Spectroscopic identification of the heme axial ligation of cytochrome b_{558} in the NADPH oxidase of porcine neutrophils

Hirotada Fujii[a], Michael G. Finnegan[b], Toshiaki Miki[a], Brian R. Crouse[b], Katsuko Kakinuma[a], Michael K. Johnson[b,*]

[a]*Department of Inflammation Research, The Tokyo Metropolitan Institute of Medical Science, 18-22 Hon-komagome 3-chome, Bunkyo-ku, Tokyo 113, Japan*

[b]*Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, GA 30602, USA*

Received 29 September 1995; revised version received 11 November 1995

Abstract The combination of electron paramagnetic resonance (EPR), near-infrared magnetic circular dichroism (NIR-MCD) and resonance Raman (RR) spectroscopies at cryogenic temperatures has been used to identify the axial heme ligation of the low spin cytochrome b_{558} component of NADPH oxidase from porcine blood neutrophils. The EPR and NIR-MCD results indicate the presence of two distinct forms in frozen solution; one with a low field g-value at 3.23 and porphyrin(π)-to-Fe(III) charge transfer maximum at 1660 nm and the other a low field g-value at 3.00 and porphyrin(π)-to-Fe(III) charge transfer maximum at 1510 nm. On the basis of these properties and the RR studies, both are attributed to forms of cytochrome b_{558} with bis-histidine axial ligation. The origin of the observed heterogeneity, the location and identity of the specific histidines involved in ligating the heme, and the role of the heme prosthetic group in O_2^- production are discussed in light of these results.

Key words: Cytochrome b_{558}; NADPH oxidase; Electron paramagnetic resonance; Magnetic circular dichroism; Resonance Raman

1. Introduction

Phagocytic leukocytes, such as neutrophils and macrophages, kill ingested microorganisms by releasing superoxide (O_2^-) and other reactive oxygen species. The enzyme which catalyzes the formation of O_2^- is NADPH oxidase, which is a multi-component electron-transfer system consisting of at least five subunits (for recent reviews, see [1,2]). Cytochrome b_{558} has been found to be missing in some patients with chronic granulomatous disease (CGD) who cannot produce O_2^-, and was identified as a component of the NADPH oxidase [3].

Cytochrome b_{558} is a heterodimer consisting of p22-*phox* (also known as the small or α subunit) and glycosylated p91-*phox* (also known as the large or β subunit) with molecular masses, derived from the cDNA sequences of their respective genes, of 21 kDa [4] and 65 kDa [5]. Dissociation of the complex destroys the cytochrome absorption spectrum and results in loss of the non-covalently bound heme. Cytochrome b_{558}, the heme of which has been reported to be the terminal electron carrier of the oxidase [6,7], is the only component of the NADPH oxidase complex to have been unambiguously identified. However, there has been uncertainty concerning the location and ligation of the heme and its role in O_2^- production.

Recently we have found that pyridine perturbs the heme environment of cytochrome b_{558} and the modified cytochrome b_{558} exhibits an EPR spectrum identical to that of cytochrome *P*-450 [8]. On the basis of sequence alignments for cytochrome *P*-450 with those of large- and small-subunits of cytochrome b_{558}, it was proposed that the heme in cytochrome b_{558} is associated with the large subunit. In the present study, we have used the combination of EPR, near-infrared magnetic circular dichroism (NIR-MCD) and resonance Raman (RR) spectroscopies, all at cryogenic temperatures, to identify the amino acid residues involved in axial ligation of the heme prosthetic group of purified cytochrome b_{558} from porcine blood neutrophils. The results indicate bis-histidine axial ligation and the specific histidines involved in heme coordination are discussed in light of the available mutagenesis and physicochemical data.

2. Materials and methods

2.1. Materials

DEAE-Sepharose CL-6B and heparin-Sepharose 6B were obtained from Pharmacia LKB Biotechnology Inc. Heptylthioglucoside (HTG) and EGTA were purchased from Dojindo Laboratories (Kumamoto, Japan). Diisopropyl fluorophosphate was purchased from Wako Pure Chemicals (Tokyo, Japan). Non-fluorescent glycerol was obtained from Nacalai Chemicals (Kyoto, Japan). Deuterated reagents, D_2O, d_3-glycerol and d_2-ethylene glycol, were obtained from Aldrich Chemical Co. Other chemical reagents were of analytical grade.

