# Circular Dichroism and Magnetic Circular Dichroism of Reduced Molybdenum–Iron Protein of *Azotobacter vinelandii* Nitrogenase†

P. J. Stephens,* C. E. McKenna, M. C. McKenna, H. T. Nguyen, and F. Devlin

ABSTRACT: Studies of the circular dichroism (CD) and magnetic circular dichroism (MCD) of the dithionite-reduced molybdenum–iron protein of *Azotobacter vinelandii* nitrogenase (Av1) are reported. CD and MCD are measurable at room temperature across a wide spectral range, from the near-UV to the near-IR. The visible–near-UV CD is insignificantly affected by moderate variations in pH, temperature, ionic strength, and buffer, providing evidence against conformational change in the range studied. $Mg^{2+}$ and ATP also cause no observable change in the visible–near-UV CD. Both CD and MCD in the visible–near-UV are unaffected by 30% inactivation by $O_2$. However, the CD and MCD spectra of uncrystallized Av1 differ very significantly from those of crystallized Av1; in particular, the MCD spectrum is very sensitive to the presence of heme impurities. The identicality in both CD and MCD spectra of the reduced molybdenum–iron proteins from *Azotobacter vinelandii* and *Klebsiella pneumoniae* shows that these proteins contain metal clusters, identical in number, structure, and protein environment. While the absorption, CD, and MCD spectra of reduced Av1 are typical in many respects of simpler iron–sulfur proteins and are most similar to the $[Fe_4S_4(SR)_4]^{3-}$ clusters found in reduced bacterial ferredoxins, significant differences exist. It is concluded, therefore, that the clusters present are not identical with those previously characterized, a conclusion earlier arrived at from electron paramagnetic resonance, Mössbauer, and EXAFS spectroscopies.

The enzyme nitrogenase ($N_2$ase)[1] is central to the process of biological nitrogen fixation (Newton & Nyman, 1976; Newton et al., 1977; Newton & Orme-Johnson, 1980; Winter & Burris, 1976; Orme-Johnson & Davis, 1977; Mortenson & Thorneley, 1979; Hardy et al., 1979). $N_2$ase activity requires two metalloproteins, one (MoFe) containing both Mo and Fe, and one (Fe) containing only Fe. In the presence of a suitable electron donor, $N_2$ase catalyzes the ATP-dependent reduction of $N_2$ to $NH_3$, as well as the reduction of certain other small unsaturated molecules (McKenna, 1980).

We are currently examining the application of chiroptical spectroscopy to the study of $N_2$ase. Our earliest results, recently published (Stephens et al., 1979, 1980), have shown that both circular dichroism (CD) and magnetic circular dichroism (MCD) (Stephens, 1974) can be observed across a wide spectral range in both MoFe and Fe proteins under normal, ambient temperature solution conditions. As a result, CD and MCD provide new techniques for the study of the metal centers present in MoFe and Fe proteins and for monitoring physical and chemical processes involving these proteins.

In this paper, we present a detailed account of the CD and MCD spectra of the dithionite-reduced MoFe protein of *Azotobacter vinelandii* (Av1).[2]

## Experimental Procedures

Crystalline Av1 was prepared as described elsewhere (McKenna et al., 1980, 1981) and stored, in 25 mM Tris buffer, pH 7.4, containing 250 mM NaCl and approximately 1 mM sodium dithionite, as pellets in liquid nitrogen. Except where explicitly indicated, all measurements reported in this paper were made on Av1 obtained in a single preparation, having 1.9 Mo and 3 Fe/mol and a specific activity (SA) of 2000–2100 nmol of $C_2H_4$ $min^{-1}$ (mg of Av1)$^{-1}$ (corrected for the slight $C_2H_2$ unsaturation, this corresponds to a specific activity of 2200–2300). Activity assays were carried out under argon in 21.5-mL rubber-stoppered vaccine bottles containing 1 mL of reaction mixture and 1 mL of acetylene gas. The reaction mixture contained 5 μmol of ATP (Sigma), 25 μmol of creatine phosphate (Sigma), 7.8 units of creatine phosphokinase (Sigma), 5 μmol of $MgCl_2$, 25 μmol of Hepes buffer (Calbiochem), pH 7.4, 20 μmol of sodium dithionite (MCB), and 0.06 mg of MoFe protein, with a 40× molar excess of Fe protein. The bottles were shaken at 30 °C in a water bath, and the reaction was started by the addition of Fe protein to the assay bottles. The reaction was stopped after 10 min by the injection of 0.25 mL of a 25% (w/v) solution of trichloroacetic acid. Ethylene formation was detected on a 2 ft × $^3/_{16}$ in. glass column packed with Porapak N by using a Varian 1440 flame detector gas chromatograph and a Varian 485 integrator. Acetylene was used as the internal standard for ethylene formation (McKenna, 1979). Protein concentration was determined by using the biuret method (Campbell et al., 1970).

All protein manipulations were carried out inside a glovebox (Van Beek Industries) flushed with dry $N_2$ that was passed through activated Ridox (Fisher Scientific). Frozen Av1 pellets are thawed under Ar and transferred via a syringe into the box by using a septum-sealed port. Protein solutions were prepared by diafiltration with an Amicon Model 8MC unit equipped with a PM-30 Diaflo membrane and pressurized with $H_2$ that was scrubbed by a catalytic hydrogen purifier (Englehard Industries). All buffer solutions were carefully

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[1] Abbreviations used: $N_2$ase, nitrogenase; MoFe, molybdenum–iron protein; Fe, iron protein; FeMoco, molybdenum–iron cofactor; CD, circular dichroism; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

[2] We use the Sussex nomenclature (Eady et al., 1972): Av1, Av2; Kp1, Kp2; Cp1, Cp2 refer to the MoFe and Fe proteins of *Azotobacter vinelandii*, *Klebsiella pneumoniae*, and *Clostridium pasteurianum*, respectively.

