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STUDIES ON THE CHELATE STRUCTURE OF THE HIGH-POTENTIAL IRON PROTEIN OF *CHROMATIUM**

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SUMMARY

According to recent experiments, applying Mössbauer spectroscopy and electron paramagnetic resonance (EPR) measurements, the high-potential iron protein (HiPIP) of *Chromatium* was distinguished from various ferredoxins by its characteristic mode of iron binding. These conclusions were substantiated by our results from circular dichroic (CD) absorption studies. Thus, the CD spectrum of high-potential iron protein differed qualitatively as well as quantitatively from that observed for other non-heme iron proteins indicating that highly specific polydentate chelate structures were involved. By analogy with the known structure of the model compound, bis-cysteinatocobaltate (III), we concluded that the unique ellipticity band centered around 230 nm was attributable to iron–SH bondings. Further evidence in support of this concept was provided by the very slow reactivity of high-potential iron protein towards sulfhydryl-group reagents as well as by the simultaneous and proportional decrease of absorption in the visible region during this reaction.

The CD spectrum revealed the absence of the n-π^* amide transition at 222 nm characteristic of the right-handed α-helix in polypeptides and proteins. This result was not unexpected in view of the tight chelate structure at the metal-binding site and of the large number of prolyl residues in close proximity to it. However, more detailed structural implications of the CD spectra will remain obscure until the electronic transitions involved can be better characterized.

Spectrophotometric titration with $K_3Fe(CN)_6$ of the fully reduced high-potential iron protein revealed that only one electron is transferred during oxidation–reduction of the protein. The nature of the electron transfer in this protein is discussed.

INTRODUCTION

In the last few years non-heme iron, electron transfer proteins have been isolated from plants, bacteria and animals. It has become increasingly evident, however, that these proteins constitute a heterogeneous group as far as physico-chemical properties are concerned and that the non-heme iron proteins of high

Abbreviations: EPR, electron paramagnetic resonance; CD, circular dichroism; PCMB, *p*-chloromercuribenzoate; DTNB, 5,5′-dithio-bis-(-nitrobenzoic acid).

* A preliminary account of some of the data was given at the 5th Meeting of the Federation of European Biochemical Societies in Prague, 1968.

oxidation-reduction potential (+0.35 V) isolated from photosynthetic purple bacteria[1,2] represent a class of compounds distinct from other bacterial non-heme iron proteins[1-4]. The high-potential non-heme iron proteins (HiPIP) isolated from the obligate photoautotroph *Chromatium*[1] and from photoheterotroph *Rhodopseudomonas gelatinosa*[2] are very similar to each other[2,3]. Both proteins contain four iron atoms, four acid labile inorganic sulfur atoms, and four cysteine residues per molecule, and they undergo reversible oxidation and reduction. However, it is not known how many electrons per mole are transferred in the process[4]; similarly, the exact position of these proteins within the photosynthetic electron transport chain as well as their function as electron carriers is not yet clear. Though the high-potential iron proteins resemble the plant ferredoxins in molecular weight, close similarity to these proteins was not observed with regard to the numbers of cysteines, of acid-labile sulfurs or of irons. They also differ from the ferredoxins in that they possess a highly positive oxidation-reduction potential[1,2]. Moreover, iron and inorganic sulfur appear to be more strongly bound than in the ferredoxins[3]. The environment of the iron atoms in high-potential iron protein is by no means clear, but electron paramagnetic resonance (EPR) measurements (G. PALMER, personal communication) and Mössbauer spectroscopy[4] have revealed that the iron atoms are bound to the labile sulfur atoms and to ligands in the polypeptide chain in a way that differs from that suggested for the binding of the iron in the ferredoxins.