2.2. Preparation of porcine neutrophil membranes

Neutrophils were obtained from porcine blood and disrupted by sonication [9]. The post-nuclear supernatant was separated from the cell sonicate and used for collection of membrane vesicles. The membrane-bound NADPH oxidase was solubilized from the membrane vesicles at 0°C with 1% HTG, 30% glycerol, 50 mM phosphate buffer, pH 7.0, as reported previously [10].

2.3. Purification of cytochrome b_{558}

Cytochrome b_{558} was more than 95% solubilized with HTG from membrane vesicles. The soluble fraction prepared from resting neutrophils was passed through a DEAE-Sepharose CL-6B column (5 ml bed volume) and then applied at a flow rate of 0.5 ml/min to a heparin-Sepharose column (10 ml of resin) equilibrated with a buffer composed of 50 mM phosphate buffer (pH 7.0), 50 mM NaCl, 10% glycerol, and 0.6% HTG [11,12]. The concentration of cytochrome b_{558} was determined from the difference spectrum of the dithionite-reduced and oxidized samples using an extinction coefficient of $\Delta\varepsilon_{(558-540\ nm)} = 21.6\ mM^{-1} \cdot cm^{-1}$ [13]. Samples for NIR-MCD studies were prepared in deuterated buffers according to the following procedure. Aqueous solutions of the purified cytochrome b_{558} (3 ml, 20–30 μM heme) were

*Corresponding author. Fax: (1) (706) 542-2353.
E-mail: johnson@sunchem.chem.uga.edu

Abbreviations: NIR-MCD, near-infrared magnetic circular dichroism; RR, resonance Raman; λ_{CT}, wavelength of the NIR-MCD maxima of the porphyrin(π)-to-Fe(III) charge transfer band; HTG, *n*-heptyl-β-thioglucoside; CGD, chronic granulomatous disease.

SSDI 0014-5793(95)01372-5

diluted with 100 ml of 50 mM phosphate (pH 7.0) prepared with 99.8% D_2O containing 0.6% HTG, and was concentrated at 5°C using Centricon-30 (Amicon). This buffer exchange was carried out 2 times. In order to obtain optical quality glasses upon freezing, purified cytochrome b_{558} preparations in deuterated buffer were mixed with 50% (v/v) d_3-glycerol or d_2-ethylene glycol. For EPR and RR studies, samples of purified cytochrome b_{558} were concentrated by centrifugation using a Centricon-30 at 5°C. The pH of the concentrated samples used for spectroscopic studies was measured using a Cosmo pH Boy-C1 at 25°C.

2.4. Spectroscopic measurements

Absorption spectra were recorded at 25°C using a Unisoku US-401 or a Shimadzu UV3101PC spectrophotometer. NIR-MCD spectra were recorded using a Jasco J-730 spectropolarimeter interfaced to an Oxford Instruments SM3 superconducting magnet (magnetic field 0–5 T; sample temperature 1.5–300 K) using the protocols described elsewhere [14]. X-Band EPR spectra were recorded on a Jeol (JES-FE) EPR spectrometer equipped with a Air Products, Model LTR-3 Heli-Tran cryostat system, or a Bruker Instruments ESP 300D spectrometer equipped with an Oxford Instruments ESR 900 flow cryostat. RR spectra were recorded by collecting scattering (90°) from the surface of a 10 μl frozen droplet of sample (20 K) on the cold finger of an Air Products Displex DE-202 closed cycle helium refrigerator. The light source was the 413.1-nm line of a Coherent 200-K2 Kr^+ laser and the scattered light was analyzed using a ISA U-1000 double monochromator fitted with a cooled RCA 31034 photomultiplier tube with photon counting electronics. Further details of the Raman spectrometer and the protocols for obtaining RR spectra from frozen samples under anaerobic conditions can be found in [15].

