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Circular Dichroism and Magnetic Circular Dichroism of Iron–Sulfur Proteins†

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ABSTRACT: Circular dichroism (CD) and magnetic circular dichroism (MCD) spectra are reported for the 2-Fe ferredoxins from *Pseudomonas putida* and *Spirulina maxima, Chromatium* HIPIP, the 4-Fe ferredoxin from *Bacillus stearothermophilus,* and the 8-Fe ferredoxin from *Clostridium pasteurianum.* The spectral range spans the near-infrared, visible, and near ultraviolet. In all cases except oxidized 2-Fe ferredoxins, electronic absorption is observed continuously from less than 5000 cm^{-1} to above 30 000 cm^{-1}. The CD spectra of the two 2-Fe ferredoxins are similar. In contrast, the CD of the 4-Fe and 8-Fe proteins, for a given 4-Fe cluster oxidation level, varies considerably with protein. MCD is less sensitive to protein environment than is CD. In the 2-Fe proteins, MCD at 5 T is appreciably smaller than the CD; in the 4-Fe and 8-Fe proteins, MCD and CD are comparable in magnitude. Both CD and MCD are more highly structured than the corresponding absorption spectra. The CD and MCD spectra reported provide a broader base than heretofore available for the characterization of iron-sulfur proteins containing 2-Fe and 4-Fe clusters and for the evaluation of electronic structural models for these clusters.

Iron-sulfur proteins are a class of nonheme iron proteins of widespread occurrence and diverse function (Lovenberg, 1973, 1977). Iron-sulfur proteins can be classified according to the number of iron atoms present. At this time, proteins containing 1,[1] 2, 4, and 8 Fe atoms have been extensively characterized and shown to contain three fundamental types of iron-sulfur cluster: $Fe(SR)_4$, $Fe_2S_2(SR)_4$, and $Fe_4S_4(SR)_4$. S and SR denote inorganic (labile) sulfide (S^{2-}) ions and protein-bound cysteine moieties (SR^-), respectively. Consistent with their function as redox proteins, the iron-sulfur clusters can attain multiple oxidation states. The 1-Fe, $[Fe(SR)_4]^{n-}$ cluster occurs in oxidized and reduced rubredoxins with $n = 1$ and 2, respectively, corresponding to Fe(III) and Fe(II) oxidation states. The 2-Fe, $[Fe_2S_2(SR)_4]^{n-}$ cluster occurs in oxidized and reduced (2-Fe) ferredoxins with $n = 2$ and 3, respectively, corresponding to $[Fe(III)_2]$ and [Fe(III), Fe(II)] formal oxidation states. The 4-Fe, $[Fe_4S_4(SR)_4]^{n-}$ cluster occurs in super-reduced, reduced, and oxidized HIPIP, and in reduced, oxidized, and superoxidized 4-Fe and 8-Fe ferredoxins with $n = 3$, 2, and 1, respectively, corresponding to [Fe(III), $Fe(II)_3$], $[Fe(III)_2, Fe(II)_2]$, and $[Fe(III)_3, Fe(II)]$ formal oxidation states. The definition of these structural and electronic characteristics has been especially aided by X-ray crystallographic studies on *Clostridium pasteurianum* rubredoxin (Watenpaugh et al., 1973), *Chromatium* HIPIP (Carter et al., 1974a,b; Freer et al., 1975), and *Micrococcus aerogenes* ferredoxin (Adman et al., 1973) and by the synthesis of convincing active-site analogue compounds (Holm & Ibers, 1977).

In this paper we discuss the electronic spectroscopy of iron-sulfur proteins. Electronic absorption spectroscopy in the visible-near-UV spectral region has been routinely used in the study of iron-sulfur proteins (Lovenberg, 1973, 1977; Tsibris

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[1] Abbreviations used: CD, circular dichroism; MCD, magnetic circular dichroism; 2-Fe, 4-Fe, and 8-Fe, two-, four-, and eight-iron, respectively.

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& Woody, 1970). The spectra are broad, and generally increasing in intensity with decreasing wavelength; the degree of resolved structure decreases with increasing number of Fe atoms. Typical extinction coefficients (ϵ) are $\sim 10^4$ and the transitions are assignable as charge transfer, rather than d → d excitations of Fe(III) or Fe(II). At the analytical level, the absorption spectrum provides a monitor of protein purity (via protein band:iron-sulfur cluster band absorbance ratios) and concentration. Absorption spectra have further been used to identify the iron-sulfur cluster type present; oxidized rubredoxins, oxidized 2-Fe ferredoxins, reduced HIPIP, and oxidized 4-Fe and 8-Fe ferredoxins, in particular, have been characterized routinely in this manner.

In principle, the comparison of an electronic spectrum with theory permits structural properties of the absorbing moiety to be inferred. Despite a variety of theoretical calculations of the molecular orbitals and electronic states of iron-sulfur clusters (Loew et al., 1971, 1972, 1974a,b; Yang et al., 1975; Norman & Jackels, 1975; Thomson, 1975; Bair & Goddard, 1977) as yet no detailed assignment of a visible-near-UV spectrum of an iron-sulfur protein has been accomplished. In view of the difficulties of treating the charge-transfer transitions, attempts have been made to observe the d → d transitions, which should be more readily analyzed through ligand-field theory. Using difference spectroscopy, Eaton et al. (Eaton & Lovenberg, 1970; Eaton et al., 1971) have observed weak d → d transitions in the near-IR ($\lambda > 1000$ nm) in reduced rubredoxin and reduced 2-Fe ferredoxins, confirming the existence of tetrahedral Fe(II) in these cases, and this work was extended to other proteins by Kimura et al. (Tang et al., 1973; Mukai et al., 1974). Cerdonio et al. (1974) have attempted to observe analogous transitions in reduced HIPIP; the lack of success was attributed to the existence of a delocalized electronic structure in which separate Fe(II) and Fe(III) oxidation states do not exist.

