Conformational Studies on Human and Rabbit Plasma Hemopexin and on the Effect of Ligands on the Rabbit Heme–Hemopexin Complex*

Ursula Muller-Eberhard† and Kärt Grizzuti‡

ABSTRACT: Studies of optical rotatory dispersion and circular dichroism in the far-ultraviolet region are reported on the hemopexins of human and rabbit. A principal feature of the optical rotatory dispersion of both species is a trough at 223 nm.

Human hemopexin is less levorotatory at pH 7.7 than at pH 9.0, while the reduced mean residue rotation, [m'], of RHx is pH independent. The circular dichroism spectra of both hemopexins consist of a strong negative band at 207 nm and a weak positive band at 232 nm. The spectra of the two proteins differ only at pH 7.7, in that the minimum of the negative peak at 207 nm of rabbit hemopexin is shifted to a lower wavelength (205 nm) with a concommitant increase in reduced mean residue ellipticity. Treatment of rabbit hemopexin with 8 M urea abolishes the optical rotatory dispersion trough at 223 nm, as well as the positive circular dichroism band at 232 nm. Both the optical rotatory dispersion curve and the circular dichroism spectrum are, however, nearly completely restored upon removal of the urea. Oxidation of rabbit hemopexin with performic acid also results in a decrease of the levorotation and of the circular dichroism peak at 232 nm and is associated with a more negative reduced mean residue ellipticity at 207 nm. Partially or fully heme-saturated rabbit hemopexin at pH 7.7 and 9.0, is more levorotatory than rabbit apohemopexin. No difference in reduced mean residue rotation at 223 nm is discerned between human hemopexin containing a molar ratio of heme: protein of 0.3 or 1.0. In the Soret region, optical rotatory dispersion and circular dichroism of the rabbit heme–hemopexin complex show a negative trough in the optical rotatory dispersion patterns at 414 nm and a positive circular dichroism band at 418 nm. Evidence is presented for ligand formation of the complex with CN, and for the reduced complex with CO and O_2.

A conformational change of apohemoglobin (Javaherian and Beychok, 1968) and apomyoglobin (Harrison and Blout, 1965; Breslow *et al.*, 1965, 1967) is observed upon combination with iron protoporphyrin IX. These changes depend on the state of oxidation of the iron as well as on the presence of ligands (Urry, 1967; Samejima and Kita, 1969). Another protein exhibiting a high affinity for heme is hemopexin (Neale *et al.*, 1958; Grabar *et al.*, 1960; Schultze *et al.*, 1961), a serum β-glycoprotein, which carries plasma heme to its site of degradation (Muller-Eberhard *et al.*, 1970). The hemopexins of human and rabbit have a similar molecular weight of approximately 65,000 (Hrkal and Muller-Eberhard, 1971) and each binds heme in an equimolar ratio (Heide *et al.*, 1964; Hrkal and Muller-Eberhard, 1971). Physicochemical characteristics and biological properties of human hemopexin (HHx)[1] and rabbit hemopexin (RHx) were recently summarized (Muller-Eberhard, 1970).

The present communication describes the optical rotatory dispersion and circular dichroism differences between HHx and RHx at two pH values, and after treatment of RHx with 8 M urea, 4 M guanidine hydrochloride, and with performic acid. Also reported is a conformational change for apo-RHx upon binding heme, and on the interaction of CO and O_2 with the reduced heme–RHx complex, as well as that of the unreduced complex with KCN.

Materials and Methods

HHx and RHx were purified according to a previously described technique (Muller-Eberhard and English, 1967), preceded by a precipitation with perchloric acid (Schultze *et al.*, 1961). Purity and homogeneity of the protein solutions were ascertained electrophoretically on polyacrylamide gel and immunologically by employing polyvalent antisera. The protein concentrations were measured utilizing the extinction coefficients at 414 nm, $\epsilon_{1cm}^{1\%}$ 23.0 (HHx), and at 413.5 nm, $\epsilon_{1cm}^{1\%}$ 23.2 (RHx) (Hrkal and Muller-Eberhard, 1971). RHx was available as the apo protein, while HHx contained 30% heme (Muller-Eberhard, 1970). Unless specifically indicated, RHx is apo protein, and HHx a heme-poor hemopexin. Heme solutions were prepared and assayed as reported earlier (Muller-Eberhard *et al.*, 1969a). Hemin was obtained from Mann Biochemical Corp. and kindly supplied by Dr. S. Granick; dithionite and KCN were reagent grade, and all solutions were made up in glass-distilled H_2O. Performic acid oxidation was carried out by the

