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Circular dichroism and magnetic circular dichroism of nitrogenase proteins*

(nitrogen fixation/chiroptical spectroscopy/molybdoenzymes/iron-sulfur proteins)

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ABSTRACT **Circular dichroism (CD) and magnetic circular dichroism (MCD) spectra of nitrogenase components (MoFe protein and Fe protein) from *Azotobacter vinelandii* (Av) and *Klebsiella pneumoniae* (Kp) have been obtained in the near infrared–visible–near ultraviolet spectral region. Previously, visible CD was reported to be absent or barely detectable in nitrogenase proteins; MCD spectra have not been reported. The chiroptical spectra can be measured in solution at room temperature, an advantage relative to spectroscopic methods requiring cryogenic sample temperatures. Absorption spectra were also obtained. The CD and MCD are markedly more structured, and thus interpretively more useful, than the corresponding absorption spectra. The dithionite-reduced MoFe proteins (Av1, Kp1) have nearly identical CD and MCD, demonstrating identical numbers and types of metal centers in similar protein environments. The CD and MCD cannot be explained solely in terms of contributions from known 4-Fe or 2-Fe clusters; the near-infrared MCD is inconsistent with the presence of known 4-Fe clusters. CD and MCD spectra of Lauth's violet-oxidized Kp1 are also reported. The reduced Fe proteins (Av2, Kp2) have similar CD and MCD, again indicating significant conservation of chromophore environment. The spectra clearly demonstrate the presence of a reduced bacterial ferredoxin-like (C^{3-}) 4-Fe cluster. No obvious evidence of additional chromophores is observed. CD, MCD, and absorption spectra of Av1-oxidized Av2 are reported. The absorption spectrum shows the expected shoulder near 390 nm. The CD and MCD are characteristic of a C^{2-} 4-Fe cluster; in particular, the diagnostic near-infrared MCD peak is observed at ≈8300 cm^{-1}. The CD of Av2 oxidized in the presence and absence of MgATP are radically different, providing the first direct evidence for MgATP interaction with Fe protein in this oxidation state.**

The enzyme nitrogenase (N_2ase) has been isolated and purified from various nitrogen-fixing organisms and is currently the subject of intensive investigations (1–4). Active N_2ase systems have been shown to consist of two essential metalloproteins—the MoFe protein (containing ≈2 Mo, ≈24–32 Fe, and ≈20–28 S^{2-}) and the Fe protein (containing ≈4 Fe and ≈4 S^{2-})—which together, in the presence of a suitable electron donor, catalyze ATP-dependent reduction of N_2 to NH_3. Existing evidence suggests that electrons derived from the primary reductant are transferred via the Fe protein to the MoFe protein, which is believed to provide the site for N_2 binding (1–5).

Despite study by various spectroscopic and other techniques, important aspects of the structure and catalytic role of the metal centers in N_2ase remain to be elucidated (1–4). Recent systematic studies of the circular dichroism (CD) and magnetic circular dichroism (MCD) of simple iron-sulfur proteins (6, 7) have shown that CD and MCD can be useful in characterizing iron-sulfur cluster type, oxidation level, and protein environment and that more information is afforded by these probes than by unpolarized absorption spectroscopy. Because the Fe and MoFe proteins evidently contain iron-sulfur clusters—albeit unconventional in many respects—we have undertaken to explore the value of CD and MCD in the study of N_2ase. Electronic spectroscopy has so far found relatively limited application in the study of this enzyme (1–4). Electronic absorption spectra of the two components are almost featureless. CD spectra have been obtained in the polypeptide absorption region (wavelengths <300 nm) (8, 9), but CD was reported to be absent at longer wavelengths in both N_2ase components from *Azotobacter chroococcum* (10) and was also undetectable in Fe protein from *Klebsiella pneumoniae* (8). Very weak visible CD has been reported in the *Klebsiella* MoFe protein; however, the spectrum was not given (8). No MCD work has been published.