3. Results

EPR spectra for the samples of purified cytochrome b_{558} used in this work for low temperature NIR-MCD studies, i.e. prepared in a deuterated phosphate buffer with 50% (v/v) d_3-glycerol or d_2-ethylene glycol, are shown in Fig. 1. Purification and buffer exchange had relatively little effect on the O_2^--generating ability. In a cell-free assay system [16], the purified cytochrome b_{558} exhibited an activity of 45–55 mol of O_2^- produced per second per mol of cytochrome b_{558}, compared to 73 mol of $O_2^- \cdot s^{-1} \cdot mol^{-1}$ for solubilized membranes. The EPR spectrum is identical to that reported in non-deuterated buffer [12] and is dominated by a low spin ferric heme resonance with a low-field positive absorption-shaped feature at $g = 3.23$, a broad derivative-shaped component centered at approximately $g = 2.10$ and weak high-field negative absorption-shaped feature centered at $g = 1.39$. In addition to a $g = 4.3$ signal attributed to adventitiously bound Fe^{3+} and other impurity signals close to $g = 2.00$, that vary in intensity for different preparations, there is a second low spin ferric heme resonance with $g = 3.00, \sim 2.20$, and ~ 1.60. This species is slower relaxing than the '$g = 3.23$ species' and is more pronounced at higher temperatures and/or lower microwave powers (data not shown). In addition, it is slightly more pronounced in samples treated with 50% (v/v) d_3-glycerol or d_2-ethylene glycol and significantly enhanced in samples at pH 5 (see Fig. 3 of [16]). However, it is a relatively minor species at neutral pH and the concentration of the '$g = 3.00$ species' was estimated to be 20% and 30% of the '$g = 3.23$ species' for samples in the absence and presence of glassing agent, respectively. The relative concentrations were assessed by fitting the low-field resonance to two Gaussian-shaped bands, integrating each under non-saturating conditions and correcting for the different transition probabilities according to the published procedure [17].

The heterogeneity that is apparent in the EPR spectra of cytochrome b_{558} is also apparent in the porphyrin(π)-to-Fe(III) charge transfer bands in the low temperature NIR-MCD spectra (see Fig. 2). Identical spectra were obtained using 50% (v/v) d_3-glycerol or d_2-ethylene glycol as the glassing agent. Low spin ferric hemes have two porphyrin(π)-to-Fe(III) charge transfer bands in the near-IR region. Both exhibit positively-signed, temperature-dependent MCD transitions and the lower energy component almost invariably has the larger intensity [18–21]. The energy of these transitions, as assessed by the wavelength of the MCD maxima (λ_{CT}), has been shown to be extremely sensitive to the nature of the axial ligands [18–21]. The NIR-MCD spectra of oxidized purified cytochrome b_{558} is clearly the composite of two species: a minor component with $\lambda_{CT} = 1510$ nm that is attributed to the $g = 3.00$ species and a major component with $\lambda_{CT} = 1660$ nm that corresponds to the $g = 3.23$ species.

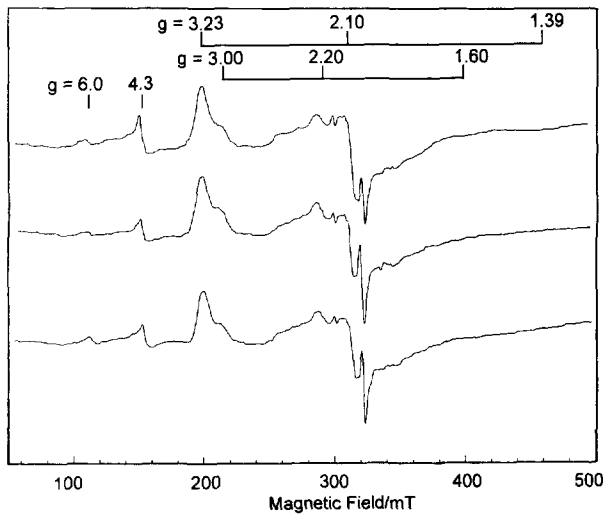

Fig. 1. EPR spectra of purified oxidized cytochrome b_{558}. The purified cytochrome b_{558} was prepared in deuterated phosphate buffer and concentrated to 110 μM (upper spectrum). The middle and lower spectra correspond to the samples used for NIR-MCD studies are after the addition of 50% (v/v) d_3-glycerol and d_2-ethylene glycol, respectively. In each case the pD of the medium was adjusted to 7.4. The instrumental settings were as follows: microwave frequency, 9.04 GHz; microwave power, 10 mW; modulation amplitude, 10 Gauss; temperature, 10 K.