Spectropolarimetric studies of some of the non-heme iron proteins have recently been undertaken by several workers[5-13]. No detailed information about the chelate structure in these proteins was obtained nor was it expected to result from such studies alone. Nevertheless, in conjunction with other physicochemical and chemical evidence, circular dichroic (CD) absorption spectra can yield valuable information on the environment of the iron atoms as well as on the secondary structure of the polypeptide chain. In an attempt to obtain further insight into the nature of the metal-binding sites and into the ligand asymmetry in *Chromatium* high-potential iron protein, spectropolarimetry as well as additional physicochemical techniques were applied. Like other non-heme iron proteins, the *Chromatium* high-potential iron protein reveals unusual physicochemical properties when compared with simple iron complexes[14]. This fact has stimulated our search for more suitable complex ion models. Since cysteine sulfhydryl-groups were thought to be bonding groups in certain non-heme iron proteins[15], transition metal complexes of cysteine were considered as possible model compounds in the hope that they might contribute to the interpretation of the complex CD spectrum and of the chelate structure of this non-heme iron protein. Iron–cysteine complexes are, however, very unstable[16]; therefore a complex of Co(III) and cysteine was used. Both metal ions, Fe(II) and Co(III), have $3d^6$ valence-electron configurations and form octahedral complexes[17]. Furthermore, cobalt readily accepts distorted geometries in model complexes[14] which consequently should reveal optically active electronic transitions.

MATERIALS AND METHODS

Non-heme iron protein

Chromatium high-potential iron protein (HiPIP) was isolated and purified from *Chromatium D* according to the procedure described by BARTSCH[1]. The main compo-

nent[3] with pI (0°) = 3.88 and pI (0°) = 3.68 in the oxidized and reduced form, respectively, was isolated by isoelectric focusing in a combined density and natural pH gradient[18] by using the isoelectric focusing equipment LKB-8100 from LKB Instruments, Stockholm.

Reagents

Deionized water was used in making up all solutions. Analytical grade salts were used without purification.

Preparation of the cobalt–cysteine complex

The green isomer of bis-cysteinatocobaltate (III), Co (III) $(Cys)_2 \cdot 2H_2O$, was synthesized as described by NEVILLE AND GORIN[19]. The green crystals thus obtained were dissolved in a 40 mM ammonium phosphate buffer at pH 6.9 and further purified by molecular-sieve chromatography on Sephadex G-10 equilibrated with the same buffer. The resulting product was essentially pure.

Circular dichroism

CD measurements were performed on a Jasco ORD/UV-5 recording spectrophotometer equipped with a CD attachment. Cells of 1-cm light path were used. The ellipticity, $[\theta]$, was calculated from the equation[20]

$$[\theta] = 2.303\ (4500/\pi) \cdot \Delta\varepsilon$$

where the units are degrees$\cdot$cm$^2\cdot$dmole^{-1}. The calculations of $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ were based on a molecular weight of 10074 (ref. 3).

The protein was prepared in the fully reduced and oxidized form using mercaptoethanol and $K_3Fe(CN)_6$, respectively; Sephadex G-10 was used for molecular-sieve chromatography in order to remove the reducing and oxidizing agents. To ensure complete oxidation of the protein, however, a small amount of $K_3Fe(CN)_6$ (10% of the total protein) was added to the solution. The resolution of the CD spectra into Gaussian curves was computed by simultaneous description of the absorption and CD curves. The total number of such curves necessary for adequate fitting was kept at a minimum.

Spectrophotometry

The concentrations of high-potential iron protein in solutions were determined spectrophotometrically by using the millimolar absorbance coefficients ε_{mM} at 388 nm (reduced) = 16.1 and ε_{mM} at 375 nm (oxidized) = 20.0 (ref. 3). Spectrophotometric titrations of reactive sulfhydryl-groups were performed by using either *p*-chloromercuribenzoate (PCMB)[21] or 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB)[22] as the sulfhydryl-blocking agent. The concentrations of bis-cysteinatocobaltate (III) in solutions were determined spectrophotometrically by using the millimolar absorbance coefficient ε_{mM} at 443 nm = 5.47 (see RESULTS). All these studies were made with a Unicam Sp. 800 recording spectrophotometer, using a temperature-controlled cell holder.

Microanalyses

Cobalt was assayed by atomic absorption spectrophotometry[23]. Amino acid analyses were performed on a Biochrom amino acid analyzer (BioCal, Germany) using

a modification (T. FLATMARK, unpublished results) of the stepwise four-buffer elution program of DUS *et al.*[24]. Cysteine was measured as cysteic acid by quantitative amino acid analysis of performic acid oxidized samples[25].