In addition to absorption spectroscopy, the techniques of optical rotatory dispersion (ORD) and circular dichroism (CD) have also been used to study iron-sulfur proteins (Lovenberg, 1973, 1977; Tsibris & Woody, 1970). Visible-near-UV ORD and/or CD have been reported for the rubredoxins from *Peptostreptococcus elsdenii* (Gillard et al., 1965; Atherton et al., 1966), *Desulfovibrio desulfuricans* (Newman & Postgate, 1968), *Pseudomonas oleovorans* (Peterson & Coon, 1968), *Clostridium pasteurianum* (Rao et al., 1972; Lovenberg & Williams, 1969; Eaton & Lovenberg, 1973), and *Chloropseudomonas ethylica* (Rao et al., 1972); the 2-Fe ferredoxins from spinach (Garbett et al., 1967; Cammack et al., 1971a; Rao et al., 1971; Sutherland et al., 1972; Palmer et al., 1967; Petering & Palmer, 1970; Ulmer & Vallee, 1963; Thomson et al., 1977), parsley (Garbett et al., 1967; Fee & Palmer, 1971), *Zea mays* (Hall et al., 1973), *Equisetum* (Hall et al., 1973; Kagamiyama et al., 1975), *Spirulina maxima* (Hall et al., 1973; Thomson, et al., 1977), *Clostridium pasteurianum* (Cardenes et al., 1976), *Azotobacter vinelandii* (Der Vartanian et al., 1969), *Halobacterium halobium* (Kerscher et al., 1976), *Pseudomonas putida* (Gunsalus & Lipscomb, 1973), and adrenodoxin (Palmer et al., 1967; Cammack et al., 1971b; Kimura & Suzuki, 1967; Kimura, 1968; Padmanabhan & Kimura, 1969, 1970; Thomson et al., 1977); *Chromatium* HIPIP (Flatmark & Dus, 1969; Hall et al., 1974); the 4-Fe ferredoxin from *Bacillus stearothermophilus* (Mullinger et al., 1975); and the 8-Fe ferredoxins from *Chromatium* Hall et al., 1974), *Peptostreptococcus elsdenii* (Gillard et al., 1965; Atherton et al., 1966), *Clostridium pasteurianum* (Gillard et al., 1965; Hall et al., 1974), *Clostridium acidi-urici* (Gillard et al., 1965), and *Clostridium thermosaccharolyticum* (Devanathan et al., 1969). In comparison to the absorption spectra, the CD spectra generally exhibit plentiful structure, demonstrating that the broadness of the absorption spectrum is due to the existence of many unresolved transitions. The greater resolution in the CD derives from the bisignate nature of the CD phenomenon. Unlike the absorption spectrum, the magnitude of CD is generally relatively constant across the visible-near-UV region.

Like the absorption spectrum, CD has been used as a monitor of protein purity and of structural changes due to environmental change (including denaturation), and as a diagnostic of iron-sulfur cluster type. CD is expected to be more sensitive than absorption spectra to changes in the cluster environment. On the one hand this makes CD an excellent probe of deviations from native protein structure; on the other hand, this can diminish the utility of CD as a diagnostic of gross cluster type. Comparison of CD among proteins containing the same cluster structure and oxidation state in many cases shows substantial similarities; see, for example, the comparisons between oxidized rubredoxins from *Clostridium pasteurianum* and *Chloropseudomonas ethylica* (Rao et al., 1972) and between oxidized and reduced 2-Fe ferredoxins from *Zea mays, Equisetum,* and *Spirulina maxima* reported by Hall et al. (1973). However, significant variations can also be found: compare, for example, the oxidized 2-Fe ferredoxins from *Clostridium pasteurianum* (Cardenes et al., 1976) and *Azotobacter vinelandii* (Der Vartanian et al., 1969) (proteins I and II) with typical plant and algal ferredoxins. Due to the paucity of data, the variation of CD with protein cannot be properly assessed for any oxidation state of 4-Fe clusters in either 4-Fe or 8-Fe proteins.

While almost all CD experiments have been restricted to the visible-UV spectral region, Eaton et al. have reported CD in the near-IR ($\lambda > 1000$ nm) for *Clostridium pasteurianum* rubredoxin (Eaton & Lovenberg, 1970; Eaton et al., 1971), spinach ferredoxin, and adrenodoxin (Eaton et al., 1971). These experiments substantiate the assignment of the near-IR absorption observed in these proteins to Fe(II) d → d transitions, in addition to being an elegant and direct manner of observing IR electronic transitions against a background of protein and solvent vibrational absorption. In the case of spinach ferredoxin, the CD spectrum was extended down to 2900 cm^{-1} by Hosein et al. (1974).

A few measurements of magnetooptical rotatory dispersion (MORD) and magnetic circular dichroism (MCD) (Stephens, 1974) have also been carried out with iron-sulfur proteins. Marlborough et al. (1969) and Sutherland et al. (1972) reported MORD and MCD, respectively, in spinach ferredoxin. Ulmer et al. (1973) presented MCD data on *Clostridium pasteurianum* rubredoxin and 8-Fe ferredoxin and spinach ferredoxin. Mason & Zubieta (1973) exhibit MCD data on the ferredoxin from *Micrococcus lactilyticus*. Most recently Thomson et al. have studied the MCD of *Clostridium pasteurianum* rubredoxin (Rivoal et al., 1977) and of the 2-Fe ferredoxin from *Spirulina maxima,* spinach ferredoxin, and adrenodoxin (Thomson et al., 1977) at low (liquid-helium) temperatures. Eaton and Lovenberg (1973) and Thomson et al. (Rivoal et al., 1977; Thomson et al., 1977) have attempted analysis of the MCD of *Clostridium pasteurianum* rubredoxin, and of the spinach ferredoxin, adrenodoxin, and *Spirulina maxima* 2-Fe ferredoxin MCD, extracting information regarding transition polarizations, excited-state degeneracies, low-symmetry splittings, spin-orbit coupling, and exchange interactions.

While the electronic spectroscopy of iron-sulfur proteins has thus been quite extensive, it is clear that a number of

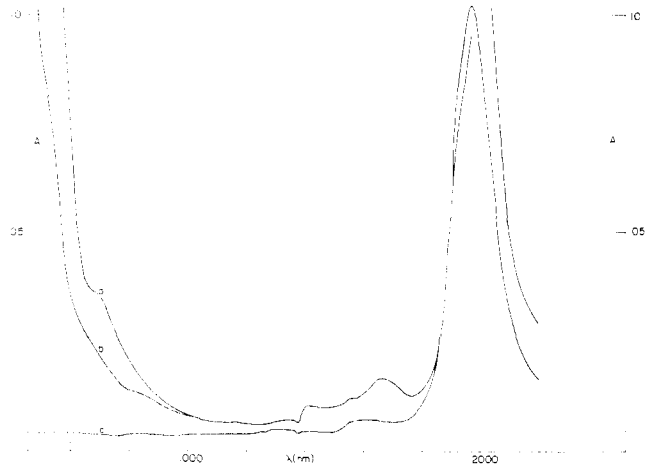

FIGURE 1: Near-IR absorption spectra of *Spirulina maxima* ferredoxin. (a) Oxidized protein, 3.1 mM, 1-mm pathlength; (b) as in a, with addition of excess dithionite; (c) baseline, D_2O and buffer. *A* is absorbance.

questions remain incompletely resolved. The goals of the current work are twofold: first, to extend the applicability of electronic spectral techniques as a diagnostic of iron–sulfur cluster structure, both geometrical and electronic; and, second, to develop additional data for comparison with theoretical calculations of electronic properties.