Low temperature (25 K) resonance Raman spectra (413-nm excitation) for as prepared and dithionite-reduced samples of cytochrome b_{558} are shown in Fig. 3. The spectra are very similar to those reported previously at low temperature (90 K) for cytochrome b_{558} in partially purified solubilized preparations and neutrophil plasma membranes [22], except that laser-induced photoreduction was not apparent in the samples used in this work thereby enabling direct determination of the spectrum of the oxidized cytochrome. The frequencies of the 'oxidation-state' marker bands, 1363 cm^{-1} for reduced and 1378 cm^{-1} for oxidized, are characteristic of the ferrous and ferric oxidation states, respectively, and the 'spin-state' marker bands at 1585 and 1646 cm^{-1} are characteristic of a low spin ferricytochrome [23]. The spectra are very similar to those observed for bis-imidazole Fe(II)/Fe(III) protoporphyrin IX complexes and the band at 273 cm^{-1} has tentatively been assigned to Fe–N stretching of a bis-histidine ligated ferricytochrome [22].

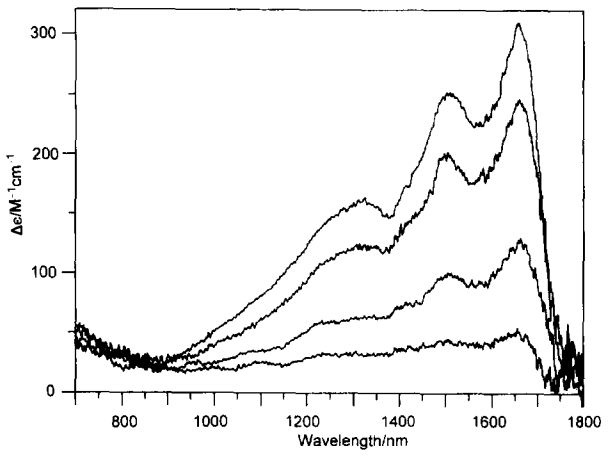

Fig. 2. Low temperature NIR-MCD spectra of purified oxidized cytochrome b_{558}. The sample contained 50% (v/v) d_3-glycerol and was the same used in Fig. 1. The spectra were recorded at a magnetic field of 4.5 T at a temperatures of 2.0, 4.2, 10, and 30 K. All transitions increase in intensity with decreasing temperature.

4. Discussion

EPR, NIR-MCD and resonance Raman studies all indicate a low spin 6-coordinate ferric heme prosthetic group in cytochrome b_{558}. However, the NIR-MCD and EPR data provide clear evidence for heterogeneity with a major species (g = 3.23, ~2.10,~1.39 and λ_{CT} = 1660 nm) and minor species (g = 3.00, ~2.20, ~1.60 and λ_{CT} = 1510 nm). The relative amount of the minor species appears to increase with sample handling, addition of 50% glycerol or ethylene glycol, and lowering the pH.

On the basis of the EPR spectra alone, it is not possible to make an unambiguous assignment of the axial ligands for the major low spin ferric heme species. There are examples of hemes with bis-histidine, histidine/methionine and histidine/lysine with similar g-value anisotropy [19]. However, the ranges of λ_{CT} values established for these types of axial ligation by NIR-MCD studies, i.e. bis-histidine, λ_{CT} = 1500–1660 nm; histidine-methionine, λ_{CT} = 1740–1950 nm; histidine-amine (lysine or N-terminal amine), λ_{CT} = 1480–1550 nm [18–21,24], argue strongly in favor of bis-histidine axial ligation. This conclusion is clearly in accord with the resonance Raman presented herein and published previously [22]. The rationale for showing resonance Raman spectra for the samples used in this work is two-fold. First, they demonstrate that the cytochrome b_{558} is identical in these two studies. Hence the apparent absence of a low spin ferric heme EPR signal that was reported by Hurst et al. [22] must be the result of instrument sensitivity problems and does not require explanation in the form of interheme magnetic interactions as previously proposed. Second, they demonstrate that this form of cytochrome b_{558} is present in vivo, since identical Raman spectra have been observed in various subcellular environments and in cellular physiological states [22]. Coupled with our earlier studies which showed that only the low spin form of cytochrome b_{558} is effective in O_2^- production in an in vitro assay [16], this indicates that this bis-histidine form of cytochrome b_{558} is functional in vivo.