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[1] Abbreviations used are: [m'] = reduced mean residue rotation, [θ'] = reduced mean residue ellipticity, HHx = human hemopexin, RHx = rabbit hemopexin, and heme = ferriprotoporphyrin IX.

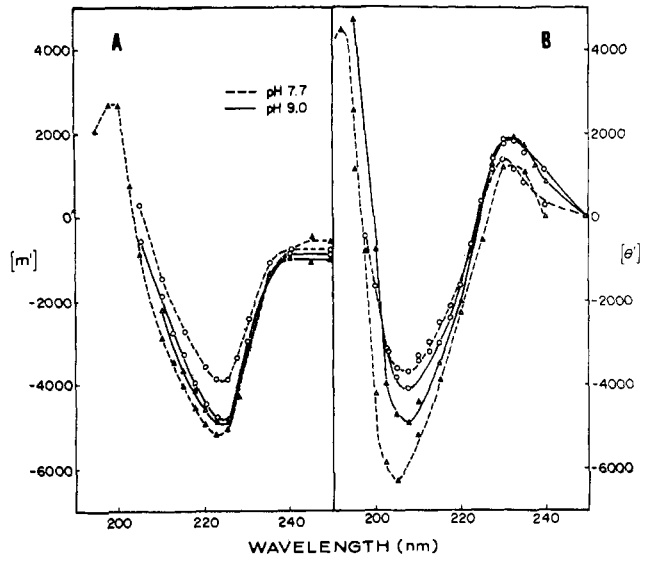

FIGURE 1: Optical rotatory dispersion (A) and circular dichroism (B) of rabbit hemopexin (Δ) and human hemopexin (○) in sodium phosphe buffer, pH 7.7, ionic strength 0.1 (---), and 0.025 M sodium borate, pH 9.0 (—) at 25°.

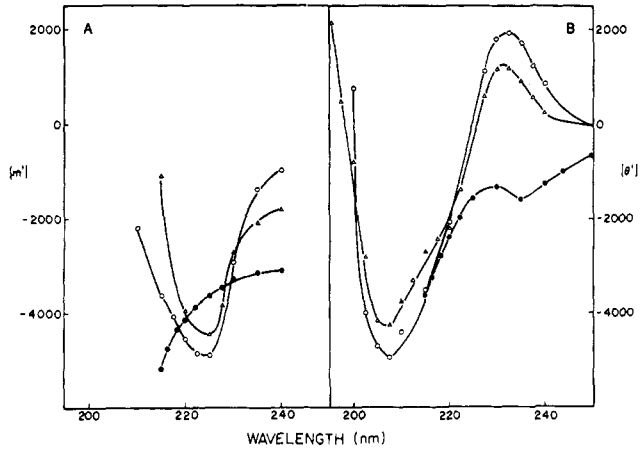

FIGURE 2: Optical rotatory dispersion (A) and circular dichroism (B) of rabbit hemopexin in 0.025 M sodium borate, pH 9.0 (○), in 8 M urea (●), and after its removal by dialysis (Δ).

