidly with DTNB (RSSR), e.g.

protein–S^- + R′SSR′ →

protein–SSR′ + $R'S^-$ (noncolored)

$R'S^-$ + RSSR → R′SSR + SR^- (colored)

This represents a possible additional path to the direct formation of SR^- by eq 1 (Wilson et al., 1980).

Addition of 100 mM perchlorate ion markedly suppressed the reaction of methemerythrin even when high concentrations (0.3 mM) of DTNB were used. However, on dilution (14-fold) of the perchlorate ion–protein incubated solution, the anion is no longer effective, and the reaction proceeds at the normal rate. The high concentration of perchlorate ion has obviously not effected an irreversible (i.e., through oxidation) suppression of –SH reactivity. The retardation by perchlorate ion of the reactivity of hemerythrin toward PHMB and NEM has been previously noted (Darnall et al., 1968; Garbett et al., 1971). If the structural data for *T. dyscritum* can be used for *P. gouldii* [and these have complete homology in residues from 46 through 59 (Loehr & Loehr, 1979)], then ClO_4^- ion is located in the inner cavity of the octamer and is apparently bonded to Lys-53 amino groups from two adjacent subunits, either electrostatically or through hydrogen bonding. The lysine side chain is thus held in a blocking position to Cys-50. As has been mentioned by Klippenstein et al. (1976), the 48–53 sequence is quite different in myohemerythrin (the monomeric protein from the retractor muscle of *Themiste zostericola*) with even the cysteine residue missing, and the question arises as to whether ClO_4^- binds to myohemerythrin. In a few experiments only, we find that DTNB reacts with both cysteine residues (at positions 34 and 99) of metmyohemerythrin and that ClO_4^- does *not* retard the reaction. If ClO_4^- binds, it obviously has no effect on the reactivity of the protein toward DTNB.

Usually the disulfide–thiol interchange rate is markedly pH dependent in the pH 7–9 region since the protein–S^- grouping

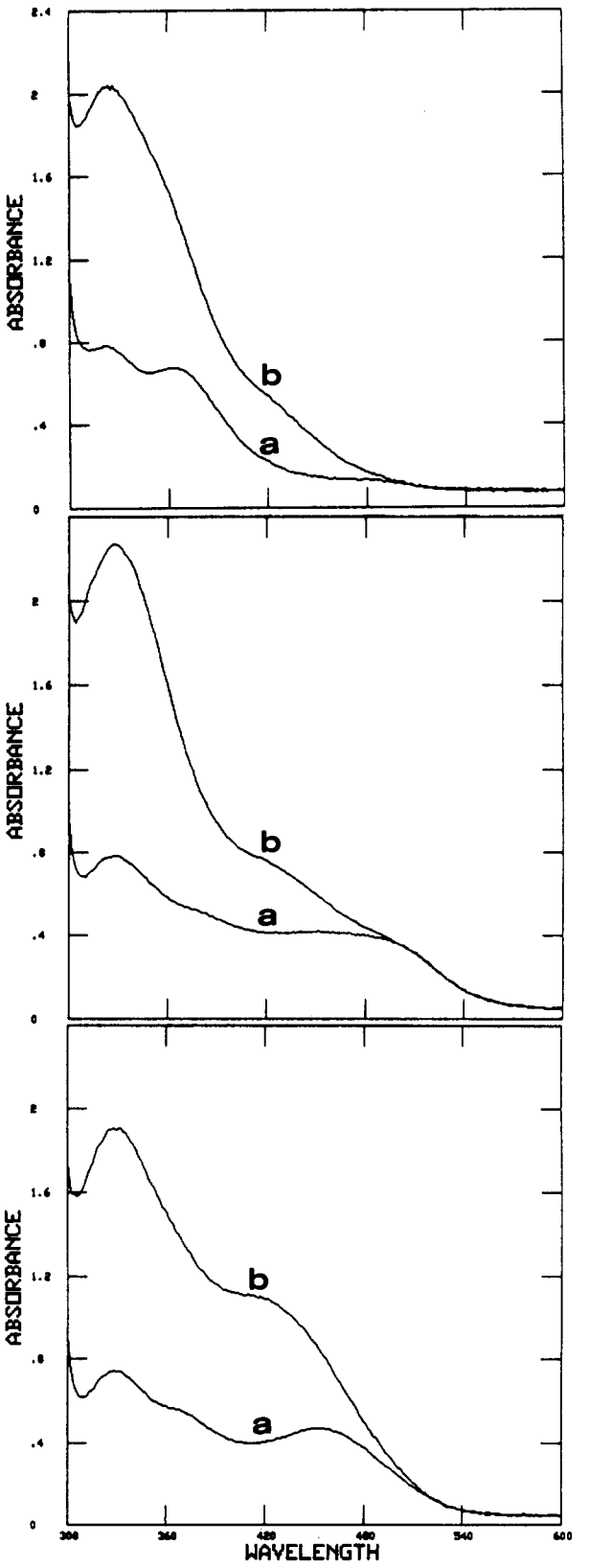


FIGURE 1: Spectra of hemerythrin species and their DTNB derivatives in 0.005 M Tris–0.05 M Na_2SO_4, pH 9.0. (Top) (a) Methemerythrin; (b) DTNB–methemerythrin. (Middle) (a) Methemerythrin–azide adduct; (b) DTNB–methemerythrin–azide adduct. (Bottom) (a) Methemerythrin–thiocyanate adduct; (b) DTNB–methemerythrin–thiocyanate adduct. Protein concentration was 0.11 mM.