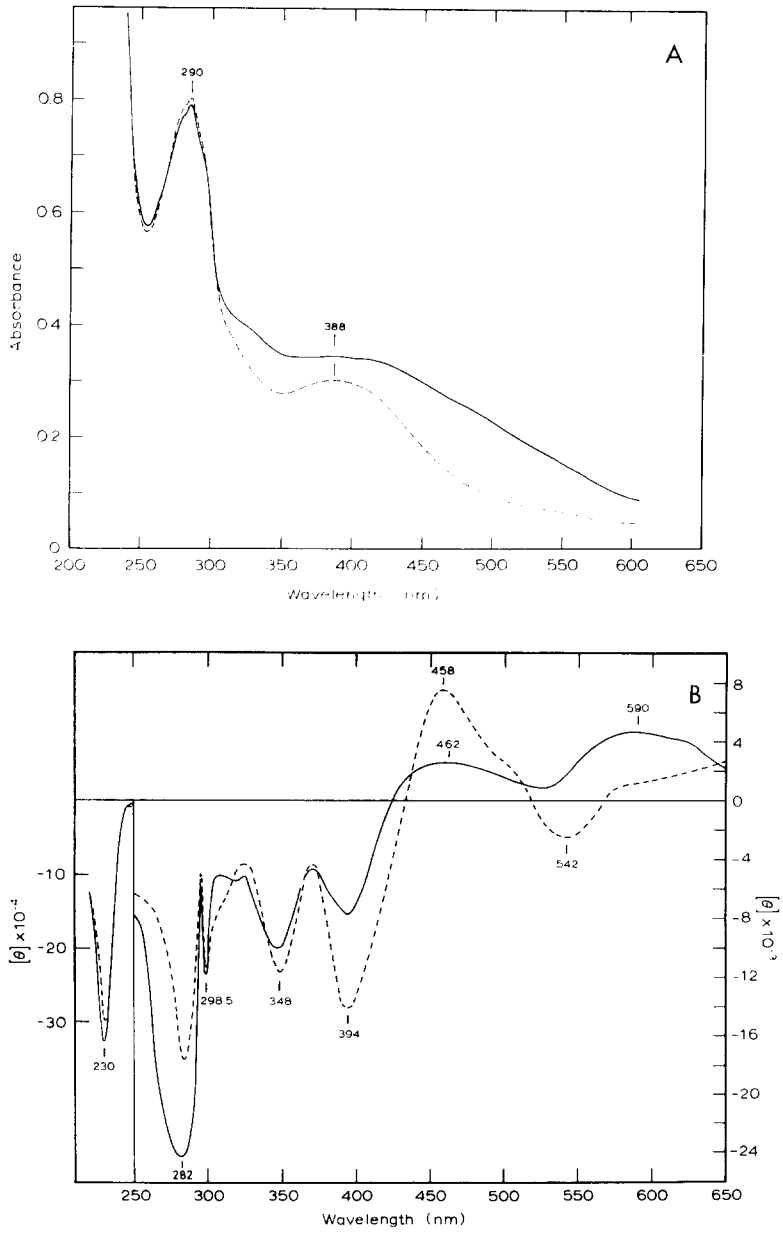

Fig. 1. *Chromatium* high-potential iron protein. (A) Absorption spectrum. (B) Circular dichroic absorption spectrum. Molar ellipticity, $[\theta]$, given in degrees·cm²·dmole⁻¹, was calculated on the basis of a molecular weight of 10074. ———, oxidized form; – – – –, reduced form, in 0.04 M phosphate buffer (pH 6.9) at 20°.