method of Hirs (1967). Urea was twice recrystallized from alcohol and added as solid to attain a final urea concentration of 8 M. Optical rotatory dispersion and circular dichroism were measured at 25° with a Cary Model 60 recording spectropolarimeter, equipped with the 6001 circular dichroism attachment. Fused quartz cells with 0.5-, 1.0-, and 10-mm light paths were used. For measurements in the far-ultraviolet region, protein concentrations varied from 0.2 to 0.5 mg per ml and for those in the Soret region the protein concentration was 5.0 mg/ml. All experiments were conducted in either sodium borate, 0.025 M, pH 9.0, or sodium phosphate buffer, ionic strength 0.1, pH 7.7. Results, corrected for refractive index, are expressed in terms of reduced mean residue rotation, $[m']$, or reduced mean residue ellipticity, $[\theta']$. A mean residue molecular weight of 112 derived from the amino acid composition of both hemopexins (Hrkal and Muller-Eberhard, 1971), was used for calculations. The carbohydrate content of the hemopexins, which is 20% of the molecule (Heimburger *et al.*, 1964; Hrkal and Muller-Eberhard, 1971) was not taken into account.

Results

Effect of pH on Optical Rotatory Dispersion and Circular Dichroism of HHx and RHx. The optical rotatory dispersion recordings in the far-ultraviolet region for HHx and RHx are shown in Figure 1A. The trough of the Cotton effect has a rotation of $[m']_{223} = -5000$ at pH 9.0 for both proteins, but the reduced mean residue rotation of HHx decreased to $[m']_{223} = -3900$ at pH 7.7. A peak at 198 nm was detected only in experiments with RHx at pH 7.7. Although HHx also showed a positive deflection, unfavorable signal-to-noise ratios due to excessive absorption, prevented our discerning a definite peak.

The corresponding circular dichroism spectra are depicted in Figure 1B. For both proteins a positive band is observed at 232 nm, a negative band at 207 nm, and a peak at 195 nm for RHx only at pH 7.7. In the latter wavelength range, no band could be recorded for HHx. The reduced mean residue ellipticity of RHx increased from $[\theta']_{207} = -4950$ at pH 9.0 to $[\theta']_{205} = -6320$ at pH 7.7, while HHx measurements remained essentially unchanged ($[\theta']_{207} = -4110$ and -3710) at these pH values.

Effect of Urea and Performate. Figure 2A,B shows the optical rotatory dispersion patterns and circular dichroism spectra of RHx in 8 M urea of apparent pH 9.0. The optical rotatory dispersion trough at 223 nm and the positive circular dichroism band at 232 nm were abolished. These effects were, however, nearly completely reversed after removal of urea by exhaustive dialysis. A very similar change in optical rotatory dispersion and circular dichroism properties was observed when RHx was treated with 4 M guanidine hydrochloride.

Figure 3A,B illustrates the effects of performic acid treatment on RHx. The optical rotatory dispersion curve assumed the shape of a broad trough in the wavelength region between 220 and 235 nm. The circular dichroism spectrum, similar

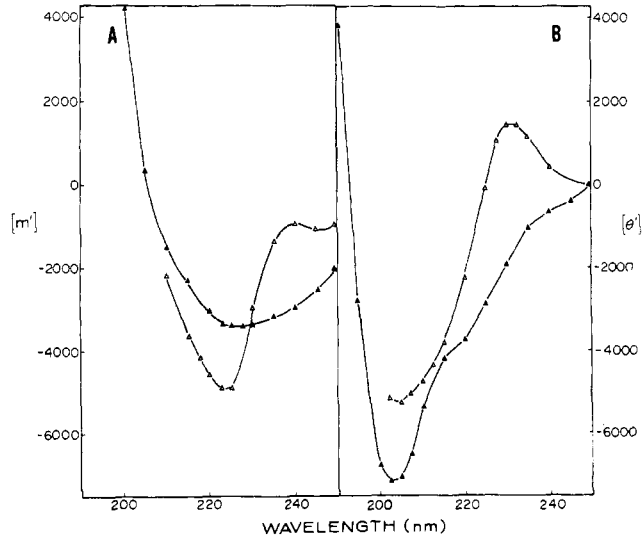

FIGURE 3: Optical rotatory dispersion (A) and circular dichroism (B) of rabbit hemopexin in 0.025 M sodium borate, pH 9.0, before (Δ) and after (▲) treatment with performic acid.