In the study of a large, and conceivably heterogeneous, iron–sulfur protein an initial goal is the identification of the number, structural type, and oxidation level of clusters present. Spectroscopic probes can accomplish this goal to the extent that they are characteristic of cluster type and oxidation state, independent of protein environment, sensitive to all clusters and oxidation levels, and quantitative. At the present time, no one spectroscopic method appears to satisfy all of these criteria and additional techniques can have value. In this work, we specifically focus on the CD and MCD techniques. As discussed above, insufficient data exist as yet to enable the diagnostic reliability of these methods to be assessed. This requires CD and MCD data over a wide, near-IR–visible–near UV spectral region for a variety of proteins of each cluster type and in all accessible oxidation states. In the case of MCD, temperature dependence is also of importance in view of the paramagnetic nature of several iron–sulfur cluster ground states. Since CD and MCD are quite independent phenomena, their value must be assessed separately. In particular, unlike natural CD, which depends for its existence on chirality and, in the case of iron–sulfur proteins, on the interaction of the achiral iron–sulfur cluster with the chiral protein moiety, MCD is an intrinsic property of a chromophore and is generally relatively insensitive to small structural perturbations. It is reasonable to expect, therefore, that MCD will be less sensitive to variations in protein composition and conformation than is CD. Via its temperature dependence, MCD is also capable of direct discrimination between diamagnetic and paramagnetic chromophores.

With regard to the analysis of the electronic spectra of iron–sulfur proteins, in view of their complexity it is clear that a maximal amount of experimental information is important. In particular, since iron–sulfur proteins appear to absorb continuously from the medium-IR to the vacuum-UV, the study of the entire spectral range is of importance. Also, MCD is here again more useful than natural CD, whose calculation poses formidable theoretical problems.

In this paper, we present the results of preliminary studies of the CD and MCD of a variety of iron–sulfur proteins. The spectral range studied includes the near-IR. However, measurements are limited to room-temperature solutions. Extension of the work to cryogenic temperatures is in progress and will be reported in future publications.

Experimental Section

Absorption spectra were measured using a Cary 17 spectrophotometer over the range 200–2600 nm. CD and MCD spectra were measuring using a Cary 61 spectropolarimeter for the 200–800-nm region and over the 700–2600-nm range using the IR CD instrument constructed in our laboratory (Osborne et al., 1973; Nafie et al., 1976). Magnetic fields up to 5 T (50 kG) were provided by a Varian super-conducting magnet. Specially constructed cylindrical quartz cells of 11-mm diameter were used to minimize the volume of protein solution required. In the case of solutions requiring anaerobic conditions solutions were manipulated and cells were filled in an argon-purged glove-box. Cells were then either septum-sealed or enclosed in a sealed cell-holder for spectroscopic measurements. In the former case, a positive pressure of argon was applied via the septum.

Optimum CD and MCD measurements require absorbances in the range 0.5–1.0. Adjustment of solution concentration and cell pathlength to satisfy this condition is straightforward in the visible and UV region (where ϵ values lie in the range 10^3 to 10^5). However, in the near-IR, the weakness of the electronic transitions (maximum ϵ values $\sim 10^2$) and the occurrence of solvent and protein vibrational absorption make this more difficult. For experiments at $\lambda \gtrsim 1000$ nm exchange of H_2O solvent by D_2O was carried out, using an Amicon diafiltration unit, or by sequential evaporation and addition of D_2O. In addition, solutions were concentrated as far as possible, while maintaining sufficient fluidity; in general, limiting concentrations lie in the 1–10 mM range. Cell path lengths were then chosen to be the maximum possible, with the absorbance limited to below 1.0. For experiments at $\lambda \gtrsim 1900$ nm, path lengths ≤ 1 mm are required; at $\lambda \lesssim 1900$ nm longer path lengths can be used. CD and MCD spectra were obtained at wavelengths in the region 1900–2500 nm, the cut-off point being determined principally by the amount of H_2O remaining in the sample.

In the case of 2-Fe, 4-Fe, and 8-Fe ferredoxins, the stable ("oxidized") protein state can be reduced (to the "reduced" state) and both oxidation states were examined. Reductions were effected using sodium dithionite, either as a concentrated solution or solid. In the latter case, the solid was mixed with solid buffer to prevent excessive local pH changes during mixing. In general, the reduction was carried out in stages; the existence of isosbestic points and the independence of spectra, after some point, to further addition of dithionite are useful criteria of a clean reduction. In some experiments, dithionite reduction was found to lead to precipitation of dark-colored material. This leads to additional background absorbance, preventing the appearance of clean isosbestic points in absorption spectra. CD and MCD spectra appeared to be much less affected. However, experiments in which appreciable deterioration of this type occurred were considered unacceptable. Near-IR absorption spectra obtained during a reductive titration of *Spirulina maxima* ferredoxin are shown in Figure 1. At $\lambda > 1300$ nm absorption features attributable to vibrational absorption obscure any broad, weak electronic absorption.

In the case of HIPIP, the stable oxidation state is the "reduced" state and this is oxidized to the "oxidized" state by $K_3Fe(CN)_6$. As with dithionite reductions, oxidation was also carried out in stages. The near-IR absorption spectra obtained

during a titration are illustrated in Figure 2.

In the case of visible-UV ($\lambda < 800$ nm) spectra of oxidized HIPIP, excess $Fe(CN)_6^{3-}$ was first removed by passage through a Sephadex G-25 column. Excess dithionite was not removed from reduced samples and, when present, spectra are limited to the region $\lambda \gtrsim 360$ nm owing to the ~ 320-nm absorption band of dithionite.

Prior to all spectral measurements, protein quality was monitored by measurement of visible-near-UV absorption spectra.

Absorption and CD/MCD spectra are reported as molar extinction coefficient ϵ and differential molar extinction coefficient $\Delta\epsilon$, respectively. In the case of MCD, $\Delta\epsilon$ values are normalized to a magnetic field of +1 T (10 kG). All measurements were made at ambient temperatures.

Spirulina maxima ferredoxin was prepared as described previously (Hall et al., 1972). Concentrations were measured using $\epsilon_{420} = 9700$ (Hall et al., 1972) for the oxidized protein.

Putidaredoxin was prepared as described elsewhere (Gunsalus & Wagner, 1978). Concentrations were measured using $\epsilon_{325} = 15\,600$ (Gunsalus & Wagner, 1978) for the oxidized protein. All putidaredoxin solutions contained dithiothreitol.