RESULTS

Circular dichroism

As seen from Fig. 1, the *Chromatium* high-potential iron protein gives a complex CD spectrum in both the oxidized and the reduced form. Most of the bands, unfortunately, have only a weak rotatory strength and arise from previously undetected electron transitions, *i.e.*, they are not resolved in the light-absorption spectrum. Thus the single, rather broad absorption band from 350 to 650 nm in the reduced form is really a composite of 5–6 transitions. The same is true for the ultraviolet region where more details are seen in the CD spectrum than in the light-absorption spectrum. The CD spectra indicate the presence of at least ten optically active transitions between 210 and 650 nm in the oxidized as well as in the reduced protein.

Among the most interesting features of the CD spectra are the qualitative differences in the region 430–650 nm between the two oxidation states. These differences show that reduction of the protein does induce significant alterations in the chromophores of the molecule. On the other hand, below 430 nm there are only quantitative differences between the two oxidation states. In this part of the spectrum it is especially interesting to note that no ellipticity band is observed around 222 nm which is characteristic of the n-π^* amide transition of the right-handed α-helix in polypeptides and proteins[26,27]. Only a negative band at 230 nm is observed (see DISCUSSION).

Sufhydryl determinations

An amino acid analysis of performic acid-treated *Chromatium* high-potential iron protein shows the presence of 4 equiv of cysteic acid per mole of protein having a molecular weight of approx 10000 (ref. 3). To determine how many of these cysteic acid equivalents were present originally as cysteine residues with a free sulfhydryl group, the protein was reduced with a large excess of mercaptoethanol and then treated with either of two reagents, DTNB or PCMB, known to react with sulfhydryl groups. The results are summarized in Table I. As the data show, none of these two reagents reacted immediately with the protein at a neutral pH which indicates that

TABLE I

SULFHYDRYL GROUPS OF *Chromatium* HIGH-POTENTIAL IRON PROTEIN

The amino acid analysis was performed on performic acid-oxidized protein[3].

Method of determination	*Moles of sulfhydryl groups per 10 kg of protein*
Amino acid analysis	4
Reaction of the native protein with DTNB*	
t = 1 min	0
Reaction of the native protein with PCMB*	
t = 1 min	0
t = 16 h	1.5**

* Described under MATERIALS AND METHODS.
** Mercaptide formation equivalent to 1.5 sulfhydryl-groups.

its cysteine residues are not present as free sulfhydryl groups. PCMB reacted very slowly with the protein at a molar ratio PCMB/high-potential iron protein of 4 (Fig. 2), but even after a reaction time of 16 h, only 1.5 moles of PCMB had reacted with 1 mole of protein. Since 2 moles of PCMB react with 1 mole of Na_2S and since

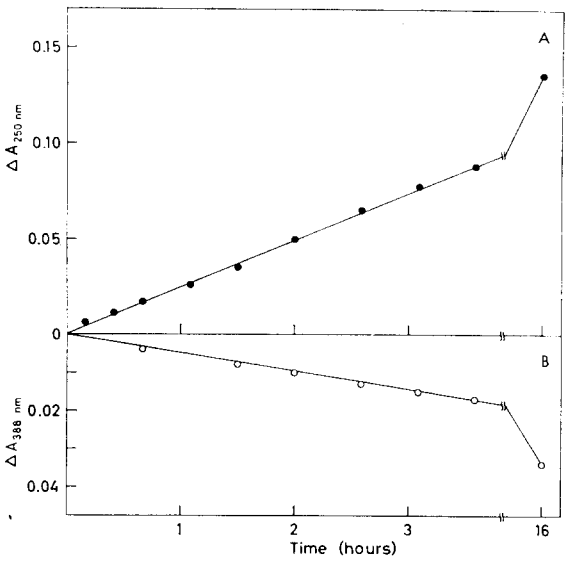

Fig. 2. Time course for the reaction of reduced *Chromatium* high-potential iron protein with PCMB. The reaction mixture contained: 43.6 μM PCMB, 11.5 μM high-potential iron protein, 0.03 M phosphate buffer (pH 7.0); 20°. The maximum corresponds to 1.5 sulfhydryl-groups per mole of high-potential iron protein.

the protein contains four cysteine residues and 4 moles of inorganic sulfide, this result demonstrates that a maximum of 1.5 of the sulfur atoms present has reacted with PCMB. Fig. 2 also shows that upon reaction with PCMB, the visible spectrum decreases simultaneously and proportionally with the formation of mercaptide.