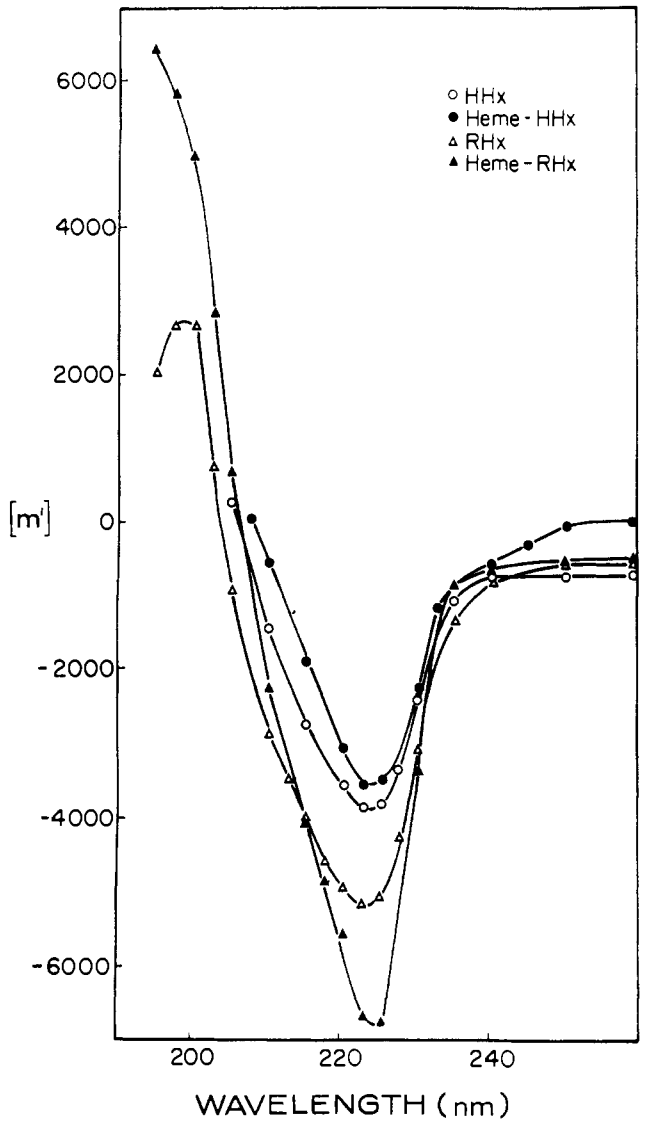

FIGURE 4: Effect of heme (equimolar amount) on human hemopexin (HHx) and rabbit hemopexin (RHx). The optical rotatory dispersion curves were examined at pH 7.7, in sodium phosphate buffer, ionic strength 0.1. RHx contained less than 3% but HHx already contained 30% heme prior to heme addition.