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deaerated with Ar and contained approximately 2 mM sodium dithionite. Hepes buffer was from Calbiochem; Tris buffer was prepared from Tris-HCl and Trizma base (Sigma); phosphate buffer resulted from mixing equimolar $K_2HPO_4$ and $KH_2PO_4$ solutions (Mallinckrodt). Except where the effects of variations in pH and salt concentrations were investigated, all solutions were at pH 7.4 and contained 250 mM NaCl. $Mg^{2+}$ and ATP were added as solutions of $MgCl_2 \cdot 6H_2O$ (Mallinckrodt) and $Na_2ATP \cdot 2H_2O$ (Sigma) in 25 mM Hepes, pH 7.4, and 250 mM NaCl. Exchange of solvent $H_2O$ for $D_2O$ was accomplished by diafiltration with buffer solutions constituted with $D_2O$ of 99.7% isotopic purity (Merck, Sharp and Dohme).

Crystalline Av1 protein of SA 2000, in 25 mM Hepes, pH 7.4, 250 mM NaCl, and ~2 mM sodium dithionite, was partially inactivated by exposure to air in a gently agitated open tube for approximately 30 min. At this time, the specific activity was 1400, corresponding to a loss of 30% of the initial protein activity. The sample tube was then flushed with argon, and, after addition of excess sodium dithionite, the solution was returned to the glovebox. The uncrystallized Av1 used [FeMo II of McKenna et al. (1981)] possessed a specific activity of 700.

CD and MCD were measured over the wavelength range 200–800 nm by using a Cary 61 spectropolarimeter. Magnetic fields up to 50 kG (5 T) were produced by a Varian superconducting magnet. Additional shielding was incorporated in the Cary 61, enclosing both the photomultiplier tube and its preamplifier, in order to minimize field-induced base-line shifts. The Cary 61 spectropolarimeter is calibrated by using the 290-nm band of *d*-camphorsulfonic acid (CSA). Our anisotropy ratios (*g* factors) are in satisfactory agreement with the results of Chen & Yang (1977).

Over the range 700–2000 nm, CD and MCD are measured by using an instrument constructed at USC. This instrument has been described at various stages of its development (Osborne et al., 1973; Nafie et al., 1976; Stephens & Clark, 1979). In the experiments reported here, a Sylvania 750-W tungsten–halogen lamp and 77 K InSb detector were used; both photoelastic modulators employed were Hinds International $CaF_2$ devices with modulation frequencies of approximately 57 and 60 kHz. Absorption spectra over the range 200–2000 nm were recorded by using a Cary 17 spectrophotometer.

Absorption, CD, and MCD spectra are reported in terms of the molar extinction coefficient ($\epsilon$), the differential molar extinction coefficient ($\Delta\epsilon$), and the differential molar extinction coefficient per tesla (10 kG) ($\Delta\epsilon$), respectively. Beer's law is assumed to be valid throughout. The Av1 molecular weight is taken to be 240 000 [see Eady & Smith (1979) and Swisher et al. (1977) for a compilation of Av1 molecular weight measurements].

In order to minimize the volume of protein solution required for a spectroscopic measurement at a given concentration and path length, cylindrical cells of small diameter and containing no dead volume were employed. The experiments reported here used neckless cells (Optical Cell Co.) of internal diameter 11 mm, path lengths varying between 0.5 and 10 mm and calibrated by using CSA CD, and either suprasil or infrasil fused quartz windows.

In order to protect the air-sensitive protein solutions, the cells were enclosed in cylindrical holders, containing double O-ring sealed fused quartz windows (Heraeus-Amersil). The anaerobic performance of these holders has been evaluated by monitoring visually the oxidation of solid chromous acetyl acetonate (generously provided by Professor C. A. Reed). In typical cell holders, no detectable change from orange toward grey-green was visible after 10 days. In addition, complete activity retention of MoFe solutions is observed after periods in excess of 24 h.

In view of the smallness of the CD of the $N_2$ase proteins, it is very important that all windows be of high optical quality. The absence of dependence of the CD base lines on rotational orientation of all cells and cell holders was therefore carefully monitored, especially in the latter case where excessive tightening of O-rings can readily induce observable strain in the windows.

In some natural CD measurements, sample temperature control over the range 5–40 °C was achieved by enclosing the cell holder in a thermostated mount. In all other cases, ambient temperature was in the range 20–28 °C.

As a result of the smallness of the CD of the $N_2$ase proteins and of the rapid increase in the extinction coefficient on decreasing wavelength, it is necessary for optimal results to measure spectra in segments, the absorbance in each segment being placed in the range 0.1–2.0 (or as close thereto as possible) by use of appropriate cell path lengths and concentrations. Concentrations in these experiments were in the range 5–85 mg/mL.

All solutions are monitored with regard to the concentration and activity immediately prior to and subsequent to their study. Absorption, CD, and MCD spectra of solutions are measured repetitively (a minimum of twice, often more frequently) to ensure that spectra are not time dependent over the span of the experiments. In cases where sample degradation causes any significant change in activity, absorbance, CD, or MCD, the measurements are discarded. Sample pH is in all cases measured immediately after spectroscopy by using a Beckman Futura Model 39505 5-mm combination electrode.

## Results

The absorption, CD, and MCD spectra of dithionite-reduced Av1 across the near-IR, visible, and near-UV spectral region (380–1900 nm) are reported in Figures 1–3. Figure 1 shows the normalized spectra. Figure 2 shows the CD and MCD anisotropy ratio (AR) spectra, obtained by dividing the CD and MCD by the absorption. Figure 3 exhibits the relative magnitudes of CD and MCD at normal magnetic field strengths (4 T) and the signal-to-noise ratio typically observed in Cary 61 experiments.