We report here spectra demonstrating that CD and MCD are observable in both N_2ase components across the near-infrared–visible–near-ultraviolet spectral region (300–2000 nm) in solution at room temperature. We have compared the spectra of N_2ase proteins from *Azotobacter vinelandii* and *K. pneumoniae*. We have examined the dependence of CD and MCD on metalloprotein oxidation state and, in the case of the Fe protein, the sensitivity to MgATP. The results presented clearly indicate that chiroptical electronic spectroscopy is valuable for probing N_2ase metal cluster structure and function.

EXPERIMENTAL

Experiments were carried out on N_2ase proteins purified from N_2-fixing *A. vinelandii* OP and *K. pneumoniae*.§ Av1 and Av2 were isolated in multigram amounts from cell-free extracts of

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Abbreviations: N_2ase, nitrogenase; CD, circular dichroism; MCD, magnetic CD; EPR, electron paramagnetic resonance; DI, difference index.

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§ We shall use the Sussex nomenclature (8) to distinguish N_2ase components with respect to provenance: Av1 and Av2 = MoFe protein and Fe protein, respectively, from *A. vinelandii*; Kp1 and Kp2 = MoFe and Fe proteins, respectively, from *K. pneumoniae*; and similarly for the N_2ase components from *A. chroococcum* (Ac), *Rhizobium japonicum* (Rj), *Clostridium pasteurianum* (Cp).

continuously cultured N_2-grown *A. vinelandii* grown in a N_2 atmosphere (procedures to be published elsewhere). Av1 was a heme-free[¶] preparation of specific activity 2000–2400 nmol of C_2H_4 produced per mg per min; values for Av2 preparations were 1900–2100 nmol of C_2H_4 produced per mg per min. Both components were essentially homogeneous by anaerobic polyacrylamide gel electrophoresis. Kp1 and Kp2 were purified by established methods (8). Kp1 and Kp2 had specific activities of 1700 and 1300 nmol of C_2H_4 produced per mg per min, respectively. The proteins were stored in liquid nitrogen and handled in a glove box under Ridox-purified N_2. Samples for spectroscopy were concentrated (and exchanged as necessary into the required buffer) on Amicon PM-30 membranes in an Amicon 8MC diafiltration unit pressurized with Deoxo-purified H_2. For near-infrared spectroscopy the solvent was replaced by ^2H_2O. The pH of actual cell samples was determined anaerobically with a Beckman Futura 5-mm combination electrode.

Absorption spectra were obtained on a Cary 17. CD and MCD spectra were measured by using a Cary 61 spectropolarimeter in the range 200–800 nm and an infrared instrument constructed at the University of Southern California (13, 14) in the region 700–2000 nm. Magnetic fields up to 50 kilogauss (5 tesla) were provided by a Varian superconducting magnet. Small-diameter cylindrical quartz cells (pathlengths 1.0–10 mm) were filled in a N_2-purged glove box and enclosed in a gastight holder to maintain an anaerobic environment during spectral measurement.

Spectra are reported in terms of ϵ (molar extinction coefficient) for absorption, $\Delta\epsilon$ for CD, and $\Delta\epsilon$ per 10 kilogauss (1 tesla) for MCD. Molecular weights for MoFe and Fe proteins were taken to be: Av1, 240,000; Kp1, 218,000; Av2, 64,000; and Kp2, 67,000 (1–4). The absolute accuracy of specific ϵ and $\Delta\epsilon$ data will reflect any uncertainty in these values and is also limited by the errors normally associated with colorimetric determination of protein concentration by the biuret and Lowry methods. All experiments were carried out at ambient temperatures (range, 20–25°C).

Because sample inactivation has sometimes led to artifactual results in spectroscopic studies of N_2ase components [cf. initial Mossbauer (15, 16) and EXAFS (17, 18) work], we carefully compared activity and protein concentration values immediately before and after all spectral measurements. Specific activity values of samples for which data are presented decreased less than 10% in most cases and never more than 15–20% during spectroscopy over periods of up to 12 hr. In addition, sample pH values were similarly monitored in representative experiments with the Fe protein; an inadvertent pH change has reportedly (19, 20) led to a misinterpretation (21) of electron paramagnetic resonance (EPR) data on Fe protein–nucleotide interactions. All spectra reported here were scanned repetitively to verify time-independence over the duration of the experiments.