Chromatium HIPIP was prepared as described previously (Dus et al., 1967; Evans et al., 1970). Concentrations were measured using $\epsilon_{390} = 16\,100$ (reduced) and $\epsilon_{375} = 20\,000$ (oxidized) (Dus et al., 1967).

Bacillus stearothermophilus ferredoxin was prepared as described previously (Mullinter et al., 1975). Concentrations were measured using $\epsilon_{390} = 15\,000$ (Rao, unpublished work) for the oxidized protein.

Clostridium pasteurianum ferredoxin was prepared as described previously (Thompson et al., 1974). Samples contained some nucleic acid, contributing UV absorption and preventing measurement of $A_{390}:A_{280}$. Concentrations were measured using $\epsilon_{390} = 30\,600$ (Hong & Rabinowitz, 1970) for the oxidized protein.

Results and Discussion

The absorption, CD, and MCD spectra obtained are shown in Figures 3-7. UV spectra are limited to the region $\lambda > 300$ nm where only the iron-sulfur clusters absorb; spectra at $\lambda < 300$ nm contain contributions from other protein moieties and are considerably more complex to interpret usefully. In addition, the spectra of dithionite-reduced proteins are limited to $\lambda \gtrsim 360$ nm when measured in the presence of excess dithionite. IR absorption spectra are limited to the region $\lambda < 1300$ nm by the presence of solvent and protein vibrational absorption at longer wavelengths (see Figures 1 and 2). Owing to the facts that vibrational natural CD is a very weak phenomenon (Nafie et al., 1976) and vibrational MCD has not yet been detected, the contributions of vibrational transitions to CD and MCD spectra can be safely ignored. CD and MCD spectra can therefore be obtained to the transmission limit imposed by solvent absorption which, with the samples and path lengths used in this work, lies in the range 1900–2600 nm. The spectra obtained make clear the importance of studying iron-sulfur proteins over the entire near-IR-visible-near-UV spectral range. Of the systems studied, only in the case of the oxidized 2-Fe ferredoxins does a region exist in which electronic excitations cannot be detected—and even here only at $\lambda > 1300$ nm.

The accuracy of the data is limited by a variety of factors. In particular, since concentrations were determined via published visible ϵ values any error in the latter is reflected in our

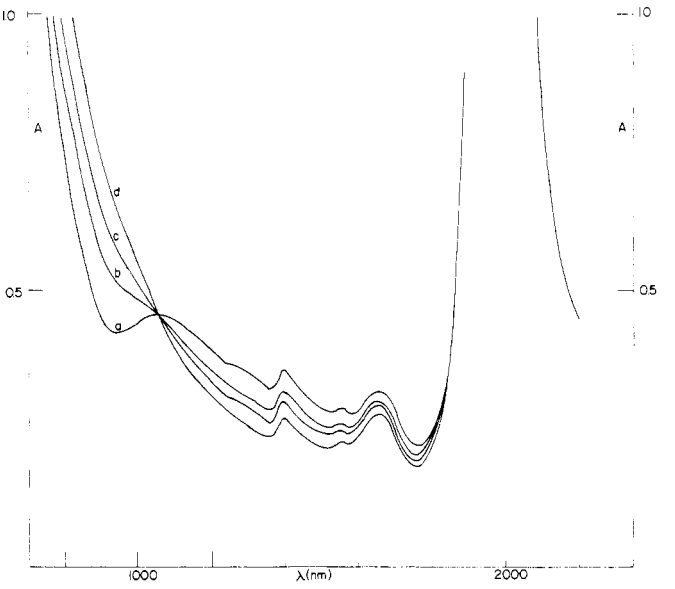

FIGURE 2: Near-IR absorption spectra of *Chromatium* HIPIP. (a) Reduced protein, 3.4 mM, 2-mm pathlength; (b-d) as in a, with addition of increasing amounts of solid $K_3Fe(CN)_6$. Curve d does not change on addition of further $K_3Fe(CN)_6$.

ϵ and $\Delta\epsilon$ values. Data for *Spirulina maxima* (Hall et al., 1972) and *Bacillus stearothermophilus* (Mullinger et al., 1975) ferredoxins are most susceptible to error in this regard, being the most recently isolated and least thoroughly studied of the proteins we have used. We would expect maximum errors from this source to be ~10–20%. In addition, less than optimal experiments were in some cases necessitated by the limited amounts of protein available. This was especially true of near-IR experiments where the weakness of the electronic absorption requires relatively large amounts of protein (typically 1–10 mg), making extensive studies of the reproducibility of data impossible.

The accuracy of MCD measurement in the case of naturally optically active systems is limited by the relative magnitudes of CD and MCD. In the 4-Fe and 8-Fe proteins at the magnetic fields used MCD was generally comparable to or somewhat larger than the CD and was easily measurable. However, in the 2-Fe proteins the MCD is *relatively* much smaller and in the near-IR was sufficiently smaller to prevent accurate measurement. We can therefore only place approximate upper limits on the MCD of *Spirulina maxima* ferredoxin and putidaredoxin: in the range $\lambda > 1000$ nm, the MCD at 1 T is no larger than 2% of the CD.

The absorption spectra of 2-Fe, 4-Fe, and 8-Fe proteins show steadily increasing absorption with decreasing wavelength, ϵ values varying from $< 10^3$ at $\lambda > 1300$ nm to $> 10^4$ at $\lambda < 400$ nm. The degree of structure varies with the number of Fe atoms per cluster and with the cluster oxidation state, the oxidized 2-Fe protein absorption being the most highly structured. In addition to well-defined absorption peaks, all systems exhibit shoulders and inflections. The natural CD spectra, in contrast to the absorption spectra, are highly structured and do not vary so greatly in magnitude across the spectral range. The many maxima and minima observed clearly demonstrate the presence of many electronic transitions, more resolved in the CD spectrum due to the bisignate nature of the CD phenomenon. The MCD spectra are intermediate between CD and absorption spectra. MCD generally increases with decreasing wavelength but is appreciably more structured than absorption.