The spectral range is limited, in the UV, by the absorption of sodium dithionite and, in the IR, by solvent absorption. Sodium dithionite exhibits an absorption band in the near-UV, peaking at ~315 nm (Dixon, 1971; Ljones & Burris, 1972). At the dithionite concentrations and path lengths used in our experiments, the contribution to absorption and MCD[3] spectra can be appreciable at wavelengths as great as 360–370 nm. The cutoff used in this work is 380 nm. In the IR, with deuterated solutions, solvent absorption is observed beginning at ~1400 nm, due to residual $H_2O$, and becomes intense at ~2000 nm due to $D_2O$.[4] The cutoff in absorption spectra used in this work is 1300 nm. Since vibrational transitions exhibit very weak CD and MCD (Nafie et al., 1976), however, the solvent absorption does not contribute to the CD and MCD spectra, and for these the cutoff used is 1900 nm.

[3] The MCD spectrum of the 315-nm band of sodium dithionite is of the form expected for a B term (Stephens, 1974); the sign of the MCD is negative, and the anisotropy ratio [normalized to 10 kG (1 T)] is $\sim 6 \times 10^{-5}$.

[4] Typical near-IR absorption spectra of deuterated solutions of proteins are exhibited in Figures 1 and 2 of Stephens et al. (1978b).

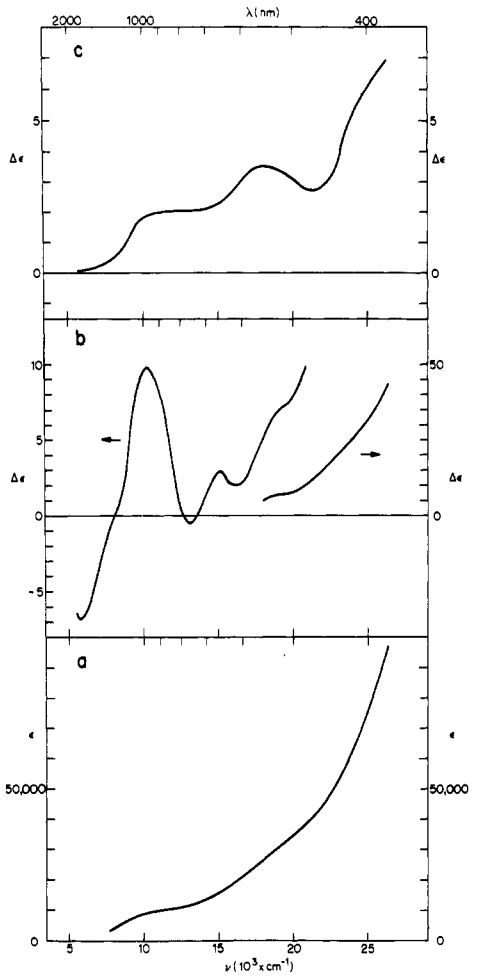


FIGURE 1: (a) Absorption spectrum of reduced Av1; (b) CD spectrum of reduced Av1; (c) MCD spectrum of reduced Av1.

The data in Figures 1–3 were obtained with Av1 in 25 mM Hepes, 250 or 750 mM NaCl, and ~1 mM sodium dithionite, pH 7.4, at ambient temperatures (20–28 °C). We have examined the effects of variation in environmental conditions with respect to buffer (Hepes, Tris, phosphate), NaCl concentration (250–750 mM), pH (6.9–8.9), and temperature (5–40 °C) on the visible–near-UV CD of Av1. We have also examined the effects of addition of excess $Mg^{2+}$ (10 mM) and excess ATP (10 mM), separately and together. Insignificant changes were observed in all cases in the AR spectrum over the entire range sampled, as shown in Figure 4. The insensitivity to temperature removed the need to carefully regulate this parameter in these Av1 CD and MCD experiments.

The effects of $O_2$ damage, leading to 30% loss of activity on the visible–near-UV CD and MCD, were also found to be very small, as exhibited in Figure 4. On the other hand, incompletely purified protein, which had not been crystallized, was found to provide a significantly different CD spectrum and a dramatically different MCD spectrum, as shown in Figures 3 and 4.

The spectra of Av1 in Figure 1 show minor quantitative differences from those reported earlier (Stephens et al., 1979). This is attributable primarily to the use of biuret determination of protein concentration in this work, in place of the Lowry method used earlier. The AR spectra, which are independent of the concentration determination, are identical with those obtained from the earlier spectra within experimental error.

## Discussion

The absorption spectrum of dithionite-reduced,[5] crystallized

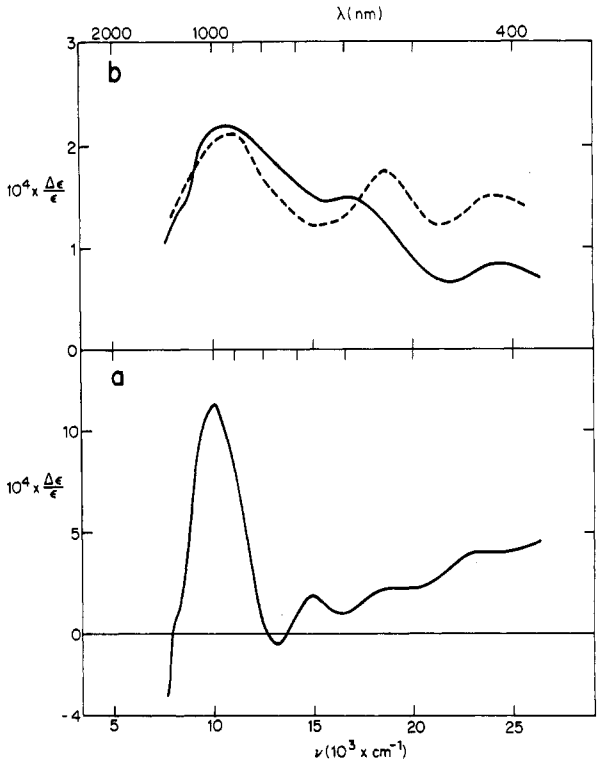


FIGURE 2: (a) CD anisotropy ratio (AR) spectrum of reduced Av1. (b) MCD AR spectrum of reduced Av1 (—) and of *Clostridium pasteurianum* ferredoxin (- - -).