The spectral range studied is limited at short wavelengths by the incursion of either $Na_2S_2O_4$ or polypeptide absorption. At long wavelengths, absorption spectra are limited to 1300 nm, beyond which appreciable solvent and protein vibrational absorption occur. This absorption does not give rise to CD or MCD (14), however, and CD and MCD spectra are measured at ≈2000 nm.

[¶] Levels of heme contamination (11, 12) having an almost imperceptible effect on visible absorption spectra of Av1 prominently contribute to the corresponding MCD. Visible MCD spectra of our Av1 samples showed no discernable heme-like features.

RESULTS AND DISCUSSION

MoFe proteins

The absorption, CD, and MCD spectra of $Na_2S_2O_4$-reduced Av1 and Kp1 are shown in Fig. 1. As reported earlier (8, 11), the absorption spectra are featureless, absorption decreasing monotonically from the near ultraviolet with increasing wavelength. Spectra have not previously been reported beyond 760 nm; as shown in Fig. 1, absorption remained far from zero at the limit of our measurements.

The CD spectra showed much more structure than did the absorption spectra. Av1 and Kp1 CD were remarkably similar. Because CD is generally very sensitive to both protein composition and structure, this indicates that the amino acid sequence and its geometrical structure around the chromophoric entities must be closely related in the two components. Sequences of MoFe proteins have not been determined to date; the amino acid compositions of Av1 and Kp1 are fairly similar, having a compositional relatedness difference index (DI) of 5.1 (with reference to a scale such that the DI of identical proteins is 0 and the mode for that of a random group of proteins is 26) (4). Cross-assays of heterologous Av/Kp component mixtures

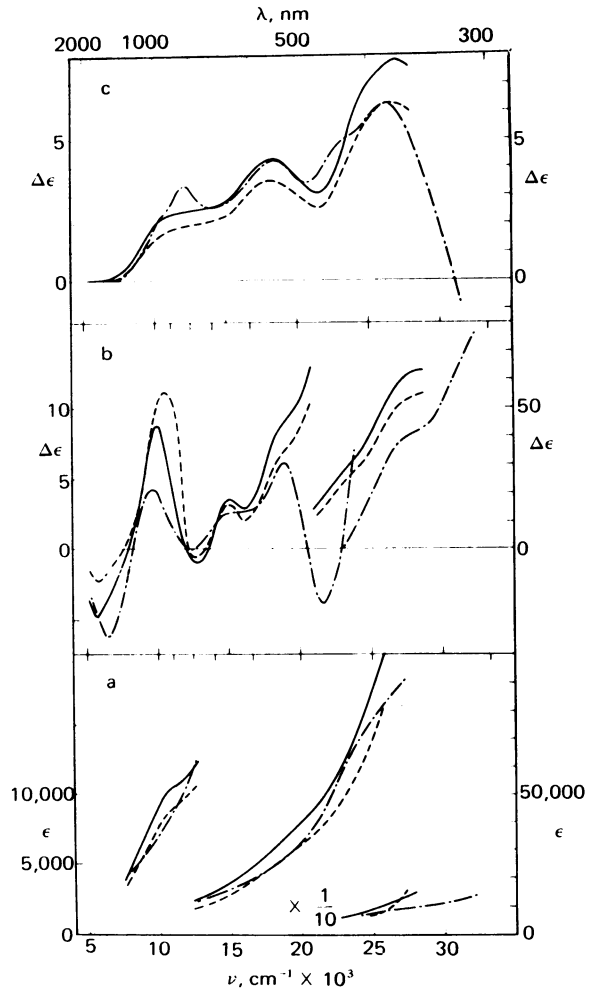

FIG. 1. Absorption spectrum (*a*), CD (*b*), and MCD (at 1 T) (*c*) of reduced Av1 (——), reduced Kp1 (- - -), and oxidized Kp1 (-·-·-) in 0.025 M Hepes, pH 7.4/0.25 M NaCl (Av1 in H_2O) or 0.50 M NaCl (Av1 in ^2H_2O; Kp1 in H_2O or ^2H_2O). Reduced samples were 1.6 mM in dithionite. Av1 and Kp1 concentrations were ≤0.25 mM. Kp1 was oxidized with excess Lauth's violet and isolated by Sephadex G-25 chromatography.