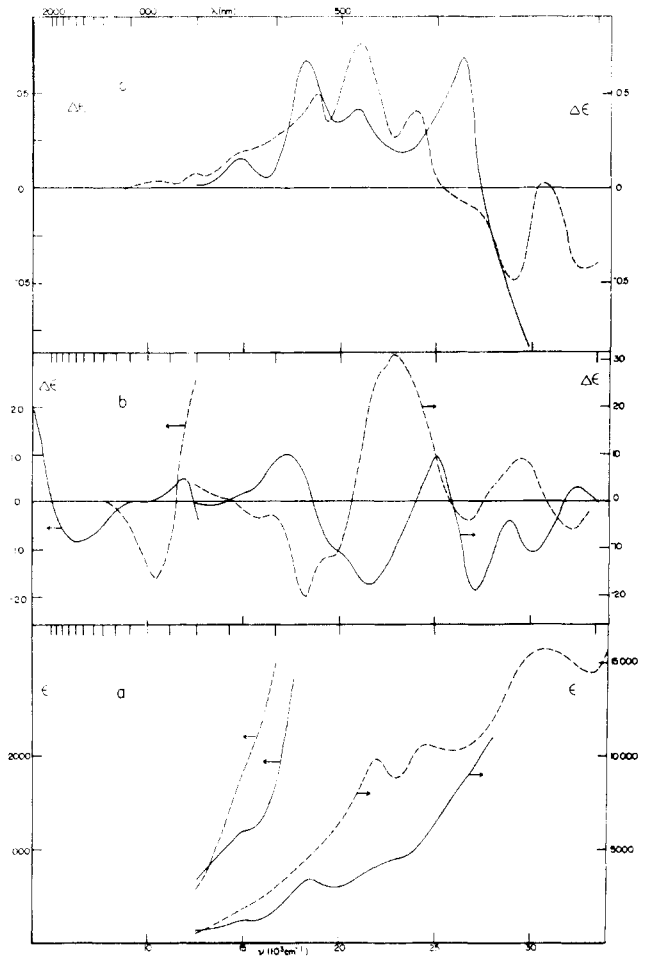

FIGURE 3: (a) Absorption spectrum; (b) CD; and (c) MCD (at 1 T) of putidaredoxin. (- - -) Oxidized; (—) reduced.

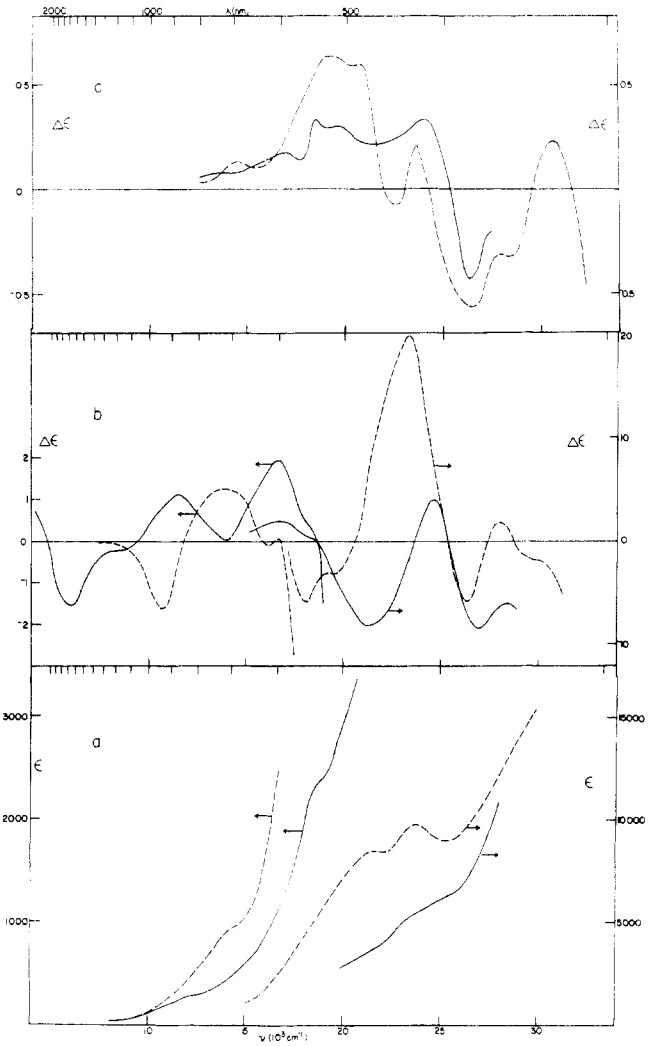

FIGURE 4: (a) Absorption spectrum; (b) CD; and (c) MCD (at 1 T) of *Spirulina maxima* ferredoxin. (- - -) Oxidized; (—) reduced.

The absorption spectra of the oxidized and reduced forms of the 2-Fe ferredoxins from *Pseudomonas putida* and *Spirulina maxima* are shown in Figures 3 and 4 and are typical of 2-Fe proteins. Our spectra are in agreement with, and more extensive than those previously reported (Hall et al., 1972; Cushman et al., 1967; Gunsalus & Lipscomb, 1973).

The CD spectra of putidaredoxin (Figure 3) and of *Spirulina maxima* ferredoxin (Figure 4) are in good agreement with previously reported spectra (Hall et al., 1973; Thomson et al., 1977; Gunsalus & Lipscomb, 1973) and extend the range of measurement down to ~4000 cm^{-1}. CD spectra below 10 000 cm^{-1} of 2-Fe proteins have been measured previously only for spinach ferredoxin and adrenodoxin (Eaton et al., 1971).

The CD of oxidized putidaredoxin and *Spirulina maxima* ferredoxin are quite similar to one another, although $\Delta\epsilon$ values and the resolution of specific features differ somewhat. The spectra of the reduced proteins are also broadly similar. The visible–near UV CD spectra of *Spirulina maxima* ferredoxin closely resemble the spectra of plant ferredoxins (Hall et al., 1973), which are themselves remarkably invariant to protein source. Greater variation is, however, apparent when the visible CD spectra of bacterial 2-Fe ferredoxins (e.g., from *Azotobacter vinelandii* (Der Vartanian et al., 1969), and *Clostridium pasteurianum* (Cardenes et al., 1976)) are included. CD spectra over the range 4000 to 12 000 cm^{-1} have now been obtained for four 2-Fe proteins from widely different sources. The spectra are remarkably similar in both reduced and oxidized proteins.

The CD anisotropy ratios ($\Delta\epsilon/\epsilon$) in 2-Fe ferredoxins are generally larger in the near-IR compared with the visible–near-UV region. It was pointed out by Eaton et al. (1971) that the very high values ($>10^{-2}$) of this ratio below 8000 cm^{-1} for the reduced proteins are consistent with assignment of the bands observed in this region to d → d transitions of tetrahedral ferrous iron.

The visible–near UV MCD of oxidized *Spirulina maxima* ferredoxin (Figure 4) is in good agreement with that previously reported by Thomson et al. (1977). Room temperature MCD data on reduced *Spirulina maxima* ferredoxin and oxidized and reduced putidaredoxin have not previously been reported. The MCD spectra of the two oxidized proteins are broadly similar in form and magnitude as are the spectra of the two reduced proteins (Figures 3 and 4), although with appreciable variation in $\Delta\epsilon$ values and in the resolution of spectral features. Considering that the ground states of the oxidized and reduced proteins are diamagnetic and paramagnetic, respectively, it is remarkable that the oxidized and reduced proteins exhibit MCD of similar magnitude. The anisotropy ratio, $\Delta\epsilon/\epsilon$ at 1 T, is $\sim 10^{-4}$ for the reduced protein which is smaller than that observed in many paramagnetic systems, where $\Delta\epsilon/\epsilon$ ratios at room temperature frequently exceed 10^{-3}.