Av1 has been reported by Shah & Brill (1973) over the range 350–650 nm and by Bulen (1976) over the range 370–700 nm. The spectrum in Figure 1 is in good agreement with these earlier spectra; values derived[6] from Figure 3 of Shah & Brill (1973) at 400, 500, and 600 nm are within 12% of our values, while Figure 1 of Bulen (1976) agrees to within 5%. Our results extend the long-wavelength limit of the earlier data to 1300 nm.

The earliest absorption spectra of reduced Av1 reported were due to Bulen & LeComte (1966) and Burns et al. (1970). The spectrum of Bulen & LeComte is qualitatively similar to the later spectra of Shah & Brill and of Bulen, but leads to $\epsilon$ values[6] lower than those of Figure 1 by 35–40%. This is consistent with the reported ~70% purity. Although obtained with crystallized protein, the spectrum of Burns et al. shows peaks at ~420, ~525, and ~557 nm, which have been ascribed to contaminating heme protein (McKenna, 1971; Shah & Brill, 1973). Peaks at these wavelengths are visible in our spectrum of uncrystallized Av1 shown in Figure 3 and, as discussed below, give rise to large MCD features diagnostic of the heme group. These features disappear from our absorption and MCD spectra on crystallization.

The absorption spectra of dithionite-reduced Kp1 [250–760 nm (Eady et al., 1972); 380–1300 nm (Stephens et al., 1979)] and Cp1 [300–660 nm (Walker & Mortenson, 1973)] are similar, qualitatively and quantitatively,[7] to that of Av1. Heme

[5] Henceforth, "reduced Av1" will be used in place of "dithionite-reduced Av1".

[6] With the assumptions that the absorbance reported is due to protein alone, the relevant base line has been subtracted, the path length is 1 cm, and the molecular weight is 240 000.

[7] $\epsilon$ values for Cp1 calculated from Figure 3 of Walker & Mortenson (1973) are 25–30% lower than our Av1 values; $\epsilon$ values for Kp1 calculated from Figure 6 of Eady et al. (1972) are 35–45% lower; however, $\epsilon$ values for Kp1 reported in Stephens et al. (1979) are within 15% over the range 380–1300 nm, presumably due to the use of Kp1 of higher purity.

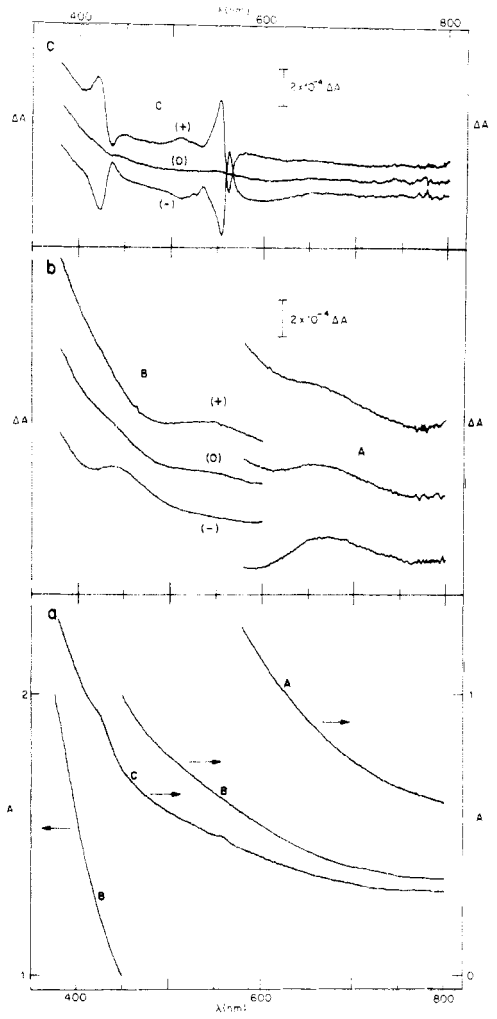


FIGURE 3: (a) Absorption spectra of reduced, crystallized, and uncrystallized Av1. Spectra A and B are of a solution containing 21.3 mg/mL crystallized Av1 (SA 2000) in 25 mM Hepes, pH 7.4, 250 mM NaCl, and ~1 mM sodium dithionite, and with path lengths 5.14 and 2.09 mm, respectively. Spectrum C is of a solution containing 11.2 mg/mL uncrystallized Av1 (FeMoII) (SA 700) in 25 mM Tris, pH 7.4, 250 mM NaCl, and ~1 mM sodium dithionite and with a path length of 5.10 mm. (b) CD and MCD spectra of reduced crystallized Av1. Spectra A and B correspond to absorption spectra A and B in (a). Spectral bandwidth is 4 nm; pen period is 10 s. (+), (0), and (-) indicate magnetic fields of +40 kG, 0, and -40 kG, respectively. (c) CD and MCD spectra of reduced uncrystallized Av1, corresponding to absorption spectrum C in (a). Spectral bandwidth is 4 nm; pen period is 10 s. (+), (0), and (-) indicate magnetic fields of +40 kG, 0, and -40 kG, respectively.

protein contamination has not been observed with these proteins.