have revealed some degree of functional complementarity (22), but the comparative CD results provide the first direct evidence that the chiral protein environment of absorbing metal centers in these two phylogenetically diverse MoFe proteins is strongly conserved. It will be of interest to extend this comparison to other MoFe proteins having smaller (Av1/Ac1) and larger (Av1/Rj1, Cp1) DI values, including ones that are functionally incompetent with Av2 (Cp1) (22). There has been no previous report of CD studies on Av1. Kp1 was reported to exhibit weak positive CD in the visible (8). Prior failure to observe appreciable CD in these and other MoFe proteins (9, 10) can be attributed to the lower sensitivity of instruments used or the use of suboptimally absorbing solutions. A notable feature of the CD spectra is their continued existence at wavelengths close to 2000 nm.

MCD spectra have not been reported for MoFe proteins. At the magnetic fields used, CD and MCD effects are generally comparable in magnitude. Like the CD, the MCD is found to be more structured than the absorption and to be similar in Av1 and Kp1. Because MCD, unlike CD, is an intrinsic chromophoric property, it is expected to be less sensitive to protein environment than is CD (6, 7). In view of the closely similar CD exhibited by Av1 and Kp1, the similarity in MCD is not surprising.

The electronic transitions responsible for the absorption, CD, and MCD of Av1 and Kp1 at wavelengths greater than 300 nm are attributable to the protein-bound Fe and Mo ions present. These are thought to exist in clusters analogous to those known to occur in simple iron-sulfur proteins (ferredoxins). The EPR (4, 20) and Mössbauer spectra (16, 20, 23) together demonstrate, however, that in MoFe proteins the iron clusters are both heterogeneous and differ significantly from those characterized in known ferredoxins. These conclusions are reinforced by the CD and MCD spectra. On the one hand, the overall magnitude and broad features are not unlike those of ferredoxins containing 4-Fe clusters, allowing for the number of Fe atoms estimated to be present; on the other hand, the spectra are not identical to those of any single previously studied ferredoxin. It is not possible to deconvolute the MoFe protein spectra into individual contributions from the various metal species present at this time. Nevertheless, two conclusions can be drawn. First, the close similarity of the Av1 and Kp1 CD and MCD spectra strongly supports the existence of identical numbers and types of metal species, despite the differences in Fe and Mo content that have been reported for these two components (1–4). Second, despite overall similarity of the visible absorption, CD and MCD of Av1 and Kp1 to those of reduced 4-Fe and 8-Fe ferredoxins (6, 7), the near-infrared MCD is definitely different, diminishing to zero at $\approx$7000 cm^{-1}. Because 4-Fe clusters in C^{3-}, C^{2-}, and C^{1-} oxidation states[‖] all exhibit appreciable MCD in the range 5000–7000 cm^{-1} (6, 7), the presence of conventional 4-Fe clusters in any of these oxidation levels appears to be excluded. Attempts to apply cluster displacement techniques (24) have not yielded straightforward results (20): about half the total extruded Fe from MoFe protein was reported to be obtained as 4-Fe clusters; there was no evidence of 2-Fe clusters (20, 25).

The spectra of Kp1 oxidized by the redox dye Lauth's violet (thionin) are also shown in Fig. 1. It is known that Lauth's violet oxidation of Kp1 leads to an EPR silent state (8), and Mössbauer studies (16) have shown that all Fe species are changed in this oxidation. The absorption spectrum is in agreement with that previously reported (8). The CD of oxidized Kp1 is substantially different from that of reduced Kp1; changes in the MCD also occur although are less striking. The generalizations already made about the reduced MCD spectra can be extended to the oxidized spectrum: the magnitude is similar to that of known 4-Fe proteins, but the spectral features cannot be explained simply in terms of normal 4-Fe clusters. A C^{4-} ferredoxin cluster is thus far unreported but conceivably might be present in reduced Kp1. We cannot be certain that such a cluster would have detectable near-infrared MCD but, if dye oxidation removed one to three electrons from such a cluster, near-infrared MCD would be predicted; however, it was not observed in the oxidized Kp1.