is much more reactive than protein–SH (pK ~8.3) (Torchinsky, 1981). However, since the cysteine-50 SH is close to several positively charged residues in hemerythrin, the pK may be substantially reduced and be largely in the anionic

(dissociated) form at both pH 8.2 and pH 9.0. The small increase in rate at pH 9 over that at pH 8.2 (Tables I and II) may therefore reflect a slightly faster conformational change in the protein (vide infra) at the higher pH. The reactivity of thiol groups in native proteins is often enhanced upon denaturing (Torchinsky, 1981). Revealing results are obtained when denatured methemerythrin (pH 9.0) is reacted with DTNB. Quite surprisingly, the 3.1 mM SDS-denatured material is unreactive toward DTNB, and this is ascribed to strong bonding by SDS at the ClO_4^- site, with similar results to that seen with perchlorate. In contrast, the 6.0 M guanadinium chloride completely denatured (after 90-min incubation) material reacts rapidly with DTNB by a second-order reaction ($k = 1.1 \times 10^5$ M^{-1} s^{-1} at pH 9.0). Even within 5 min of contact with this denaturing reagent however, the thiol group has been opened up to second-order attack by DTNB ($k \sim 9 \times 10^3$ M^{-1} s^{-1}).

Methemerythrin reacts with a number of anions to form adducts. Those with N_3^- and SCN^- are particularly stable (Keresztes-Nagy & Klotz, 1965; Meloon & Wilkins, 1976; Olivas et al., 1979). The reactions of the azide and thiocyanate adducts with DTNB show interesting contrast with that of unliganded methemerythrin. The first-order rate constant for the reaction of met-azide is slightly larger than that for met, and again, the value is independent of DTNB concentration. The thiocyanate complex reacts about 50 times slower. With the azide adduct, perchlorate has *no* effect on the rate of the thiol–disulfide interchange. With the thiocyanate complex, perchlorate has a small retarding effect. In this, and other reactions of the protein (Garbett et al., 1971; Z. Bradič and R. G. Wilkins, unpublished results), the effect of perchlorate is much more striking on methemerythrin in the absence of N_3^- and SCN^- ions. Other derivatives were cursorily examined with DTNB (Table II). A few experiments at pH 9.0 on octameric methemerythrin and metazidohemerythrin from *T. zostericola* showed similar rates and behavior patterns toward DTNB as shown by *P. gouldii* protein.

Treatment of *P. gouldii* hemerythrin (usually as metazidohemerythrin) with PHMB results in complete conversion of native octamer to monomers (Kereztes-Nagy & Klotz, 1963; Rao & Keraztes-Nagy, 1973; Clarke et al., 1979). In contrast with the thiol–disulfide interchange, the reactions of methemerythrin and metazidohemerythrin with CMNP are relatively fast and show overall second-order kinetics (Table I). Experiments were carried out at pH 8.2 only since there were turbidity problems at lower pH. From the linear dependence of k_{obsd} vs. [CMNP], in excess, formation (M^{-1} s^{-1}), dissociation (s^{-1}), and equilibrium (M^{-1}) constants (pH 8.2) are 220, 5×10^{-3}, and 4.4×10^4 (met) and 227, 1.2×10^{-3}, and 1.9×10^5 (met–N_3^-). For PHMB, a formation rate constant of approximately 2 M^{-1} s^{-1} is estimated (Garbett et al., 1971). Although there is evidence for binding of mercurial reagents at sites in addition to Cys-50 (Klapper, 1970), the reagent is highly specific for –SH groups (Torchinsky, 1981), particularly at the low concentrations of mercurial used. One mole of PHMB is bound per subunit of metazidohemerythrin from spectral titrations (Clarke et al., 1979).