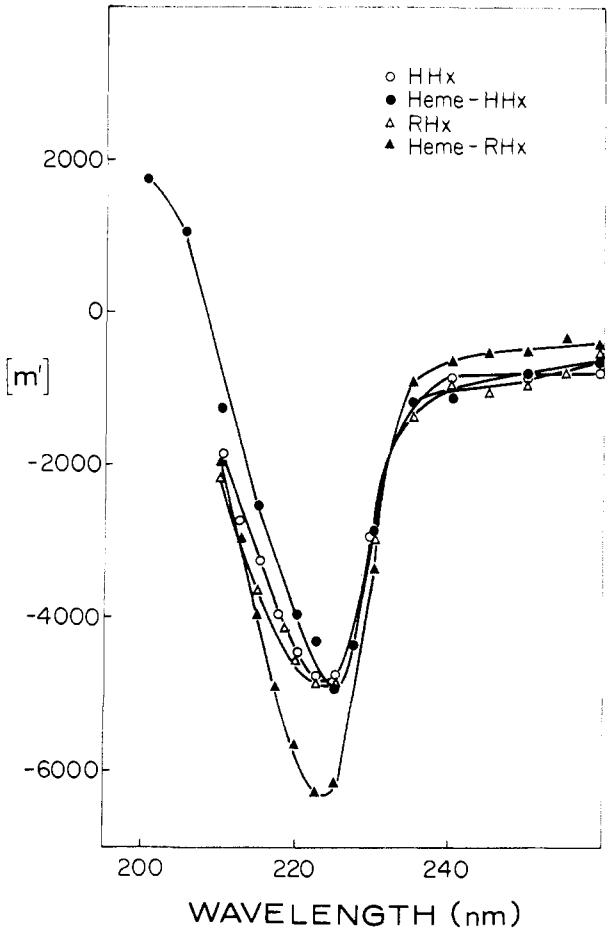

FIGURE 5: Optical rotatory dispersion of human and rabbit hemopexin at pH 9.0, 0.025 M sodium borate, conditions otherwise identical with those described in Figure 4.

to that of urea-treated RHx, demonstrated a loss of the peak at 232 nm and an altered reduced mean residue ellipticity which changed from $[\theta']_{207} = -5220$ to $[\theta']_{207} = -7020$.

Effect of Heme Binding on Optical Rotatory Dispersion of RHx. The interaction of heme with the hemopexins, at either pH value, increased the levorotation of RHx at 223 nm but did not alter its circular dichroism spectrum.

Figure 4 shows the optical rotatory dispersion measurements obtained at pH 7.7 and Figure 5, those at pH 9.0. The levorotation of RHx increased by a value of $\Delta [m']_{223} = -1500$ at the lower, and by $\Delta[m']_{223} = -1400$ at the higher pH value.

Optical rotatory dispersion changes of the same magnitude were observed in separate experiments comparing apo-RHx with RHx which contained heme:protein ratios ranging from 0.2 to 2.0. The heme-poor and heme-saturated HHx, on the other hand, showed the same reduced mean residue rotation (Figures 4 and 5).

Effect of Dithionite, CO, O_2, and KCN on Heme-RHx. The heme-RHx complex shows (Figure 6A,B) in the Soret region an optical rotatory dispersion trough at 414 nm and a positive circular dichroism band at 418 nm when examined at pH 9.0. Addition of sodium dithionite to the complex resulted in a markedly decreased reduced mean residue rotation and decreased reduced mean residue ellipticity; the latter change was associated with a shift of the circular dichroism maximum from 418 and 426 nm. Exposure of the reduced heme-RHx to CO strikingly increased the reduced mean residue rotation and shifted the trough from 414 to 410 nm. Furthermore, the reduced mean residue ellipticity decreased and the circular dichroism maximum shifted to 416 nm. The complex was then exposed to O_2 which reversed the CO effect to a great extent. An even closer approximation of the original optical rotatory dispersion curve and circular dichroism spectrum resulted from subsequent exhaustive dialysis against borate buffer.

In a separate experiment, KCN was added in a 1.1 molar ratio to heme-RHx causing a significant diminution of levorotation at 414 nm, a decreased ellipticity, and a shift of the circular dichroism band maximum to 416 nm.

Discussion

The described optical rotatory dispersion curves and circular dichroism spectra of the hemopexins do not, to our knowledge, resemble any hitherto reported spectra. The presence of

six and five S–S bonds, respectively, in HHx and RHx (Hrkal and Muller-Eberhard, 1971) may account in part for the structural characteristics of the molecules. Whether the high carbohydrate content contributes to the peculiarities of the curves and spectra remains to be explored. The hemopexins contain a considerable amount of hexosamines and 6–8% sialic acid (Heimburger *et al.*, 1964; Hrkal and Muller-Eberhard, 1971). A high content of N-acetylated hexosamines affects the optical rotatory dispersion and circular dichroism of glycoproteins (Beychok and Kabat, 1965). Moreover, proteins with a sialic acid content exceeding 10% show a weak positive Cotton effect at 195 nm (Jirgensons and Springer, 1968).

A slight but conspicuous species difference between the hemopexin molecules of these two mammals was revealed at pH 7.7 in that HHx had less rotatory power and RHx increased its negative reduced mean residue ellipticity as compared to pH 9.0. That RHx, but not HHx, showed a conformational change upon interaction with heme, probably does not reflect a species difference. Two facts may explain why no change occurred: first, the available HHx contained a heme:protein ratio of 0.3; and secondly, the spectral change of RHx was already maximal when partially saturated with heme.