Fe proteins

The absorption, CD, and MCD spectra of $Na_2S_2O_4$-reduced Av2 and Kp2 are shown in Fig. 2. As reported (8, 11) the absorption spectra are featureless, absorption decreasing monotonically from the near ultraviolet with increasing wavelength. Spectra have not previously been reported beyond 760 nm; as shown in Fig. 2, in Av2 absorption remained far from 0 at 1300 nm.

The CD spectra are significantly more structured than the

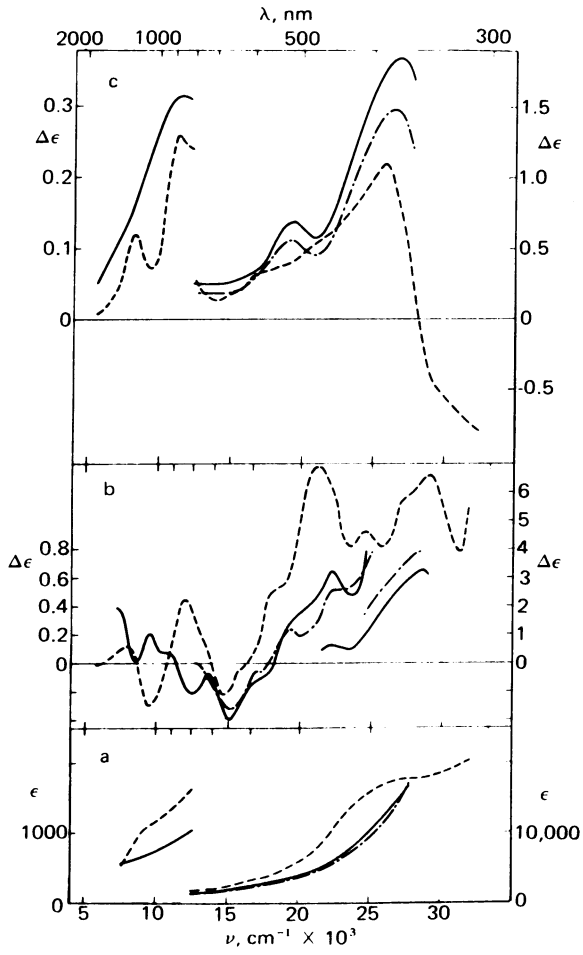

FIG. 2. Absorption spectrum (*a*), CD (*b*), and MCD (at 1 T) (*c*) of reduced Av2 (——), reduced Kp2 (- - -), and post-steady-state oxidized Av2 (- · -) in 0.025 M Hepes pH 7.0–7.4 buffer. Reduced samples contained 0.1 mg of dithiothreitol per ml, 2–4 mM dithionite, and 2 mM Mg^{2+}. Oxidized Av2 samples initially contained 1% (mol/mol) Av1, 0.1 mg of dithiothreitol per ml, 1.6 mM dithionite, 12 mM Mg^{2+}, 10 mM ATP, 20 mM creatine phosphate, and 1 mg of creatine kinase per ml. Dithionite exhaustion was determined spectroscopically. Av2 and Kp2 concentrations were $\leq$1 mM.

[‖] We shall use the following designations for 4-Fe cluster oxidation states: $[Fe_4S_4(SR)_4]^{3-} \equiv C^{3-}$; etc. (6, 7).

absorption spectra. Av2 and Kp2 CD are noticeably similar over the range where both have been measured, indicating, as with the MoFe proteins, considerable conservation of protein structure in the region of the chromophore [the DI of Av2 and Kp2 is 9.2 and both have some cross-activity with the reciprocal MoFe proteins (4, 22)]. There has been no report of CD studies on Av2. CD has been reported in Fe proteins for Kp2 (8), but only at wavelengths below 250 nm; visible CD was reported to be absent in Kp2 and also in Ac2 (10).