It is interesting to compare these room temperature data with the MCD data of Thomson et al. obtained at liquid helium temperatures on *Spirulina maxima* ferredoxin, spinach ferredoxin, and adrenodoxin (Thomson et al., 1977). The MCD

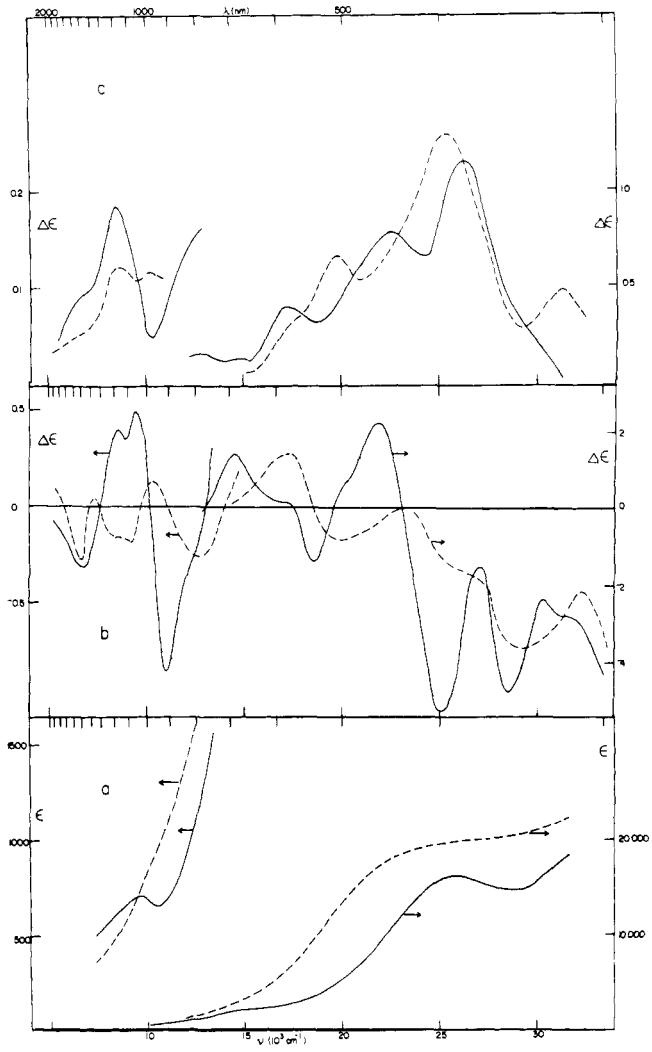

FIGURE 5: (a) Absorption spectrum; (b) CD; and (c) MCD (at 1 T) of *Chromatium* HIPIP. (- - -) Oxidized; (—) reduced.

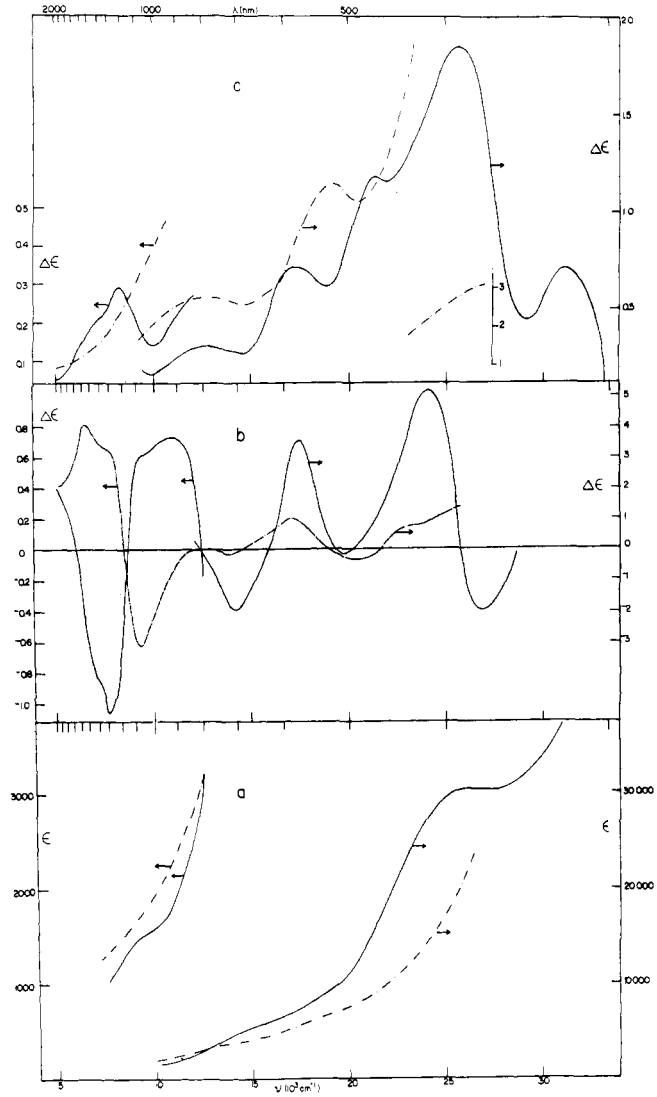

FIGURE 6: (a) Absorption spectrum; (b) CD; and (c) MCD (at 1 T) of *Clostridium pasteurianum* ferredoxin. (—) Oxidized; (- · - ·) reduced.

of oxidized *Spirulina maxima* ferredoxin at room temperature is very similar in both form and magnitude to the low-temperature spectra of oxidized spinach ferredoxin. In the case of the reduced proteins, the MCD of *Spirulina maxima* and putidaredoxin at room temperature broadly resemble the 6 K spectra of spinach and *Spirulina maxima* ferredoxins and adrenodoxin, with an intensity enhancement of ~10 and a considerable sharpening of spectral features in the latter.

Owing to the large values of the CD anisotropy ratio, $\Delta\epsilon/\epsilon$, in the near-IR we have been unable to obtain unambiguous MCD spectra for the reduced 2-Fe ferredoxins. The relative smallness of the MCD is consistent with MCD anisotropy ratios comparable to or smaller than those in the visible–near UV.

The 4-Fe clusters of 4-Fe and 8-Fe proteins exist in one of three possible oxidation levels which we will refer to as C^- (oxidized HIPIP), C^{2-} (reduced HIPIP, oxidized *Bacillus stearothermophilus*, and *Clostridium pasteurianum* ferredoxins), and C^{3-} (reduced *Bacillus stearothermophilus*, *Clostridium pasteurianum* ferredoxins).