The effect of 8 M urea, 4 M guanidine hydrochloride, and performic acid treatment on RHx are as expected. Remarkable only is the reversibility of both the altered levorotation and the loss of the circular dichroism band at 232 nm encountered in high concentrations of denaturating agents.

A conformational change was observed on interaction of heme with apomyoglobin. Harrison and Blout (1965) measured $[R']_{233} = -7300$ for apomyoglobin at 5° and $[R']_{233} = -9000$ for myoglobin at 30° in water. Breslow *et al.* (1965) reported a levorotatory change of $\Delta[m']_{233} = -1310$ when apomyoglobin binds heme at 25° in borate buffer, pH 9.4. These changes in reduced mean residue rotation compare well with those reported here for apo-RHx and heme–RHx, *i.e.*, $\Delta[m']_{232} = -1500$. Urry (1967) cautions that these alterations should not necessarily be attributed to a change in helical content because they may instead depend on the optical activity of coordinating imadazole groups. In this connection, it is of interest that both apomyoglobin and apohemopexin are equally effective in removing heme from a cytochrome of the P-450 type (Muller-Eberhard *et al.*, 1969b). Thus, their affinity for heme is probably very similar.

The reported optical rotatory dispersion and circular dichroism values in the Soret region indicate strongly that CN is bound by the heme–RHx complex and that, after reduction of the latter, CO is ligated. Ligand formation of the complex with O_2 is less certain, since both the optical rotatory dispersion trough and the circular dichroism bands were not shifted, although the height of their minima and maxima was diminished. Circular dichroism findings in the Soret region reflected changes in the extent of residue ellipticity as well as shifted wavelengths. Optical rotatory dispersion measurements, however, with the exception of those on CN ligand formation, showed no alteration in wavelengths. This is possibly due to changes in Cotton effects removed from the wavelength region under investigation.

Recently, Samejima and Kita (1969) described distinct Soret Cotton effects and circular dichroism spectra for carbonyl-, oxy-, azide-, and cyanomyoglobin. Derivatives of ferromyoglobin showed a greater increase in Soret Cotton effect and in circular dichroism alteration than those derived from ferric heme. Contrary to our observations on the heme–RHx complex, $[\theta]$ values were alike for the circular dichroism bands of myoglobin and reduced myoglobin, although the latter was red shifted. A sizable difference in the ellipticity value was measured between ligated CO- and O_2-myoglobin which was not encountered for heme–RHx.

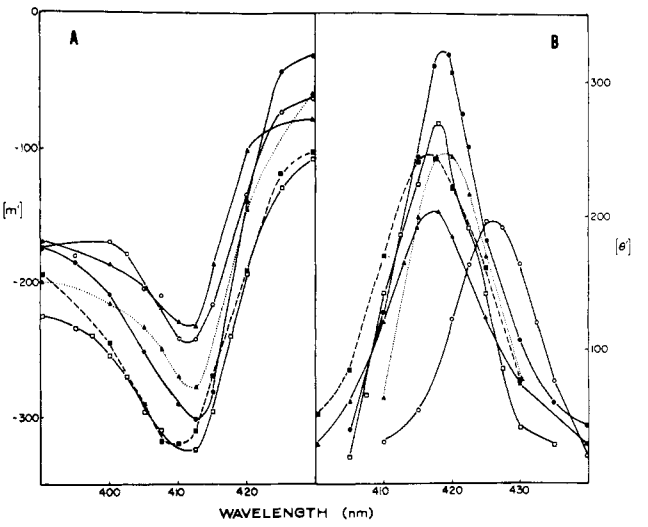

FIGURE 6: Optical rotatory (A) and circular dichroic (B) measurements in the region of the Soret band for rabbit hemopexin in 0.025 M sodium borate, pH 9.0: heme–RHx (●—●); reduced (○—○); ligated with CO (■---■); O_2 (▲---▲); CN (△—△); and reoxidized (□—□).