The MCD spectra also show more structure than do the absorption spectra. Av2 and Kp2 MCD closely resemble each other. At the magnetic fields used, MCD is generally somewhat larger than the CD.

The EPR of reduced Fe proteins has been compared to the EPR of spinach ferredoxin, a 2-Fe protein (26); however Mossbauer (16), linear electric field effect (LEFE) (20), and cluster displacement (20, 27) studies point to the presence of one 4-Fe cluster. The strength of the Mossbauer evidence has been questioned (20). The MCD of reduced Av2 and Kp2 are similar in form and magnitude to the MCD of reduced (C^{3-} state) 4-Fe clusters in 4-Fe and 8-Fe ferredoxins (6, 7), definitively establishing that these Fe proteins contain one 4-Fe cluster, not two 2-Fe clusters. The absorption spectra and CD are not inconsistent with this conclusion. The absorption and CD are comparable in magnitude to those of other C^{3-} 4-Fe clusters. The CD is not similar in form, but it has been found that the CD of 4-Fe clusters varies appreciably with protein (6, 7). In view of the latter, the close resemblance of Av2 and Kp2 is especially remarkable.

The quantitative agreement among the MCD (per 4-Fe cluster) of Av2, Kp2, and other well-characterized 4-Fe and 8-Fe ferredoxins eliminates the possibility that the low integrated intensity of the Fe protein EPR (1, 4) can be attributed to incomplete reduction of the cluster. Recently, it was suggested that a second paramagnetic center is present, interacting with the 4-Fe cluster and providing a modified lineshape and integrated intensity (20, 28). No independent evidence has yet been adduced for the existence of such a center, and our optical studies have not so far shown any effects attributable to additional chromophores. MCD, EPR, or magnetic susceptibility studies, at lower temperatures than used thus far, might yield more positive evidence, however, and would be of considerable interest.

Fig. 2 also shows absorption, CD, and MCD spectra of Av2 oxidized by Av1 after exhaustion of $Na_2S_2O_4$ in solutions permitting enzyme turnover. In accord with previous observations (4, 20), oxidation leads to the development of a "390 nm" band in the absorption spectrum,** characteristic of C^{2-} state 4-Fe clusters. The CD changes markedly and has a magnitude (although not a form) comparable to that of previously studied 4-Fe clusters in this oxidation state (6, 7). The MCD is also generally consistent in magnitude and form with that observed in C^{2-} 4-Fe clusters (6, 7). In particular, the diagnostic peak appearing just below 10,000 cm^{-1} is observed. For reasons that remain to be clarified, the structure in the visible region is not as clearly resolved as in the "model" ferredoxins.

Because MgATP is necessarily present in the MoFe-protein oxidation of Fe protein under the conditions used, this procedure does not allow the spectroscopic effects of MgATP to be determined. In the case of Av2, however, we have been able

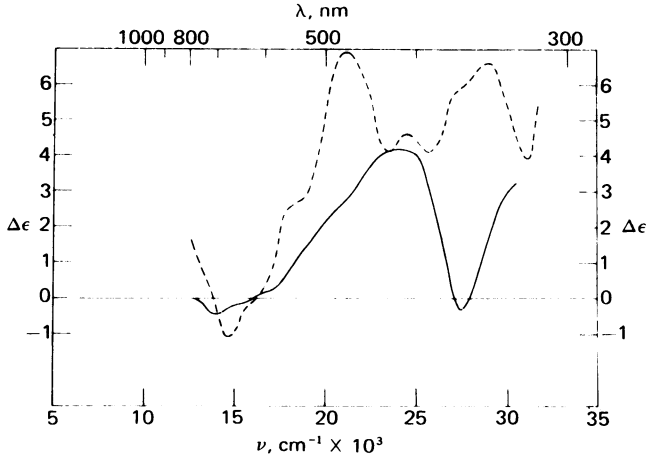

FIG. 3. CD of Av2 oxidized in the absence (—) and presence (- - -) of MgATP. "Self-oxidized" Av2 samples were in 0.025 M Hepes pH 7.0–7.4 buffer, and contained 0.1 mg of dithiothreitol per ml and 2 mM Mg^{2+}. Dithionite exhaustion and oxidation was followed spectroscopically. Post-steady-state oxidized Av2 samples are described in the legend in Fig. 2.