Stoichiometry of electron transfer reaction

The exact number of electrons transferred per mole in the oxidation–reduction of *Chromatium* high-potential iron protein is not clear. In his first studies BARTSCH[1] found that a change of one electron was indicated for the interaction between protein and titrant ($Fe(CN)_6^{3+}$–$Fe(CN)_6^{4+}$ mixtures as redox buffers). A different conclusion was arrived at by MOSS *et al.*[4] by titrating the freshly oxidized protein with a limiting amount of NADH in the presence of a catalytic quantity of *N*-methylphenazine metasulfate. 2 or 3 oxidation–reduction equivalents were indicated from these experiments; however, the authors did not arrive at any definite conclusion due to the incomplete removal of O_2 from the reaction mixture and left the problem open to further investigation. Since Mössbauer spectroscopy[4] showed that the electron configurations of the four iron atoms of *Chromatium* high-potential iron protein are identical and that all are altered upon oxidation–reduction of the protein, it is important that this question be answered.

Because the reaction of *Chromatium* high-potential iron protein with the

sulfhydryl-group reagents, PCMB and DTNB, is extremely slow, the fully reduced protein was titrated spectrophotometrically with $K_3Fe(CN)_6$ in the presence of O_2. The formation of oxidized high-potential iron protein was followed by the maximum absorption increase at $\lambda = 480$ nm at which wavelength the absorption of excess

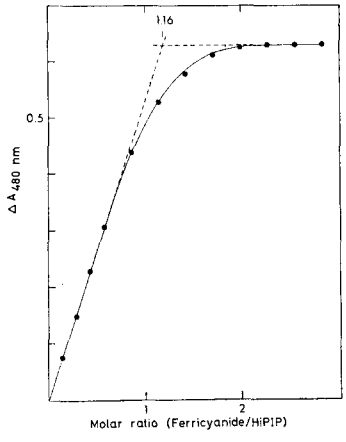

Fig. 3. Spectrophotometric titration of freshly reduced *Chromatium* high-potential iron protein (HiPIP) with increments of 1 mM $K_3Fe(CN)_6$ in the presence of O_2 (278 μM). The initial reaction mixture contained: 79.3 μM high-potential iron protein, 0.04 M phosphate buffer (pH 6.9); 20°.

$K_3Fe(CN)_6$ is zero. It is seen from Fig. 3 that the initial part of the titration curve followed a straight line giving an extrapolated value of 1.16 for the molar ratio where the fully oxidized form was reached. The deviation from this line in the last part of the curve is easily explained by the small difference (about 100 mV) in oxidation–reduction potential between the two reactants. Therefore the result indicates that only one electron is transferred during the oxidation–reduction of the protein as originally suggested by BARTSCH[1].

Physicochemical properties of the cobalt–cysteine complex

The isolated complex contained 1.91 moles of cysteine (determined as cysteic acid following performic acid oxidation) per mole of cobalt, and this result confirms the isolation of a pure product of bis-cysteinatocobaltate (III)[19]. None of the sulfhydryl groups reacted with PCMB at a neutral pH. The complex migrated on the amino acid analyzer column like an acidic compound in the same region where aspartic acid usually is recovered. Although the crystal structure of the complex is not known, our results confirm a previous conclusion[19] that both the sulfhydryl and the amino groups of the cysteine residues are coordinated to the central cobalt atom.

From Fig. 4A, it is seen that the absorption spectrum of the model compound reveals three pronounced bands centered around 443, 350 and 280 nm; in addition there are shoulders in the visible region and around 230 nm. All are optically active (Fig. 4B), indicating a high degree of asymmetry in the structure involved. This spectrum can be resolved into at least ten Gaussian functions, including two bands around 353 and 443 nm which are also well resolved in the absorption spectrum. Therefore these bands served as reference wavelengths in resolving the CD spectrum.