The absorption spectra of HIPIP, *Clostridium pasteurianum*, and *Bacillus stearothermophilus* ferredoxins (Figures 5, 6, and 7) agree well with previous reports (Dus et al., 1967; Buchanan et al., 1963) in the visible–near UV and are typical of 4-Fe and 8-Fe proteins. Below 10 000 cm^{-1} only the 77 K spectrum of reduced HIPIP has previously been reported (Cerdonio et al., 1974). The 9600-cm^{-1} band in the spectrum of reduced HIPIP (Figure 5) is also clearly visible in the absorption spectra of oxidized *Bacillus stearothermophilus* ferredoxin (Figure 7) and *Clostridium pasteurianum* ferredoxin (Figure 6), although less well resolved in the latter two cases. The ϵ values at 9600 cm^{-1} and throughout the spectrum are comparable in all three proteins, allowing for the presence of two clusters in the ferredoxin from *Clostridium pasterianum*. Although they do not exhibit resolved maxima in the near-IR, oxidized HIPIP, reduced *Clostridium pasteurianum* ferredoxin and reduced *Bacillus stearothermophilus* ferredoxin continue to show absorption to below 8000 cm^{-1}.

Visible–near UV CD have previously been reported for HIPIP (Flatmark & Dus, 1969; Hall et al., 1974) and for *Clostridium pasteurianum* (Hall et al., 1974) and *Bacillus stearothermophilus* (Mullinger et al., 1975) ferredoxins. Our data for reduced HIPIP (Figure 5), oxidized *Clostridium pasteurianum* ferredoxin (Figure 6), and oxidized and reduced *Bacillus stearothermophilus* (Figure 7) are in good agreement with the earlier reports. The spectrum of oxidized HIPIP (Figure 5) agrees with that of Hall et al. (1974) but differs from that reported by Flatmark & Dus, (1969) which we attribute to incomplete oxidation of their sample. The visible–near UV CD of *Clostridium pasteurianum* ferredoxin (Figure 6) diminishes markedly on reduction and the spectrum of the

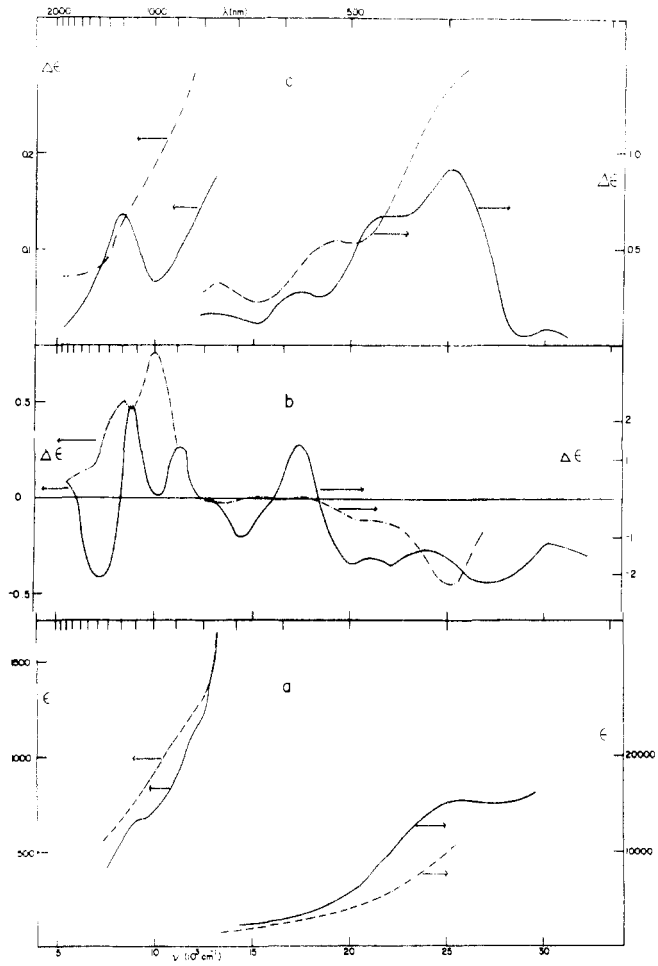

FIGURE 7: (a) Absorption spectrum; (b) CD; and (c) MCD (at 1 T) of *Bacillus stearothermophilus* ferredoxin. (—) Oxidized; (- - - -) reduced.

reduced protein previously reported by Hall et al. (1974) is too indistinct to exhibit its form. Near-IR CD has not previously been reported for any 4-Fe or 8-Fe protein.

The CD spectra of the C^{2-} state of three different proteins, namely, HIPIP, *Bacillus stearothermophilus* ferredoxin, and *Clostridium pasteurianum* ferredoxin, are very different in form, although comparable in magnitude across the entire spectral region measured. In this respect they differ from the 2-Fe proteins. This generalization also applies to the CD spectra of the C^{3-} states of *Clostridium pasteurianum* ferredoxin (Figure 6) and *Bacillus stearothermophilus* ferredoxin (Figure 7). The highly structured CD spectra of all three oxidation levels contrast sharply with the almost featureless absorption spectra. Throughout the spectral region studied, $\Delta\epsilon$ values are generally significantly lower than those observed with the 2-Fe proteins.

The only previous reports of MCD for proteins containing 4-Fe clusters are those of Ulmer et al. for *Clostridium pasteurianum* ferredoxin (Ulmer et al., 1973) and Mason and Zubieta for *Micrococcus lactilyticus* ferredoxin (Mason & Zubieta, 1973), in both cases data being obtained only in the visible–near UV. Our data differ considerably from those reported for both ferredoxins. No near-IR MCD data have been published previously. On account of the smaller CD anisotropy ratios of the 4-Fe and 8-Fe proteins, the MCD is much more readily detected in these proteins than in 2-Fe proteins.

MCD magnitudes are roughly comparable in C^{1-}, C^{2-}, and C^{3-} oxidation states, which is surprising in view of the ground state diamagnetism of the C^{2-} level and paramagnetism of the C^{1-} and C^{3-} states. It is also striking that the MCD of all oxidation states remains positive throughout the entire spectral range studied, a virtually unprecedented situation for such highly symmetric molecules.

The MCD spectra of the three proteins studied in the C^{2-} oxidation state are closely similar, with only minor differences in relative magnitudes of peaks. In particular the band at 9600 cm^{-1} in the absorption spectrum exhibits a well-resolved MCD band in all three proteins. The $\Delta\epsilon$ at 1 T for this band in *Clostridium pasteurianum* ferredoxin is 0.30 compared with values of 0.18 in reduced HIPIP and 0.14 in oxidized *Bacillus stearothermophilus* ferredoxin. In the two cases studied, the MCD in the C^{3-} oxidation level is also very similar in different proteins.