Studies of CD in MoFe proteins have been reported previously. Eady et al. (1972) exhibited CD spectra of reduced and oxidized Kp1 over the range 210–250 nm and reported that weak CD existed for reduced Kp1 at visible wavelengths. Chen et al. (1973) reported CD in reduced and oxidized Cp1 in the range 200–230 nm. Yates & Planqué (1975) reported the absence of observable CD in Ac1 in the range 300–600 nm. Prior to our work, no studies of Av1 CD or the MCD of any MoFe protein have been described.

Our results demonstrate that CD and MCD can be observed in reduced Av1 across a wide wavelength range from the near-UV to the near-IR. We have previously demonstrated very similar CD and MCD spectra in reduced Kp1 (Stephens et al., 1979). It is clear, therefore, that the prior absence of CD spectra at wavelengths greater than 250 nm reflects the sensitivity of earlier measurements, rather than an intrinsic lack of chirality in MoFe proteins. Optimum signal-to-noise ratio in the CD measurement in turn requires optimum instrument sensitivity and sample absorbance (in the range 0.1–1.0 for most instrumentation). The Cary 61 spectropolarimeter and our IR CD instrument are designed for high sensitivity and are capable of noise levels in $\Delta A$ of $<10^{-5}$ at practical bandwidths and time constants. Earlier work used less sensitive instrumentation. Since the extinction coefficients of MoFe proteins decrease continuously with increasing wavelength from 300 nm, optimization of sample absorbances requires increasing protein concentrations. Our near-IR experiments used concentrations as high as 80–85 mg/mL at path lengths of 1–5 mm. It is possible that earlier CD experiments were unsuccessful due to the use of insufficiently absorbing samples.

As shown in Figures 3 and 4, the CD and, especially, the MCD spectra of Av1 preparations are sensitive to the presence of impurity proteins. The MCD spectrum of uncrystallized Av1 clearly shows the presence of heme protein impurity (McKenna et al., 1980). With the assumption that the heme MCD is identical in magnitude with that of ferrocytochrome *c* (Sutherland & Klein, 1972), in the sample studied the heme mole percentage is ~1%. The heme Soret and Q ($\alpha$, $\beta$) bands can be only just discerned in the absorption spectrum. Figure 3 clearly demonstrates the considerable superiority of MCD in detecting the presence of heme impurities.

Although the nature of impurity proteins in nitrogenase protein preparations varies with the bacterial source, our results with Av1 show that an additional factor militating against observation of CD in previous work may have been the presence of significant levels of absorbing impurity proteins.

The specific activity of Av1 reflects not only its purity but also the degree to which $O_2$ has inflicted damage on the protein during purification. Even when impurities are reduced to an undetectable level, it is important to minimize $O_2$ damage. The high activity of the Av1 used in this work indicates that $O_2$ damage is minimal during purification. In addition, the careful monitoring of activity prior and subsequent to spectroscopic measurements enables any loss of activity during these to be detected. At ambient temperatures, it is routinely found that solutions of Av1 survive our handling procedures for times as long as 10 h with no loss of activity. Further evidence that $O_2$ damage does not perturb our spectroscopic data is provided by the lack of sensitivity of both CD and MCD AR spectra to $O_2$ damage, leading to 30% activity loss (Figure 4).

Since the CD of metalloproteins at wavelengths >300 nm usually reflects the chirality induced in intrinsically achiral metal-containing chromophores by the protein moiety, CD is both expected and found to be particularly sensitive to variations in protein structure, either due to environmental perturbations on a specific protein or as found in a series of related proteins from varied sources. Both absorption and MCD spectra, in contrast, are generally an intrinsic property of the chromophores and relatively insensitive to protein structural modification. We have made use of the CD phenomenon to examine the effects of a variety of external perturbations of Av1. In view of its independence on concentration and path length, the AR spectrum, rather than the CD spectrum itself, is the most sensitive criterion of such changes. As seen in Figure 4, essentially no effects are observed in the visible–near-UV CD over the range of perturbations studied. This clearly indicates that the chromophoric groups are in quite rigid environments, which undergo no conformational change in the temperature, ionic strength, and pH ranges covered.

Very few studies of isolated MoFe proteins as a function of temperature, ionic strength, and pH have been reported. Yeng et al. (1970) reported some pH dependence in the absorption spectrum of reduced Cp1 over the pH range 5.6–7.2, Smith et al. (1973) observed a pH-induced transition in the EPR spectrum of reduced Kp1 with $pK \sim 8.7$. In addition, a discontinuity has been reported in the activation energy of Av $N_2$ase at 21 °C (Burns, 1969) and in the Kp1–Kp2 association constant at 17 °C (Thorneley et al., 1975); these have suggested the possibility of a conformational change in either MoFe or Fe protein near ambient temperatures. The current CD studies of Av1 do not provide evidence for related pH and temperature dependences.

The majority of studies of the effects of ATP on MoFe proteins have found no evidence of binding. Gel binding studies have been carried out on reduced Cp1 by Bui & Mortenson (1968) and by Tso & Burris (1973) with negative results; no effect on the EPR of reduced Cp1 and *Chromatium* MoFe protein was found by Zumft et al. (1972, 1973) and by Evans et al. (1973), respectively. Walker & Mortenson (1974) reported no enhancement of the rate of reaction of Cp1 with $\alpha,\alpha'$-bipyridyl on addition of ATP. On the other hand, Biggins & Kelly (1970) obtained evidence of binding of ATP to Kp1 by using gel filtration, Orme-Johnson et al. (1972) reported an effect of ATP on the amplitude of the reduced Cp1 EPR signal, and, recently, Miller et al. (1980) reported ATP binding to Kp1 at very high ATP levels. The absence of any observable perturbation of the CD of Av1 by $Mg^{2+}$ and ATP, separately or together, up to 10 mM, provides additional evidence that there is no binding, at least in the neighborhood of the chromophoric groups.