It is evident from Figs. 1 and 4A that the CD spectra of the cobalt complex and of high-potential iron protein are remarkably similar especially in the far ultraviolet region. Furthermore, in the CD spectrum of L-cysteine we observed two of these transitions centered around 284 and 353 nm; however, in the free amino acid these bands had a very low rotatory strength of less than 1/30 of that of the cobalt-cysteine complex. On the other hand, below 250 nm the ellipticity is positive in L-cysteine while negative in the cobalt complex.

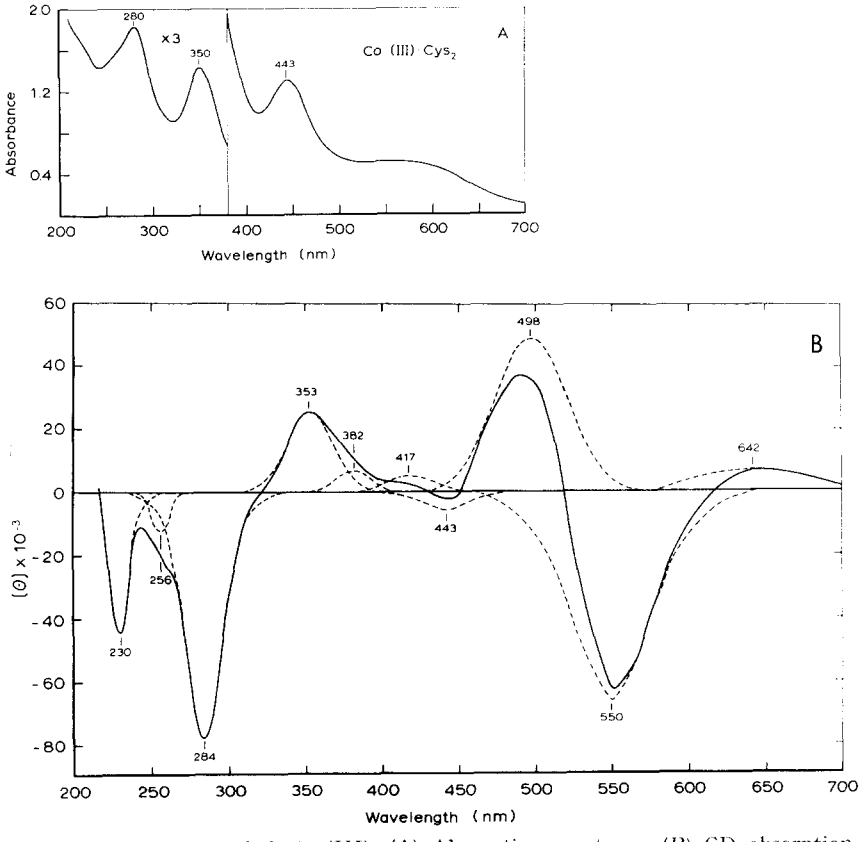

Fig. 4. Bis-cysteinatocobaltate (III). (A) Absorption spectrum. (B) CD absorption spectrum; ———, recorded curve; – – – –, possible Gaussian analysis of the CD curve.

DISCUSSION

Based on the determination of amino acid sequences and other chemical data, several models have been proposed for the chelate structure of non-heme iron proteins of the ferredoxin group[15]. None of the proposed structures, however, have been confirmed by X-ray crystallographic studies. Although information on the primary structure of *Chromatium* high-potential iron protein is not yet complete[28], various physical studies of the protein permit us to draw certain conclusions with respect to its three-dimensional structure. Thus, the CD spectra (Fig. 1) give no evidence for the presence of any α-helical structure in high-potential iron protein. This may be due to

restraints imposed by the tight chelate structure and by the adjacent prolyl residues[28]. It should also be noted that X-ray crystallographic studies by STRAHS AND KRAUT[29] on the fully reduced protein have indicated that the four iron atoms are unresolvable at 4 Å resolution and therefore must be part of a very compact three-dimensional structure. This interpretation is further supported by some recent chemical studies[3] which have shown that the high-potential iron proteins have more stable chelate structures than were observed for the ferredoxins. In addition, the inaccessibility to chemical reagents of the cysteine sulfhydryl groups in *Chromatium* high-potential iron protein (Fig. 2) also supports this concept.