Next we turn our attention to the origin of the minor species that is apparent in the EPR and NIR-MCD data of the purified cytochrome b_{558} samples. Thomson and coworkers have elegantly demonstrated that the intensity of the NIR-MCD porphyrin(π)-to-Fe(III) charge transfer bands and the low-field g-value of bis-histidine ferric hemes reflect the rhombic distortion at the iron site [20,21]. Perpendicular imidazole rings provide the most axial environment which leads to very intense NIR-MCD charge transfer bands ($\Delta\varepsilon$ values up to 560 $M^{-1} \cdot cm^{-1}$ at 4.2 K and 5 T) and low field g-values approaching 4.0. b-Type cytochromes with properties approaching this extreme are found in membrane bound environments in which the cytochrome bridges across two membrane spanning helices, e.g. in the cytochrome bc_1 complex [24] and in the membrane anchor subunits of succinate dehydrogenases [25,26]. The majority bis-histidine ligated low spin ferric heme species in cytochrome b_{558} is clearly in well-defined environment somewhere between the extremes of parallel and perpendicular imidazole rings. Denatured, bis-histidine b-type cytochromes in which the constraints of the native structure have been removed due to removal from the membrane and or subunit dissociation, exhibit electronic properties identical to those of bis-imidazole ferric protoporphyrin model complexes, i.e. g = 2.95, 2.25, 1.60 and λ_{CT} = ~1510 nm (maximal $\Delta\varepsilon$ values 200–250 $M^{-1} \cdot cm^{-1}$ at 4.2 K and 5 T) [24,26–28]. Since these properties are very similar to those of the minor species observed in purified sam-

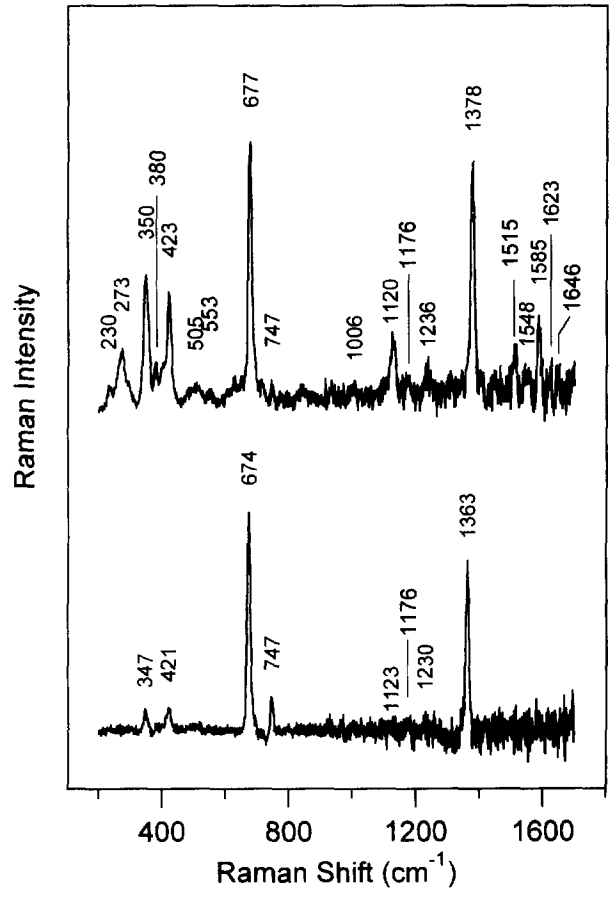

Fig. 3. RR spectra of purified cytochrome b_{558}. Upper spectrum: oxidized sample, 200 μM in heme, pH 7.4. Lower spectrum: dithionite-reduced sample, 180 μM in heme, pH 7.4. Conditions: sample temperature, 25 K; laser excitation: 413.1 nm; laser power at sample, 10 mW; spectral resolution, 6 cm^{-1}; spectra collected by photon counting for 1 s every 1 cm^{-1} and each is the sum of 12 scans. In both cases a linear ramp has been subtracted to correct for background fluorescence.