The chromophoric groups responsible for the absorption, CD, and MCD spectra of Av1 at wavelengths greater than 300 nm undoubtedly involve the metal ions of the protein. These have been extensively studied in Av1, Kp1, and Cp1 (Newton & Nyman, 1976; Newton et al., 1977; Newton & Orme-Johnson, 1980; Winter & Burris, 1976; Orme-Johnson & Davis, 1977; Mortenson & Thorneley, 1979; Bottomley & Burns, 1979) by using EPR (Smith et al., 1972, 1973; Zumft et al., 1972, 1973; Orme-Johnson et al., 1972; Palmer et al., 1972; Mortenson et al., 1973), Mössbauer (Smith & Lang, 1974; Münck et al., 1975; Zimmermann et al., 1978; Huynh et al., 1979), and, most recently, EXAFS (Cramer et al., 1978a,b; Hodgson, 1980) spectroscopies. In addition, a Fe- and Mo-containing moiety has been extracted [MoFe cofactor (FeMoco)] and separately studied (Shah & Brill, 1977; Rawlings et al., 1978; Cramer et al., 1978b), and the cluster extrusion methodology has been applied to MoFe proteins (Wong et al., 1979; Kurtz et al., 1979). As a result of these studies, the metal ions are believed to be grouped in clusters, related to, but not identical with, those characterized in simple ferredoxins. While the exact number and nature of these clusters remain uncertain, recent Mössbauer (Zimmermann et al., 1978) and extrusion (Kurtz et al., 1979) studies have concluded that two types of cluster predominate: a Fe- and Mo-containing cluster [M (Münck et al., 1975)], responsible for the EPR of reduced MoFe proteins and retained essentially unmodified in FeMoco, and a Fe-containing cluster [P (Zimmermann et al., 1978)], involving four Fe atoms, and a close relative of the 4-Fe clusters found in 4-Fe and 8-Fe ferredoxins. In this analysis, two M and four P clusters are present in the intact protein.

The absorption, CD, and MCD spectra of reduced Av1 are consistent with the presence of clusters of the iron–sulfur type but show no features definitely ascribable to specific, known clusters. These spectra thus confirm earlier general conclusions regarding the clusters.

The absorption spectrum of reduced Av1 is quite similar to that of 4-Fe clusters, found in 4-Fe and 8-Fe ferredoxins, in the 3– oxidation level (hence denoted $C^{3-}$). The ratio of the spectrum of reduced Av1 to that of reduced *Clostridium pasteurianum* ferredoxin (CpFd) (Stephens et al., 1978a,b) (which contains two $C^{3-}$ 4-Fe clusters) over the range 10 000–26 000 $cm^{-1}$ is fairly constant at $3.9 \pm 0.7$ (Figure 5). The absorption spectrum would not be inconsistent, therefore, with the presence of $8 \pm 2$ $C^{3-}$ 4-Fe clusters. This in turn requires $32 \pm 8$ Fe/molecule, which is compatible with reported Fe analyses (Orme-Johnson & Davis, 1977; Zimmermann et al., 1978; Eady & Smith, 1979).

The absorption spectrum of the dithionite-reduced Av1 cofactor, FeMoco, has been reported over the range 400–700 nm (Shah & Brill, 1977). Since it is featureless and similar in shape to that of Av1, it follows that this must also be the case for the noncofactor clusters. Unfortunately, the featureless nature of the Av1, FeMoco, and "apoprotein" absorption spectra renders any deductions of cluster type and number therefrom less than definitive. In particular, the presence of reduced 2-Fe clusters, found in 2-Fe ferredoxins, which exhibit similar, though somewhat more featured, absorption spectra to $C^{3-}$ 4-Fe clusters (Stephens et al., 1978a,b), cannot be ruled out. However, it can be said that the absorption spectrum of Av1 shows no sign of the characteristic peaks of either oxidized ($C^{2-}$) or superoxidized ($C^{1-}$) 4-Fe clusters (Stephens et al., 1978a,b) or oxidized 2-Fe clusters (Stephens et al., 1978b), making the presence of such clusters and oxidiation levels unlikely.

The CD spectrum of reduced Av1 is not obviously similar to that of any other ferredoxin (Stephens et al., 1978a,b). The CD spectra of 2-Fe ferredoxins, oxidized and reduced, have been found to be surprisingly similar over a wide range of protein sources (Stephens et al., 1978a,b). In addition, peak $\Delta\epsilon$ values in the visible region in both oxidized and reduced states are typically 10–30. The presence of even one such cluster should therefore be noticeable in the visible CD of reduced Av1; the absence of such CD is strong evidence against the presence of any 2-Fe clusters. The near-IR CD of Av1 bears a qualitative resemblance to that of reduced *Spirulina maxima* ferredoxin (Stephens et al., 1978b). However, the magnitude is several times greater in Av1, and the difference in visible–near-UV CD rules out assignment of the near-IR CD to 2-Fe clusters.

The CD spectra of 4-Fe clusters, in all three known oxidation levels ($C^{3-}$, $C^{2-}$, and $C^{1-}$), are considerably weaker in amplitude than those of 2-Fe clusters (Stephens et al., 1978a,b). Further, all proteins studied to date exhibit unique spectra, unrelated to those of any other protein. The qualitative difference between Av1 CD and previously reported 4-Fe cluster CD is therefore neither inconsistent with nor diagnostic of the presence of 4-Fe clusters in Av1. Like the 4-Fe ferredoxin clusters studied, Av1 shows strong near-IR CD and weaker visible–near-UV CD, as gauged by the AR spectrum.