Both the CD and MCD spectra in the near-IR region clearly show the presence of electronic transitions to below 5000 cm^{-1} in all oxidation levels of all 4-Fe clusters studied. The CD in particular reveals a multiplicity of bands in the regions where electronic absorption is weak and obscured by vibrational transitions. Previous workers have been unable to observe electronic transitions in this region in the case of reduced HIPIP and erroneously concluded they were absent (Cerdonio et al., 1974). Conclusions about the appropriateness of theoretical electronic structural models drawn from the absence of transitions in this region must therefore be reexamined.

Conclusion

The data reported here permit a preliminary assessment of the utility of room temperature absorption, CD and MCD spectroscopies in the study of iron–sulfur proteins containing 2-Fe and 4-Fe clusters. As was expected, we have found MCD to be generally less sensitive to protein environment, for a cluster of given type and oxidation level, than is CD. (The relative invariance of the CD of 2-Fe clusters to the nature of the surrounding protein remains a striking exception to this generalization). By contrast MCD spectra are more distinctive than absorption spectra. As a result, MCD should constitute a more reliable tool than both absorption and CD in the diagnosis of iron–sulfur cluster type in an uncharacterized protein. On the other hand, we have found MCD to be a less dramatic function of cluster structure and oxidation level than was anticipated. In particular, the constancy in sign and order of magnitude on changing oxidation level in both 2-Fe and 4-Fe clusters is remarkable (and at this time unexplained). In addition, the MCD is not particularly large in any system, creating some practical difficulties in measuring the phenomenon against an appreciable CD background. The practical analytical application of MCD will thus clearly depend on the particular system under study. At the present time, it appears likely that MCD can be most useful in the characterization of systems containing 4-Fe clusters, where the absorption spectra are most diffuse and the CD smallest and least characteristic.

Our results demonstrate the additional information obtainable from absorption, CD, and MCD by extension of the spectral region to include the near-IR. For some systems, the use of the near-IR region may well provide the most definitive characterization. The near-IR may also be of importance in proteins containing additional chromophores which absorb in the visible–near-UV region.

The present work is limited in two dimensions. First, the proteins examined are small in number and extension of these studies to a larger and more diverse set of proteins is highly desirable. Second, measurements have been made only at ambient temperatures. In the case of paramagnetic systems,

it is important to extend the MCD studies to cryogenic temperatures in order to determine the temperature dependence of the spectra. While some studies have been carried out on 1-Fe and 2-Fe proteins (Rivoal et al., 1977; Thomson et al., 1977) as yet no low-temperature MCD spectra exist for 4-Fe clusters. In the event that the MCD at liquid helium temperatures is dominated by paramagnetic (T^{-1}-dependent) effects, the differentiation of diamagnetic and paramagnetic clusters will be greatly facilitated. As an additional advantage, the MCD will also be larger than at room temperature and easier to measure.

The use of CD and MCD for iron-sulfur cluster characterisation provides both advantgaes and disadvantages relative to other commonly employed techniques, such as EPR, NMR, and Mössbauer spectroscopies. We shall not attempt here to enumerate these in detail; we merely emphasize that measurements are possible on proteins under physiological conditions of temperature and solvation and are equally possible for diamagnetic and paramagnetic systems. In addition, denaturing solvent conditions are not required as is the case for the core extrusion methodology (Holm & Ibers, 1977).

Our data provide additional information regarding the excited electronic states of iron-sulfur clusters which must be accounted for by satisfactory theories of the electronic structure of these clusters. Further interpretation of the spectra and evaluation of theoretical models will be presented subsequent to the completion of experiments at cryogenic temperatures.

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Synthesis of Peptides of Arginine Chloromethyl Ketone. Selective Inactivation of Human Plasma Kallikrein†

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ABSTRACT: Synthetic procedures have been developed for the preparation of peptides of arginine chloromethyl ketone and applied in the preparation of affinity labels which correspond to the -Pro-Phe-Arg- C terminus of bradykinin, a physiological cleavage site of kallikrein in kininogen. Two such reagents, Ala-Phe-ArgCH_2Cl and Pro-Phe-ArgCH_2Cl, proved to be highly effective as well as selective affinity labels for human plasma kallikrein. For example, Pro-Phe-ArgCH_2Cl inactivates plasma kallikrein 50% in 24 min at a concentration of 2×10^{-8} M, while other trypsin-like proteases are less susceptible in inactivation than kallikrein, differing by a factor of 48 for plasmin and factors of 10^2–10^5 for factor X_a, thrombin, and urokinase. The affinity of human plasma kallikrein for Ala-Phe-ArgCH_2Cl (K_i = 0.078 μM) is about 60 times that for Ala-Phe-LysCH_2Cl (K_i = 4.9 μM), whereas human plasmin exhibits about the same affinity for the former affinity label (K_i = 1.3 μM) as for the latter (K_i = 0.83 μM). The rate constants for the irreversible step of the affinity labeling reaction, k_2, are similar for all affinity labels tested with the individual proteases: 0.35 min^{-1} for plasma kallikrein and 0.18 min^{-1} for plasmin.

An increasing number of serine proteases have been characterized which have very specialized physiological roles that are often limited to the cleavage of one or two peptide bonds (Neurath and Walsh, 1976). Other serine proteases have recently been identified by their biological activities, while their structure and function remain uncertain. Inhibitors are helpful in deducing or confirming the physiological role of enzymes. For example, diisopropyl fluorophosphate and phenylmethanesulfonyl fluoride are useful in the initial characterization of an enzyme as a serine protease, while more specialized inhibitors acting as affinity labels are capable of greater discrimination. Affinity labeling by substrate-derived chloromethyl ketones has permitted further characterization of the activities of serine proteases into chymotryptic, tryptic, and elastolytic (Shaw, 1975). Of these, the serine proteases of trypsin-like specificity comprise a large group of enzymes, including proteases of blood coagulation, fibrinolysis, and fertilization. We have selected this group of proteases, having a common primary specificity, to determine the extent to which selectivity in inactivation can be achieved by affinity labeling.

In earlier work (Coggins et al., 1974; and Shaw, 1975), it was shown that peptides of lysine chloromethyl ketone such as Ala-Phe-LysCH_2Cl inactivate both plasma kallikrein and plasmin at micromolar concentrations of reagent, distinguishing these proteases from thrombin. This selectivity was attributed to differences in the subsites for normal substrate binding. The amino acid sequences at the cleavage sites of the physiological substrates have been determined for a number of regulatory, trypsin-like proteases, revealing that in most

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