ples of cytochrome b_{558} and the relative amount of this species increases with sample handling, we attributed this to some denatured, bis-histidine *b*-type cytochromes. Moreover, on the basis of the additional intensity at 1510 nm ($\Delta\Delta\varepsilon \approx 60\ M^{-1} \cdot cm^{-1}$ at 4.2 K and 5 T), we conclude that this species corresponds to approximately 25% of a single heme, in excellent agreement with the EPR quantitations which indicate that this species accounts for between 20–30% of the total low spin heme resonance.

In a previous study [8], we have shown that the heme is localized at the large subunit of cytochrome b_{558} near the amino-terminal region, residues 100–200. For bis-histidine coordination in cytochrome b_{558}, there are six candidate histidines, residues 101, 111, 115, 119, 209, and 210 [29]. Bolscher et al. [30] reported that amino acid substitutions in the large subunit of cytochrome b_{558} of neutrophils from X-linked chronic granulomatous disease patients caused complete absence of the heme. In the cytochrome b_{558} obtained from these patients, His^{101} and His^{209} were replaced by Arg and Tyr, respectively. Taken together, the spectroscopic data reported herein and these point mutation data, implicate His^{101} and His^{209} as the most likely residues ligating the heme of neutrophil cytochrome b_{558}. Further support for this conclusion comes from analysis of the hydrophilicity index and surface probability in cytochrome b_{558} which show that these two histidine residues are in the hydrophobic region [31].

Dinauer et al. [32] have shown that there is only one invariant histidine residue in the small subunit, His^{72}, of cytochrome b_{558}. From these and our spectroscopic data which clearly show bis-histidine coordination, it is therefore extremely unlikely that the heme resides exclusively in the small subunit, as predicted by Nugent et al. [33] and Yamaguchi et al. [34]. While we cannot rule out the last possibility, i.e. that the heme bridges the small and large subunits, the absence of significant changes in the EPR and resonance Raman properties of cytochrome b_{558} accompanying isolation from the membrane-bound cellular environment [16,22], tends to argue against such an arrangement.

In conclusion, the heme in neutrophil cytochrome b_{558} is ligated via two histidines. On the basis of the spectroscopic and point mutation data, His^{101} and His^{209} in the large subunit are considered the most probable heme ligands, although it is not yet possible to rule out an arrangement in which the heme bridges the two subunits. While this heme prosthetic group is essential for the O_2^- generating ability of the neutrophil NADPH oxidase, bis-histidine ligation indicates this heme group is likely to be involved in electron transfer to the active site rather than the site of O_2^- production.

Acknowledgements: This work was supported by grants from the Ministry of Education, Science and Culture of Japan and the National Institutes of Health (GM51962 to M.K.J.).