CD in FeMoco of Av1 has so far been found to be unmeasurable.[8] However, this result cannot be immediately extrapolated to the protein-bound FeMoco, since its CD is surely strongly influenced by the protein environment. At this time, therefore, the contributions of different clusters to the Av1 CD cannot be disentangled.

[8] Unpublished studies at USC; this result is also found for Kp1 FeMoco.

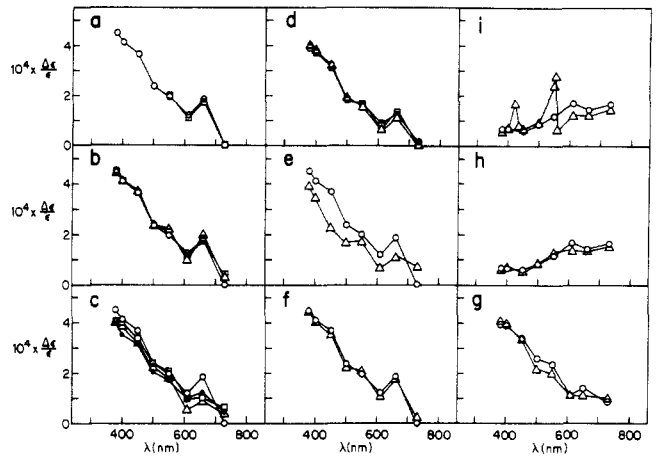


FIGURE 4: (a) CD anisotropy ratio (AR) spectrum of reduced Avl as a function of NaCl concentration: (○) 250 mM; (□) 750 mM. (b) CD AR spectrum of reduced Avl as a function of buffer: (○) 25 mM Hepes; (△) 25 mM phosphate; (□) 25 mM Tris. (c) CD AR spectrum of reduced Avl as a function of pH: (●) 6.9; (○) 7.4; (□) 7.9; (△) 8.9 (in 25 mM Tris). (d) CD AR spectrum of reduced Avl in the presence of $Mg^{2+}$ and ATP: (○) no $Mg^{2+}$ or ATP; (□) 10 mM $Mg^{2+}$; (△) 10 mM $Mg^{2+}$ and 10 mM ATP. (e) CD AR spectrum of reduced Avl as a function of protein purity: (○) crystallized (SA 2000); (△) uncrystallized (FeMoII, SA 700). (f) CD AR spectrum of reduced Avl as a function of activity: (○) SA 2000; (△) SA 1400. (g) CD AR spectrum of reduced Avl as a function of temperature: (○) 5 °C; (△) 40 °C. (h) MCD AR spectrum of reduced Avl as a function of activity: (○) SA 2000; (△) SA 1400. (i) MCD AR spectrum of reduced Avl as a function of purity: (○) crystallized (SA 2000); (△) uncrystallized (FeMoII, SA 700). Unless otherwise indicated, Avl is crystallized, SA 2000, and in 25 mM Hepes, pH 7.4, containing 250 mM NaCl and ~1 mM sodium dithionite.

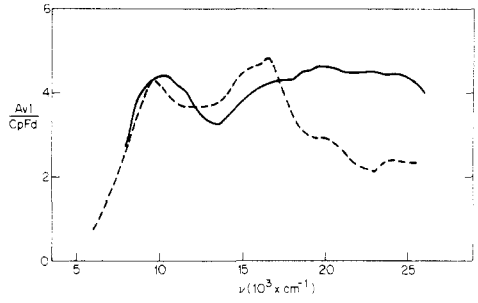


FIGURE 5: Comparison of absorption and MCD spectra of reduced Avl and reduced *Clostridium pasteurianum* ferredoxin CpFd: (—) ratio of $\epsilon$ for Avl and CpFd; (- - -) ratio of $\Delta\epsilon$ for Avl and CpFd.

The MCD of reduced Avl is similar to that of 2-Fe and 4-Fe clusters (Stephens et al., 1978a,b) in being positive in sign throughout the spectral range studied. As with the MCD of ferredoxins, more structure is apparent than in the absorption spectrum. The MCD of reduced Avl is most similar in form to that of the $C^{3-}$ 4-Fe clusters of reduced *Clostridium pasteurianum* and *Bacillus stearothermophilus* ferredoxins[9] (Stephens et al., 1978a,b). However, an appreciable difference exists, as demonstrated by comparison of the AR spectrum to that of CpFd (Figure 2) and by calculation of the ratio of $\Delta\epsilon$ for Avl and CpFd (Figure 5). The most noticeable difference occurs in the near-IR region, where the Avl MCD decreases very much more rapidly than does the MCD of the ferredoxins. The near-IR MCD of Avl thus appears to be inconsistent with the presence of normal $C^{3-}$ 4-Fe clusters.[10]

[9] The MCD spectrum of the reduced Fe protein of nitrogenase can also be included in this comparison (Stephens et al., 1979).

Because of its smallness and the impracticality of achieving Avl concentrations at and above the millimolar level (as is practicable with the simpler ferredoxins), it is difficult to completely exclude the presence of one or two 4-Fe clusters. However, the magnitude of the MCD is clearly inconsistent with the presence of four $C^{3-}$ 4-Fe clusters.

We have previously demonstrated an extremely close similarity in the absorption, CD, and MCD spectra between dithionite-reduced Avl and Kpl (Stephens et al., 1979). All conclusions drawn from the spectra of Avl therefore apply equally to Kpl. In addition, it must also be concluded that the number and nature of the chromophoric metal clusters are identical in the two proteins. The considerable variation in metal content reported (Orme-Johnson & Davis, 1977; Zimmermann et al., 1978; Eady & Smith, 1979) has left open the possibility that different proteins do not possess identical numbers of metal atoms per molecule. Our conclusion is in agreement with that reached from Mössbauer spectroscopy (Smith & Lang, 1974; Münck et al., 1975; Zimmermann et al., 1978).