The large number of electronic transitions which occur in non-heme iron proteins[5-13] is not understood at the present time. In our studies on *Chromatium* high-potential iron protein, special interest has been focused on the ultraviolet region. At present, it is impossible to interpret the spectrum below 250 nm in terms of any known polypeptide conformation[30]. On the other hand, it appears that the ellipticity band centered around 230 nm is associated with the iron ligand chromophore. This conclusion is corroborated by the fact that the octahedral cobalt complex, bis-cysteinatocobaltate (III), also exhibits this unique transition and that this transition is nearly identical to that observed in high-potential iron protein with respect to frequency, sign and amplitude. These comparative studies provide firm evidence that this unique transition is attributable to a highly specific metal coordination, *i.e.*, the existence of iron–SH or of cobalt–SH bonding. The very slow reactivity of high-potential iron protein towards sulfhydryl-group reagents and the finding that the visible absorption decreases simultaneously and proportionally with the mercaptide formation also substantiate this conclusion. In this context it should also be mentioned that per mole of metal and cysteine the rotatory strength of the 230-nm transition of the high-potential iron protein ($[\theta]_{230\,nm} = -20.7 \cdot 10^3$) and the cobalt complex ($[\theta]_{230\,nm} = -21.3 \cdot 10^3$) is remarkably similar. The small difference observed may be due to differences in the orientation of the cysteine residues and to the asymmetry of the complex.

In the region of aromatic absorption, the negative ellipticity band centered around 282 nm undergoes a significant alteration in rotatory strength upon oxidation–reduction, whereas the transition at 298.5 nm does not change. It is generally accepted[30] on the basis of studies on polypeptides and proteins that such transitions are due to asymmetrical interactions between the side chains of aromatic amino acids and the polypeptide chain. In this particular protein, however, the presence of optically active transitions other than those associated with the side chain chromophores of aromatic amino acids have to be considered. Thus, the light absorption of high-potential iron protein reveals an absorbance at 280 nm which is twice that expected on the basis of its content of aromatic amino acids[3], indicating the presence of a metal ligand absorption band in this wavelength region similar to that observed for the model complex bis-cysteinatocobaltate (III) (Fig. 4). Furthermore, in the cobalt complex this transition is optically active and generates a negative ellipticity band at around 284 nm. This fact supports the assignment of the 282-nm ellipticity band in high-potential iron protein to the chelate structure. Thus the 0.5-fold increase in the rotatory strength of the 282-nm transition upon oxidation of the protein appears to result from electron transfer from molecular orbitals of the iron ligand unit(s) and not from a change in the conformation of the polypeptide chain. It is

interesting that the 284-nm transition in the cobalt complex is the largest ellipticity band in the whole spectrum. This clearly demonstrates that in certain metalloproteins, transitions in the aromatic region may be assigned to the metal binding site and not only to the optical acitivity of the side chain chromophores of the polypeptide chain.

The prediction, made on the basis of Mössbauer spectroscopy[4] and EPR measurements (G. PALMER, personal communication), that the binding of iron in high-potential iron protein should be different from that of the ferredoxins is substantiated by our CD studies. Thus, the ellipticity bands of high-potential iron protein differ qualitatively as well as quantitatively from those observed for other non-heme iron proteins. However, at the moment the whole CD spectrum cannot be interpreted in terms of a specific chelate structure.

Oxidation–reduction titrations (Fig. 2) revealed that *Chromatium* high-potential iron protein exhibits a single electron transfer between the oxidized and the reduced form. The fact that Mössbauer spectroscopy[4] of high-potential iron protein shows the electron configurations of the four iron atoms to be identical in both oxidation states of the protein suggests that the bonding of iron in high-potential iron protein involves an interaction between these four ions; thus, one electron is equally shared between four iron nuclei. This conclusion is in good agreement with a recent crystallographic study by STRAHS AND KRAUT[29] on the fully reduced protein which indicated that the four iron atoms are unresolvable at 4 Å resolution and, therefore, must be part of a very compact three-dimensional structure.

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