Slow Changes Dominating Hemerythrin Chemistry. Obviously, the cysteine group in hemerythrin is well protected against direct disulfide attack. This could arise from steric inaccessibility or because the SH group forms intramolecular bonding with neighbors (Torchinsky, 1981). Charged groups near the SH group and responsible for perchlorate ion binding would not however hinder the approach of unionized reagent. A persuasive case can be made that the reaction is controlled by a conformational change in the protein and that the thiol–disulfide rate is not slow per se. Indeed, within a few minutes contact with guanadinium chloride the cysteine is exposed to rapid second-order attack of DTNB with a rate constant similar to that of simple thiol anions (Wilson et al., 1980). Examination of a number of varied reactions of hemerythrin studied by our group shows the prevalence of a slow reaction with first-order rate constants of similar magnitude. These include the slow interconversion of the acid and base forms of methemerythrin (3.3×10^{-3} s^{-1}, pH 8.2; Bradič & Wilkins, 1983), the reactions of methemerythrin with SCN^- and $S_2O_4^{2-}$ ions (5.0×10^{-3} s^{-1} and 2.7×10^{-3} s^{-1}, respectively, at pH 9.0; Bradič & Wilkins, 1983), the conversion of semimethemerythrin (obtained by one-electron oxidation of methemerythrin) to the other form (obtained by one-electron reduction of methemerythrin) and its oxidation by $Fe(CN)_6^{3-}$ (1.3×10^{-3} s^{-1}, pH 8.2; Bradič et al., 1980; Armstrong et al., 1983) and intramolecular disproportionation within the octameric unit of semimethemerythrins from *T. zostericola* (2.2×10^{-3} s^{-1} at pH 8.2; Harrington & Wilkins, 1981), and finally in this work the thiol–disulfide interchange (2×10^{-3} s^{-1} at pH 9.0). It is difficult at this stage to define the conformational change responsible for this common effect, but it is hoped to make modifications of the protein at various sites (Wilkins & Harrington, 1983) and thus map the approximate location of the effect. A referee has suggested that octamer dissociation and association, and not a protein conformational change, may be the process controlling these slow reactions of hemerythrin. Certainly, equilibria between octamer and lower oligomeric forms have been established with unmodified hemerythrin (Keresztes-Nagy et al., 1965; Tan et al., 1975). However, slow first-order kinetics are also observed in a number of reactions of metmyohemerythrin, including the interconversion of the semi-metmyo forms (Wilkins & Harrington, 1983) and the first-order transformation of acid and base forms of metmyohemerythrin [$k = 0.01$ s^{-1} at pH 7.5, 25 °C, and $I = 0.15$ M (Z. Bradič and R. G. Wilkins, unpublished results)]. Clearly, an explanation involving dissociation of the octamer is not applicable to the behavior of a monomer, and we therefore favor a conformational change as an explanation for these common slow steps involving both octamer and monomer.

Registry No. DTNB, 69-78-3; 2-PDS, 2127-03-9; 4-PDS, 2645-22-9; Cys, 52-90-4.

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Complex Formation and O_2 Sensitivity of *Azotobacter vinelandii* Nitrogenase and Its Component Proteins†

Z.-C. Wang,‡ A. Burns, and G. D. Watt*

Battelle—C. F. Kettering Research Laboratory, Yellow Springs, Ohio 45387
Received March 15, 1984

ABSTRACT: The O_2 stability of the MoFe protein, the Fe protein, a 1:1 mixture of these proteins, and a 1:1 mixture in the presence of the *Azotobacter vinelandii* FeS-II protein has been studied as a function of time under controlled O_2 partial pressures. The Fe protein is much more sensitive to O_2 exposure than is the MoFe protein. The presence of the FeS-II protein at a 1:1 ratio with the component proteins measurably increases the O_2 stability of the MoFe and Fe proteins. O_2 inactivation of the MoFe protein was studied in some detail and found to be quite complex. At least three partially overlapping reactions are suggested. The first is the reversible oxidation of the metal clusters of the MoFe protein to the combined extent of 12 electrons with full retention of activity. The second phase consists primarily of activity loss with little increase in the extent of reversible oxidation. The third phase continues to decrease the protein activity but is also accompanied by formation of a $g = 2.0$ EPR signal and more extensive oxidation. Ultracentrifugation studies of the FeS-II protein at a 1:1:1 ratio with the Fe and MoFe proteins do not support the formation of the Bulen complex. The formation of other O_2-stable complexes is discussed.

Nitrogenase is an O_2-sensitive, two-component protein system found in several biological organisms ranging from blue-green algae and rapidly respiring aerobic bacteria, where high concentrations of O_2 are likely to be present, to microaerophylic and anaerobic bacteria, where O_2 concentrations are relatively low. The nitrogenases from all of these biological sources are remarkably similar (Mortenson & Thorneley, 1979) and all catalyze the reduction of N_2 to ammonia. How these organisms cope with the problem of protecting the nitrogenase enzyme from O_2 inactivation is of considerable interest but is still without satisfactory explanation. One approach to studying this problem is to examine the O_2 sensitivity of both component proteins comprising nitrogenase and the isolable protein complex consisting of the strongly associated MoFe protein (M_r 230 000, 30 Fe and 2 Mo), the Fe protein (M_r 65 000, 4Fe), and an iron-sulfur protein FeS-II (ferredoxin II, Shethna protein; M_r 24 000, 2Fe). There is

† Battelle—C. F. Kettering Research Laboratory Contribution No. 841.
‡ Present address: Institute of Botany, Peking, The People's Republic of China.

0006-2960/85/0424-0214$01.50/0