The identicality in natural CD is extremely strong evidence that the metal clusters are identical not only in primary coordination but also in protein environment, since the latter is reponsible for the existence of the CD. It can thence be deduced that the protein composition in the neighborhood of the clusters is very highly conserved between these two proteins.[11] The extent to which this conclusion can be extrapolated to other MoFe proteins remains to be demonstrated.

## Conclusions

Our principal conclusions are that the CD and MCD spectra of dithionite-reduced Avl are (a) observable, (b) insensitive to minor environmental perturbations, and (c) generally ferredoxin-like, yet differentiable from those of known iron–sulfur clusters.

The observability of CD and MCD permits these techniques to be added to the armamentarium of physical probes of nitrogenase proteins. CD and MCD have the advantages of being observable in aqueous solution at ambient temperatures and in all oxidation states, whether paramagnetic or diamagnetic.

The insensitivity of these optical properties to variations in such parameters as temperature, pH, and ionic strength adds to their analytical utility. Furthermore, in combination with the previously demonstrated identicality not only of absorption and MCD but also of CD between reduced Avl and Kpl, strong evidence is provided of highly conserved, rigid environments of essentially identical metal clusters in different proteins. It is likely, therefore, that CD and MCD will be equally useful in the case of all nitrogenase MoFe proteins.

The further elucidation of the nature of the metal clusters of Avl requires, first, the deconvolution of the optical spectra into their component parts. This can be undertaken in several ways, including redox titration (since different clusters undergo oxidation/reduction at different potentials), variable-temperature MCD (which will enable the spectra of paramagnetic and diamagnetic clusters to be differentiated), cluster extrusion (since different clusters extrude in different ways and under

[10] It is theoretically possible that the rapid decrease in MCD with increasing wavelength could be due to a negative MCD contribution by other clusters in the protein. However, we have found that the near-IR MCD of Avl FeMoco is instead very similar in form to that of Avl. This possibility therefore appears very unlikely.

[11] Ruvkun & Ausubel (1980) have recently demonstrated close homology between $N_2$ase genes from Kp and Av.

different conditions), and study of the FeMoco species. The spectra of individual clusters will then be directly comparable with those of other "model" clusters, including both protein systems and synthetic analogue complexes. Such studies are in progress in our laboratories.

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# Synthesis and Properties of Acrylamide-Substituted Base Pair Specific Dyes for Deoxyribonucleic Acid Template Mediated Synthesis of Dye Polymers†

H. Bünemann,* N. Dattagupta,‡ H. J. Schuetz,§ and W. Müller§

ABSTRACT: We have tried to construct synthetic polymers for sequence-specific recognition and complexation of longer deoxynucleotide sequences. For this purpose, we developed a method of template-directed polymerization of base pair specific DNA ligands such as basic dyes. The template-directed polymerization consists in a copolymerization of various dyes of different specificities staying simultaneously in a binding equilibrium with DNA. In the present paper, we describe the synthesis and the properties of base pair specific monomers especially designed for performance of radical chain polymerization reactions in aqueous medium at room temperature. Different acrylamide derivatives of well-known dyes, such as AT-specific malachite green and GC-specific phenyl neutral red, were synthesized and studied for their ability of base pair specific complex formation with DNA of different base composition. Partition equilibrium dialysis and dye titration agarose gel electrophoresis were used to ensure for several dyes that they may be incorporated into different polymers via copolymerization of their acrylamido derivatives with various small base-unspecific monomers without substantial change of their binding parameters. Furthermore, we demonstrate that acrylamide and other small acrylamide derivatives can be used as building blocks for the synthesis of polymeric links between base pair specific monomers. The results and their consequences for template polymerization reactions are discussed.

In the past two decades, a large number of antibiotics, dyes, and other substances have been isolated and studied intensively to elaborate the molecular structure of their complexes with nucleic acids, especially with double-stranded DNA. The main emphasis of these investigations has been to elucidate the mechanisms involved in the binding specificity of naturally occurring complexes between biological macromolecules, e.g., proteins and DNA. Despite these efforts, no generally accepted model for the recognition process between proteins and DNA could be derived, however. We tried to use our knowledge about base pair specific dyes of sufficient DNA affinity for an alternative, abiotic approach toward the synthesis of oligomeric compounds with "repressor-like" properties. Recent publications similar to our approach deal with attempts to improve the binding affinity of DNA-specific ligands by their di- and oligomerization (Le Pecq et al., 1975; Wakelin et al., 1976; Le Bret et al., 1977; Kuhlmann et al., 1978). These experiments mainly revealed that multiplication of the original binding parameters of the monomers after incorporation into a polymer is only observed if the dyes in their polymeric state are allowed to interact with the DNA without any constrictions caused by the polymer backbone. We reached the same conclusion when we studied polypeptide-linked dimers of phenoxazon, the dye chromophor of actinomycin, some years ago (Bünemann, 1971).

At that time, we decided to circumvent the time-consuming conventional synthesis of long dye polymers of a defined sequence in favor of structurally similar polymers which could be obtained in a much simpler way by polymerization reactions. The advantages of each polymerization procedure are founded in its versatility and velocity. Within a short time, different polymers from various combinations of monomers can be obtained and tested for the influence of different monomers on the properties of the resulting polymers. It is clear that the polymerization procedures yield polymers in which the strictly defined distances and sequences of the effective subunits along the chain as in a conventionally synthesized linear macromolecule are replaced by average values and

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‡ Present address: Department of Chemistry, Yale University, New Haven, CT 06520.
§ Present address: Allgemeine Biologie, Universität Bielefeld, D-4800 Bielefeld, Federal Republic of Germany.

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