References

[1] Chanock, S.J., Benna, J.E., Smith, R.M. and Babior, B.M. (1994) J. Biol. Chem. 269, 24519–24522.
[2] Thrasher, A.J., Keep, N.H., Wientjes, A. and Segal, A.W. (1994) Biochim. Biophys. Acta 1227, 1–24.
[3] Segal, A.W. and Jones, O.T.G. (1978) Nature 276, 515–517.
[4] Parkos, C.A., Allen, R.A., Cochrane, C.G. and Jesaitis, A.J. (1987) J. Clin. Invest. 80, 732–742.
[5] Dinauer, M.C., Orkin, S.H., Brown, R., Jesaitis, A.J. and Parkos, C.A. (1987) Nature 327, 717–720.
[6] Cross, A.R., Jones, O.T.G., Harper, A.M. and Segal, A.W. (1981) Biochem. J. 194, 599–606.
[7] Isogai, Y., Iizuka, T., Makino, R., Iyanagi, T. and Orii, Y. (1993) J. Biol. Chem. 268, 4025–4031.
[8] Fujii, H., Yonetani, T., Miki, T. and Kakinuma, K. (1995) J. Biol. Chem. 270, 3193–3196.
[9] Kakinuma, K., Kaneda, M., Chiba, T. and Ohnishi, T. (1986) J. Biol. Chem. 261, 9426–9432.
[10] Fujii, H. and Kakinuma, K. (1991) Biochim. Biophys. Acta 1095, 201–209.
[11] Miki, T., Yoshida, L.S. and Kakinuma, K. (1992) J. Biol. Chem. 267, 18695–18701.
[12] Miki, T., Fujii, H. and Kakinuma, K. (1992) J. Biol. Chem. 267, 19673–19675.
[13] Cross, A.R., Higson, F.K., Jones, O.T.G., Harper, A.M. and Segal, A.W. (1982) Biochem. J. 204, 479–485.
[14] Johnson, M.K. (1988) in: Metal Clusters in Proteins (L. Que ed.) ACS Symposium Series Vol. 372, pp. 326–342, American Chemical Society, Washington, D.C.
[15] Hamilton, C.L., Scott, R.A. and Johnson, M.K. (1989) J. Biol. Chem. 264, 11605–11613.
[16] Fujii, H., Johnson, M.K., Finnegan, M.G., Miki, T., Yoshida, L.S. and Kakinuma, K. (1995) J. Biol. Chem. 270, 12685–12689.
[17] Aasa, R. and Vänngård, T. (1975) J. Magn. Reson. 19, 308–315.
[18] Simpkin, D., Palmer, G., Devlin, F.J., McKenna, M.C., Jensen, G.M. and Stephens, P.J. (1989) Biochemistry 28, 8033–8039.
[19] Gadsby, P.M.A. and Thomson, A.J. (1990) J. Am. Chem. Soc. 112, 5003–5011.
[20] Thomson, A.J. and Gadsby, P.M.A. (1990) J. Chem. Soc., Dalton Trans., 1921–1928.
[21] Cheesman, M.R., Greenwood, C. and Thomson, A.J. (1991) Adv. Inorg. Chem. 36, 201–255.
[22] Hurst, J.K., Loehr, T.M., Curnutte, J.T. and Rosen, H. (1991) J. Biol. Chem. 266, 1627–1634.
[23] Spiro, T.G. (1983) in: Iron Porphyrins (A.B.P. Lever and H.B. Gray eds.) part 2, pp. 91–159, Addison-Wesley, Reading, MA.
[24] Finnegan, M.G., Knaff, D.B., Qin, H., Gray, K.A., Daldal, F., Yu, L., Yu, C.-L., Kleis-San Francisco, S. and Johnson, M.K. (1995) Biochim. Biophys. Acta (in press).
[25] Fridén H., Cheesman, M.R., Hederstedt, L., Andersson, K.K. and Thomson, A.J. (1990) Biochim. Biophys. Acta 1041, 207–215.
[26] Crouse, B.R., Yu, C.-A., Yu, L. and Johnson, M.K. (1995) FEBS Lett. 367, 1–4.
[27] Salerno, J.C., McGill, J.W. and Gerstle, G.C. (1983) FEBS Lett. 162, 257–261.
[28] Salerno, J.C. and Leigh, J.C (1984) J. Am. Chem. Soc. 106, 2156–2159.
[29] Orkin, S.H. (1989) Annu. Rev. Immunol. 7, 277–307.
[30] Bolscher, B.G.J.M., Boer, M., Klein, A. and Roos, D. (1991) Blood 77, 2482–2487.
[31] Imajoh-Ohmi, S., Tokita, K., Ochiai, H., Nakamura, M. and Kanegasaki, S. (1992) J. Biol. Chem. 267, 180–184.
[32] Dinauer, M.C., Pierce, E.A., Bruns, G.A.P., Curnutte, J.T. and Orkin, S.H. (1990) J. Clin. Invest. 86, 1729–1737.
[33] Nugent, J.H.A., Gratzer, W. and Segal, A.W. (1989) Biochem. J. 264, 921–924.
[34] Yamaguchi, T., Hayakawa, T., Kaneda, M., Kakinuma, K. and Yoshikawa, A. (1989) J. Biol. Chem. 264, 112–118.