to study MgATP effects by utilizing a previously unreported property of some of our Av2 preparations. We have found that, in concentrated solutions of Av2, excess $Na_2S_2O_4$ slowly disappears with no change in the protein spectra. Subsequently, the protein oxidizes, reaching a stable state in a time that is concentration dependent and can be made to be several hours. No loss of activity accompanies this process. The absorption and MCD spectra of the "self-oxidized" protein are intermediate between those of reduced Av2 and of Av1-oxidized Av2 and are closer to those of the latter. However, the CD of "self-oxidized" Av2 is different from that of either reduced Av2 or Av1-oxidized Av2 (Fig. 3), showing that MgATP binding must also occur with *oxidized* Av2 under the experimental conditions, with significant structural perturbation. Considerable evidence has been adduced for the interaction of reduced Fe proteins and MgATP. Gel equilibrium studies have directly demonstrated MgATP binding to reduced Fe protein (33). Chemical titration experiments indicate that MgATP binding influences iron ion and thiol group reactivity, implying an induced conformational change (26, 31, 34). MgATP has no apparent effect on reduced Fe protein Mossbauer spectra (16) and, at pH 7 (corresponding to maximal activity), addition of MgATP did not alter the shape of the Cp2 EPR spectrum (19, 20), although changes are seen at higher pH in this and other Fe proteins (4, 20). No direct evidence for MgATP effects with oxidized Fe protein has previously been obtained, however. Our results suggest that this may be of importance in the modification of the Fe protein redox potential by MgATP. Studies of the "self-oxidation" of Av2 and of the interaction of reduced and oxidized Av2 with MgATP will be reported in detail elsewhere.

CONCLUSION

We have demonstrated the existence of CD and MCD arising from the metal centers in active N_2ase components from *A. vinelandii* and *K. pneumoniae*. Prior failures to observe CD can be ascribed to technical insufficiencies rather than to any intrinsic absence of the chiroptical phenomena in N_2ase. Close similarities in CD and MCD spectra of corresponding heterologous components (Av1, Kp1; Av2, Kp2) demonstrate that metal center protein environments, as well as the absorbing metal centers themselves, are virtually the same. CD and MCD

** From Fig. 2, the increase in ϵ at 425–430-nm caused by post-steady-state oxidation is ≈5500 for Av2. Values of 2900 for Ac2 at 425 nm (29) and 4500 and 6600 for Cp2 at 430 nm (30, 31) can be compared with a value of ≈7000 obtained (30) for bacterial 4-Fe ferredoxins (32). The latter differences have been explained in terms of variation in active iron content in the Fe proteins (20, 30).

allow the structures adopted by metal ions in these centers to be investigated, by reference to well-characterized iron-sulfur proteins. The MCD of reduced Av1 and Kp1 indicates that "normal" 4-Fe clusters are not present. Further elucidation of the structure of these proteins requires more detailed examination of individual clusters. MCD shows that the Fe proteins Av2 and Kp2 unquestionably contain 4-Fe clusters in C^{3-} and C^{2-} states when reduced and oxidized, respectively.

The sensitivity of CD and MCD to protein composition, oxidation state, and conformation provides a new monitor of processes that affect these properties. We have shown that the CD of the Fe protein is sensitive to the presence of MgATP. CD can therefore be used to study the stoichiometry, thermodynamics, and kinetics of MgATP binding. Similarly, sensitivity to oxidation from the $Na_2S_2O_4$-reduced state in both MoFe and Fe proteins can be exploited for further study of these processes. Most importantly, CD and MCD can be studied under near-physiological conditions of temperature and pH, unlike spectroscopic techniques requiring cryogenic temperatures. In addition, all oxidation states, whether diamagnetic or paramagnetic, are accessible. Finally, CD and MCD have obvious potential as useful physical probes of coordinated metal species possibly relevant to or derived from N_2ase—specifically, Fe- and Mo-containing model compounds and the MoFe protein cofactor (25, 35–38).

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