The structural resemblance of human and rabbit hemopexins is reflected in the pronounced similarities of their optical rotatory dispersion curves and circular dichroism spectra. The magnitude of the levorotatory increase observed for RHx in the far-ultraviolet region when combining with heme is comparable to that of myoglobin. The changes in Soret circular dichroism spectra of heme–RHx upon reduction, however, are more pronounced than in myoglobin. Moreover, the differences in ellipticity values between CO- and O_2-ligated and nonligated heme–RHx are smaller than found among myoglobin derivatives and point to distinct molecular differences between these two heme binding proteins.

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Purification and Some Properties of Molybdoferredoxin, a Component of Nitrogenase from *Clostridium pasteurianum**

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ABSTRACT: A method for the purification of molybdoferredoxin (Mo-Fe protein), a component of the nitrogenase complex from *Clostridium pasteurianum*, is presented. This method yields about 2 g of pure protein and can be prepared within 2 days from 1 kg of dried cells (170 g of crude protein). The molecular weight of the protein is 168,000 and has a partial specific volume based on sedimentation equilibrium studies of 0.72 ml/g. The protein contained the following metals and sulfur groups (moles/mole of protein): Mo, 1.0; Fe, 14.0; SH, 23; S^{2-}, 16. The protein showed a tendency to aggregate if oxidized or stored for several months in liquid nitrogen. Significant changes in both the visible and paramagnetic resonance spectra occurred as well as a 70% loss in acetylene reduction activity after 1-hr exposure to air. Less loss of activity resulted from short-term exposure to air even though the spectral change was optimal.

The nitrogenase complex from *Clostridium pasteurianum* has been resolved into two oxygen-sensitive protein components (Mortenson, 1965), named molybdoferredoxin (MoFd[1]) and azoferredoxin (AzoFd) (Mortenson *et al.*, 1967). AzoFd, a cold-sensitive protein, exists in solution as a dimer of molecular weight 55,000 and has been reported to contain four nonheme iron atoms and four acid-labile sulfide groups per dimer (Nakos and Mortenson, 1971a).

This paper describes the method currently used to purify MoFd and reports a revision in the metal content of the protein. A careful investigation of the molecular weight of MoFd was undertaken since values previously reported (Mortenson *et al.*, 1967; Dalton and Mortenson, 1970) were only estimations which ranged between 100,000 and 200,000. Several other properties of this protein are reported.

Experimental Section

Materials. Cacodylic acid, Tris (tris(hydroxymethyl)aminomethane), mersalyl (sodium *O*-[3-hydroxymercuri-2-methoxypropyl)carbamyl]phenoxyacetate), and ATP (sodium salt) were obtained from Sigma Chemical Co. Protamine sulfate was obtained from General Biochemical Co. Sephadex G-25, G-100, and G-200 were obtained from Pharmacia Fine Chemical Co. Other reagents were obtained from common commercial sources.

Methods. CELLS AND CELL-FREE EXTRACTS. *Clostridium pasteurianum* W5 cells were grown, harvested, and dried as described previously by Mortenson (1964). Batches of 2×10^3 to 3×10^3 g of dried cells were thoroughly mixed and distributed in 60-g portions, which were then sealed in bottles and stored under H_2 at $-20°$. This mixing minimized variation in the purification procedure from one preparation to another. Cell-free extracts were prepared by autolysis of the dried cells in anaerobic 0.05 M Tris-HCl buffer, pH 8.0, as described by Moustafa and Mortenson (1969).

ASSAYS. Protein concentration was estimated with the biuret reagent with bovine serum albumin (Sigma) as the standard. Acetylene reduction was used to measure enzymatic activity. The ethylene produced was analyzed quantitatively by gas chromatography as described by Bui and Mortenson (1968). Assays were carried out in 10-ml glass vials fitted with

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[1] Abbreviations used are: MoFd, molybdoferredoxin; AzoFd, azoferredoxin; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PSE, protamine sulfate extract; PSO$_4$, protamine sulfate.